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Microdialysis sampling for investigations of tetramethylpyrazine following transdermal and intraperitoneal administration

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ABSTRACT

The pharmacokinetic behaviors of tetramethylpyrazine (TMP) were investigated in rat plasma and microdialysates of various sites such as brain, blood and skin after tetramethylpyrazine hydrochloride (TMPH) transdermal or intraperitoneal (i.p.) administration. Samples were collected at timed intervals for the measurement of TMP by a quick and sensitive HPLC-UV method. The pharmacokinetic parameters were calculated by non-compartmental analysis using DAS 2.0. The results of pharmacokinetics indicated that the C_{max} in brain and plasma after transdermal administration (20 mg/cm^2, 1.2 cm^2) was similar to that after i.p. administration (40 mg/kg). The value of C_{max} after i.p. administration in brain, blood microdialysates and plasma were 8.17 ± 2.06, 11.58 ± 2.66 and 15.54 ± 3.87 mg/l, respectively. After gel transdermal administration, the value of C_{max} in brain, blood, skin microdialysates and plasma were 7.29 ± 2.65, 8.53 ± 1.98, 43.39 ± 29.57 and 15.50 ± 2.99 mg/l, respectively. Compared with traditional administration, gel transdermal administration is a promising alternative to transport TMP to the brain.

1. Introduction

Tetramethylpyrazine (TMP), an active ingredient extracted from *Ligusticum chuanxiong Hort*, has been widely used in China as a drug for the treatment of ischemic cerebrovascular disease (Guo et al., 1983). Many studies have shown that TMP has good curative effect on ischemic brain injury, and the mechanism of TMP protects the brain might be related to reduction of nNOS expression, stabilization of mitochondrial function, suppression of inflammatory reaction, reduction of neuronal apoptosis, and prevention of neuronal loss (Kao et al., 2006; Li et al., 2010; Xiao et al., 2010). Since TMP has many appropriate characteristics, with a low molecular weight of 136.2, a melting point of 76–78 °C, a water solubility of about 11 mg/ml and the partition coefficient ($\log K_{oct/water}$) of 2.3 (Han et al., 2012), it can be easily permeate through the skin.

Currently, the commercially marketed preparations of TMP are intramuscularly, intravenously or orally (tablet, capsule and drop pill) administered. However, after oral administration of 174.5 mg dose, TMP was absorbed rapidly, distributed widely in the body, and also eliminated at a fairly rapid rate with a low bioavailability (Cai et al., 1989). After intravenous injection of TMP (10 mg/kg), drug concentration was achieved peak within 10–20 min but eliminated rapidly. TMP was almost undetectable after 120 min (Tsai and Liang, 2001). Transdermal administration was reported recently (Liu et al., 2011; Zhao et al., 2011). Compared with conventional pharmaceutical dosage forms, transdermal administration offers an alternative pathway with many important advantages, including elimination of the hepatic first-pass metabolism, enhancement of therapeutic efficiency, reduced side effects and improved patient compliance (Patel and Kavitha, 2011; Selvam et al., 2010). In these studies, in order to study brain distribution of TMP, samples were extracted by homogenate method. And the preparation of emulsion-based or ethosome transdermal delivery system was complicated and difficult for industrialized production. Thus, more suitable dosage form and sampling method are required.

In comparison to traditional sampling methods, microdialysis is a unique technique for *in vivo* sampling without withdrawal of biological fluids and involving minimal disturbance of physiological function (Holmgaard et al., 2010; Tsai, 2003). Conventional total drug samples include both unbound drugs and protein bound drugs. However, protein-free drugs are useful for pharmacokinetics and related to pharmacologic action. Although several techniques have been used to determine protein unbound drugs from biological fluids, including ultrafiltration, equilibrium dialysis and microdialysis, only microdialysis allows simultaneous sampling of protein unbound chemicals from plasma, tissues and body fluids such as the bile juice and cerebral spinal fluid for pharmacokinetic and pharmacodynamic studies (Tsai, 2003). The application of this technology can be combined for multi-site synchronization of microdialysis experiments, and provide valuable information

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about drug concentration change in different sites for clinical application.

Although there were studies available concerning pharmacokinetics of TMP after transdermal administration, the present study was undertaken to investigate the concentration profiles of TMP in three sites (blood, brain and skin) following dosing gel by using microdialysis technique in rats. The aim of this study was to characterize and compare the pharmacokinetics of TMP in different sites after transdermal and intraperitoneal administration.

2. Materials and methods

2.1. Chemicals

TMP(>99%) and TMPH(>99%) were obtained from Zelang Pharmaceutical Co. (Jiangsu, China). Azone was purchased from Guangzhou fine chemical industry Co. (Guangzhou, China). Peppermint oil was provided by Hengcheng natural perfume oil refinery (Jiangxi, China). Sodium carboxymethylcellulose was obtained from Sinopharm chemical reagent Co. (Shanghai, China). HPLC-grade methanol was obtained from Ludu industrial Co. (Shanghai, China). All other reagents were of analytical grade and commercially available.

2.2. Animals

Male Sprague-Dawley rats (250–300 g) were obtained from Zhejiang Laboratory Animal Center (Hangzhou, China). Animals were acclimatized for at least one week before use. They were kept in a climate controlled room with temperature maintained at 24 ± 1 °C and a light-dark cycle of each 12 h. Water and standard laboratory food were available ad libitum until 12 h before the experiments, at which time only water was given. All experiments were performed according to the guidelines for the care and use of animals as established by Zhejiang Academy of Medical Sciences.

2.3. Preparation of gel

Transdermal gel was composed of azone (5%, w/w), peppermint oil (5%, w/w), sodium carboxymethylcellulose (8%, w/w), tetramethylpyrazine hydrochloride (8%, w/w) and water. Drug was dissolved with purified water in a glass bottle and other excipients were added successively, stirring until mixed uniformly.

2.4. Animal experiments

2.4.1. In vivo evaluation of probe recovery

The *in vivo* recovery of TMP was determined by retrodialysis methods (Shinkai et al., 2011; Xie et al., 2000). The rats were anesthetized with an intraperitoneal dose of 20% (w/v) urethane (1 g/kg) and the body temperature were kept 37 °C by a heating pad. Throughout the experiment, they were kept under anesthesia. The skin of right jugular region was shaved and a concentric dialysis probe (CMA 20Elite, CMA, Sweden) of length 10 mm was inserted into jugular vein, then the rat was fixed onto a stereotaxic apparatus (MD3000, BAS, USA). The skull was exposed and a small hole was drilled to allow the implantation of a intracerebral probe (CMA 12Elite, membrance length 4 mm, CMA, Sweden) at the frontal cortex (coordinates: AP 2.1, ML 2.0, DV 5.0) according to the Paxinos and Watson atlas (Paxinos and Watson, 2007). The skin of dorsum was shaved before and a concentric dialysis probe, which was the same type as blood dialysis probe inserted into skin. After implantation, the inlet tube of the probe was connected to a microinjection pump (MD-1001). Ringer's solution (144 mmol/l Na^+, 1.5 mmol/l Ca^{2+}, 4.0 mmol/l K^+ and 2.3 mmol/l Mg^{2+}), consisting of 4 μg/ml of TMP was pumped through the microdialysis probe. Following stabilization for 1 h, dialysate samples were collected every 30 min for 120 min when flow-rate was at 1.0 μl/min, and the probe recovery was applied to transdermal administration; dialysate samples were collected every 15 min for 60 min when flow-rate was at 2.5 μl/min, and the probe recovery was applied to i.p. administration. The concentrations of TMP in dialysate (C_{dial}) or in perfusate solution ($C_{perfusate}$) were determined by HPLC. The *in vivo* recovery ratio of TMP (R_{dial}) was calculated as $R_{dial} = (C_{perfusate} - C_{dial})/C_{perfusate}$.

2.4.2. In vivo pharmacokinetics study of TMP

For the determination of TMP concentrations following intraperitoneal injection in rats, microdialysis probes were inserted into both jugular vein and brain as described above. Probes were perfused with Ringer's solution at a flow-rate of 2.5 μl/min. After an equilibration period of 60 min, TMP (40 mg/kg) was then intraperitoneal administered. At 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 135, 150, 165, 180, 195, 210, 225, 240, 270, 300 min after administration, brain and blood dialysate samples were collected. At 0, 2, 5, 10, 20, 40, 60, 90, 120, 150, 180, 240, 300 min, 0.1 ml blood sample was taken from the tail vein and placed in a heparinized polyethylene (PE) conical tube, then centrifuged at 10000 rpm for 6 min, 40 μl plasma was obtained.

For the determination of TMP concentrations following transdermal administration in rats, microdialysis probes were inserted into three sites (jugular vein, brain and skin) as described above. Probes were perfused with Ringer's solution at a flow rate of 1.0 μl/min. After a recovery period of 60 min, gel containing TMP 20 mg/cm^2 was applied at dorsum skin (1.2 cm^2) where the microdialysis probe was implanted. Dialysate was collected at 30 min intervals for up to 600 min after dosing (0, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, 420, 450, 480, 510, 540, 570, 600 min). Blood sample was collected at 60 min intervals for up to 600 min (0, 60, 120, 180, 240, 300, 360, 420, 480, 540, 600 min). All samples were stored at −20 °C and assayed as soon as possible.

2.5. Analytical procedures

Drug concentration was determined with high performance liquid chromatography, the microdialysates (brain, blood and skin) were analyzed directly, but the plasma needed to pretreat. After 60 μl acetonitrile was added to 40 μl plasma sample, the mixture was vortexed for 2 min before centrifugation at 10,000 rpm for 6 min. The supernatant was transferred to another PE tube.

The HPLC system contained of LC-10A pump, SPD-10A UV detector and N2000 chromatographic workstation (Intelligent Information Engineer Ltd. of Zhejiang University). A Diamonsil (C18) column (4.6 mm × 150 mm, 5 μm, Dikma) was used for analysis with mobile phase containing methanol-water (55:45, V/V) at a flow- rate of 1.0 ml/min. The UV detection wavelength was 281 nm and the temperature of column oven was maintained at 35 °C. The retention of TMP was 4.0 min. Linearity was assessed by analyzing 12 standards with concentrations over the range of 0.03–63.38 μg/ml in Ringer's solution and 10 standards over the range of 0.12–63.39 μg/ml in plasma.

2.6. Data analysis

Pharmacokinetic software DAS 2.0 program (Mathematical Pharmacology Professional Committee of China, Shanghai, China) was applied to calculate pharmacokinetic parameters. The apparent clearance (CLz/F) and apparent volume of distribution (Vz/F) were calculated as CLz/F = Dose/AUC and Vz/F = CLz/F/Zeta, respectively (Wan et al., 2013). And Zeta was the parameter of non-com-

Table 1
The non-compartmental pharmacokinetic parameters for TMP in rat plasma, blood and brain microdialysates following i.p. administration (40 mg/kg) in rats (mean ± SD, $n = 7$).

Parameter	Brain	Blood	Plasma
$AUC_{(0-t)}$ (mg/l·min)	614.38 ± 147.84**	907.69 ± 211.80**	2028.89 ± 394.13
$t_{1/2z}$ (min)	35.65 ± 3.04*	48.38 ± 4.36	63.25 ± 19.83
T_{max} (min)	17.86 ± 4.88	17.86 ± 4.88	27.14 ± 12.54
CLz/F (l/min/kg)	0.067 ± 0.016**	0.046 ± 0.014**	0.020 ± 0.0050
Vz/F (l/kg)	3.46 ± 0.77**	3.19 ± 0.87**	1.69 ± 0.34
C_{max} (mg/l)	8.17 ± 2.06**	11.58 ± 2.66*	15.54 ± 3.87
$AUC_{brain/blood}$ (%)	68.32 ± 8.22		
$AUC_{brain/plasma}$ (%)	30.61 ± 7.28		
$AUC_{blood/plasma}$ (%)	44.95 ± 9.12		

* $P < 0.05$ compared with plasma.
** $P < 0.01$ compared with plasma.

partmental model, which was calculated from the slope of the last points belonging to the elimination phase of concentration versus time curve. All data were expressed as mean ± SD values. Student's *t*-test was used to study the statistical difference and a value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Validation of analytical method

The HPLC method was rapid and sensitive for measuring TMP in microdialysates and plasma. The retention time of TMP was about 4.0 min, and no interference was found from endogenous compounds.

To assure the reliability of assay, the analytical method was validated using blank Ringer's solution and plasma samples in which different concentrations of TMP were spiked. The HPLC method showed good linearity throughout the concentration range of 0.03–63.38 μg/ml in Ringer's solution and 0.12–63.39 μg/ml in plasma. The regression equations were $Y = 34659X + 619.24$ ($n = 5$, $R^2 = 1$) and $Y = 15892X + 1324.5$ ($n = 5$, $R^2 = 1$), respectively. Intra-day and inter-day variabilities were determined, and all of the R.S.D were less than 15% with good accuracy between 91.19-113.62%.

3.2. In vivo evaluation of probe recovery

At a flow-rate of 1.0 μl/min, the mean value of recovery for TMP of microdialysis probes (blood, brain and subcutaneous) were 0.728 ± 0.0250, 0.483 ± 0.007 and 0.808 ± 0.016, respectively. At a flow-rate of 2.5 μl/min, the mean value of recovery for TMP of microdialysis probes (blood and brain) were 0.446 ± 0.010 and 0.239 ± 0.015, respectively. Recovery with dialysis membrane of 10 mm length was much higher than those of 4 mm length. Recovery with flow-rate of 2.5 μl/min was lower than those of 1.0 μl/min. The microdialysis recoveries of TMP were independent of TMP concentrations according to our previous research (Xu et al., 2012). So we used 4 μg/ml perfusate solution in this experiment.

3.3. In vivo pharmacokinetics study of TMP

The concentration-time profiles in plasma and dialysates (brain, blood and skin) following transdermal (40 mg/kg) or i.p. administration (20 mg/cm^2, 1.2 cm^2) of TMP were presented in Tables 1 and 2 and Fig. 1. After i.p. injection, the TMP peak concentration in dialysate appeared at about 18 min and then reduced gradually, while T_{max} in plasma was about 27 min. After 300 min, the concentration of the drug was almost undetectable. The drug concentrations of plasma were higher than those of microdialysates (from brain and vein). There were significant differences in $AUC_{(0-t)}$ between microdialysates (brain and blood) and plasma ($P < 0.05$). After transdermal administration, the TMP peak concentration in dialysates (brain and blood) and plasma appeared at about 2.5 h and then reduced slowly, while the maximum value was about 3.5 h for skin microdialysates. The drug concentrations of skin microdialysates were much higher than those of microdialysates (from brain and vein). There were significant differences in $AUC_{(0-t)}$ and C_{max} between microdialysates (brain and blood) and skin ($P < 0.05$).

4. Discussion

It is important to quantify the drug which has central action in brain and study its neuropharmacokinetics. AUC_{brain}/AUC_{blood} is used to describe the brain penetration (de Lange et al., 1997; Tsai, 2001; Tsai and Liang, 2001). In this study, the AUC_{brain}/AUC_{blood} of unbound TMP in brain to in blood was 68.32 ± 8.22% after i.p. administration (40 mg/kg), and the value was up to 77.69 ± 13.91% after transdermal administration (20 mg/cm^2, 1.2 cm^2) which was a little higher. The results of this experiment suggested that TMP could penetrate the blood brain barrier (BBB) readily, in accordance with the earlier researcher (Tsai and Liang, 2001). But in their study, the AUC_{brain}/AUC_{blood} was much higher than our result, which maybe due to different method of administration or different dosage administration. The AUC of TMP in skin was 13358.65 ± 8931.65 mg/l·min, which was much higher than the unbound drug in brain and blood, and AUC_{skin}/AUC_{blood} was 607.28 ± 368.14%. The high level of TMP in skin indicated the characteristics of transdermal drug delivery system. TMP permeate the skin into the bloodstream then to brain after transdermal administration, so the concentration of drug $AUC_{skin} > AUC_{blood} > AUC_{brain}$. The AUC of plasma was higher than that of microdialysate (blood or brain), which is because protein-binding drug cannot permeate

Table 2
The non-compartmental pharmacokinetic parameters for TMP in rat plasma, blood, brain and skin microdialysates following transdemal administration (20 mg/cm^2, 1.2 cm^2) in rats (mean ± SD, $n = 7$).

Parameter	Brain	Blood	Skin	Plasma
$AUC_{(0-t)}$ (mg/l·min)	1893.20 ± 463.59**	2449.68 ± 441.04**	13358.65 ± 8931.65*	4483.53 ± 694.56
$t_{1/2z}$ (min)	147.85 ± 89.14	127.00 ± 29.23	188.56 ± 82.10	138.12 ± 41.63
T_{max} (min)	169.29 ± 40.36	152.14 ± 29.28	225.00 ± 174.93	162.86 ± 41.63
CLz/F (l/min/kg)	0.041 ± 0.0090**	0.031 ± 0.0060**	0.0070 ± 0.0050**	0.017 ± 0.0020
Vz/F (l/kg)	8.92 ± 5.87*	5.75 ± 2.12*	1.47 ± 0.51**	3.42 ± 1.35
C_{max} (mg/l)	7.29 ± 2.65**	8.53 ± 1.98**	43.39 ± 29.57*	15.50 ± 2.99
$AUC_{brain/blood}$ (%)	77.69 ± 13.91			
$AUC_{brain/plasma}$ (%)	41.81 ± 4.58			
$AUC_{blood/plasma}$ (%)	54.93 ± 8.90			

* $P < 0.05$ compared with plasma.
** $P < 0.01$ compared with plasma.

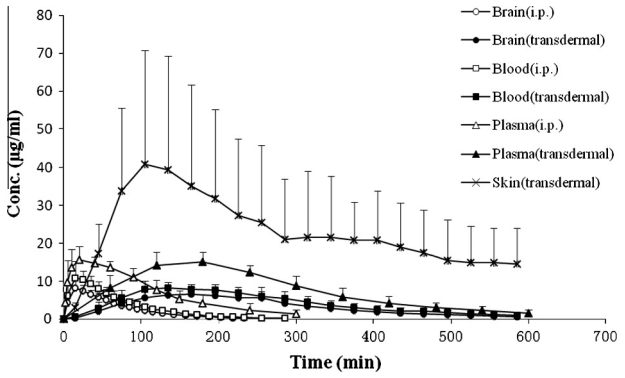

Fig. 1. The concentration versus time profiles of TMP in rat plasma, blood, brain and skin microdialysates after i.p. administration (40 mg/kg) or transdermal administration (20 mg/cm^2, 1.2 cm^2) in rats (mean ± SD, n = 7).

the microdialysis membrane and only free drug can be measured. Pharmacologic action is related with the concentration of free drug, so detecting the concentration of free drug is more meaningful.

In order to improve the safety and bioavailability of TMP and to avoid the first-pass hepatic effect of oral administration, the transdermal drug-delivery system has been studied (Liu et al., 2011; Zhao et al., 2011). The area administration of gel was only one fifth when compared to micro-emulsion patch on which a mean amount of 10 mg TMP was applied to 6 cm^2 skin area (Zhao et al., 2011). And after micro-emulsion administrated, the TMP concentrations in rat brain homogenate and plasma were all below 0.5 μg/ml, while in our study the drug concentration could be achieved to 7 μg/ml after gel administration. In another transdermal drug-delivery system study (Liu et al., 2011), the concentration of the drug was adjusted to 10 g/l, and the dose of TMP administered was 100 mg/kg. For the sake of administration dosage, it must need a large area to administer. So our preparation gel was simple and convenient to application, and could provide higher concentrations *in vivo* while smaller area administration compared with those two transdermal preparations. In pharmacodynamics and pharmacology research, many researchers found that TMP could protect the brain against ischemic insult as evidenced by the reduction in infarction volume, preservation of neurons, and decrease in brain edema. TMP administered before occlusion can significantly reduce the infarct volume (Liao et al., 2004; Zhu et al., 2009). According to earlier reports, the doses level of TMP were focused on 10 mg/kg and 4 mg/kg (Tsai and Liang, 2001; Wang et al., 2011; Yan et al., 2012; Lai et al., 2011), but the treatment dose was about 40 mg/kg. So it was meaningful to study the dose of 40 mg/kg. In order to achieve the same C_{max} level *in vivo*, we adopted the corresponding gel. The C_{max} in brain microdialysate and plasma were no significant difference between i.p. administration and transdermal administration. But C_{max} in blood microdialysate had a little significant difference between the two groups (P = 0.032), maybe more numbers of animals could eliminate the difference.

In conclusion, the present study showed that it was feasible to simultaneously sampling TMP from different sites (brain, blood and skin) using three microdialysis probes. The C_{max} in brain and plasma after transdermal administration (20 mg/cm^2, 1.2 cm^2) was similar to that after i.p. administration (40 mg/kg). Compared with traditional administration, gel transdermal administration is a promising alternative to transport TMP to the brain. There is also a need for further research in awake animals because sampling time-limited when animals under anesthesia. The microdialysis technique can be used to investigate pharmacokinetics of TMP, and provide valuable pharmacokinetic parameters for clinical applications.

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