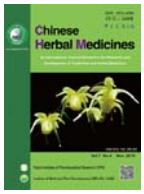

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Original article

Pharmacokinetics of Tetramethylpyrazine Hydrochloride in Rabbits Blood after Intranasal Administration

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ABSTRACT

Objective To study the pharmacokinetic behavior of tetramethylpyrazine hydrochloride (TMPH) in plasma of rabbits after intranasal administration and the relationship between absorption and dosage, furthermore, to illustrate the effects of borneol and musk used in combination with TMPH on the plasma concentration profile of TMPH in rabbits. **Methods** The concentration of TMPH was determined by RP-HPLC method. Coumarin was used as an internal standard. Sample preparation was carried out by extraction and precipitation with methanol. The pharmacokinetic parameters were computed by software program DAS.3.1.4. **Results** Blood pharmacokinetics of TMPH fitted best to a non-compartment model. After intranasal administration with single dose at 10, 20, and 40 mg/kg of TMPH, the average values of C_{max} were 8.075, 16.537, and 33.115 μg/mL, and the average values of AUC_{0-t} were 228.93, 399.273, and 728.917 mg/(L·min), respectively. C_{max} of TMPH in plasma was increased by 31.136% and 38.786% compared with those without borneol and musk, and intranasal bioavailability were increased by 21.587% and 40.633% after intranasal administration of TMPH in combination with borneol, or with borneol and musk. **Conclusion** Borneol and musk could enhance the intranasal absorption of TMPH and increase the concentration of TMPH in blood of rabbits, especially in the early period. This work also shows the rational compatibility between borneol and musk.

Key words

borneol; intranasal administration; musk; pharmacokinetics; tetramethylpyrazine hydrochloride

1. Introduction

Tetramethylpyrazine (TMP), also called ligustrazine, is a biologically active ingredient isolated from the traditional herbal medicine *Ligusticum chuanxiong* Hort (Pang et al, 1996). TMP can help to promote hematopoietic function, protect vascular endothelium, inhibit platelet aggregation, and improve microcirculation, etc (Sun et al, 2002; Yin et al, 2002; Wang et al, 2000; Li et al, 2001). It has been widely used in China for the treatment of patients with angina pectoris, ischemic

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vascular diseases, and gastric lesion (Liu et al, 1990; Sheu et al, 1997; Wan et al, 1998). Therefore, the determination of tetramethylpyrazine hydrochloride (TMPH) in preparations and blood samples has clinical applications.

Borneol is a kind of Chinese materia medica (CMM). It has been commonly used for its antibacterial, analgesic, and antiinflammatory effects and also can promote drug transdermal absorption and permeability through the blood-brain barrier and improve the bioavailability (Lu and Lin, 2002). Musk is from dry secretion of mature males of Cervidae animals such as *Moschus berezovskii* F., *M. sifancus* P., and *M. moschiferu* L. (Yu et al, 1989). It has the effects of inducing resuscitation, activating blood circulation, reducing swelling, and alleviating pain. Modern research has shown that musk has obvious effects on central nervous system and cardiovascular system (Chen and Gao, 1981). HP-β-CD is a kind of pharmaceutic adjuvant with good stability, security, low surface activity, and hemolysis activity. It is mainly used to increase the water-solubility of insoluble drugs and drug stability, improve bioavailability, reduce the adverse reactions of drugs, etc (Wang et al, 2007). Due to the low boiling point, sublimation at room temperature, and instability during the storage period, borneol and musk were used in the combination with HP-β-CD to improve the efficacy.

The protective effects of TMP on cognitive function are of significant importance for its clinical application. However, TMP of oral dosing has the specific pharmacokinetic characteristics with short biological half-life of 0.5–2 h and low oral bioavailability of 10%–30% (Feng et al, 2009). In recent years, systemic drug delivery through the nasal route has received considerable attention. Intranasal administration not only can be used for the treatment of nasal cavity, but also for the treatment and prevention of systemic diseases (Chen, 2007). Drug is rapidly absorbed into the systemic circulation through nasal mucosa after the intranasal administration. The intranasal administration offers some advantages including rapid absorption, avoidance of hepatic first-pass metabolism, and preferential drug delivery to brain via the olfactory region. It also can avoid the destruction of the drug caused by gastrointestinal digestive juices, which is helpful to improve the bioavailability of drugs (Xu and Wei, 2004).

Several studies have reported quantitive methods and pharmacokinetic studies on TMP. The pharmacokinetics of TMPH following intranasal administration also has been investigated using the brain micro-dialysis technique (Wang et al, 2004; Liang et al, 1999; Tsai and Liang, 2001; Xiao et al, 2007; Li et al, 2006; Xu et al, 2013; Meng et al, 2014). However, the method of RP-HPLC for the determination of TMPH in plasma of rabbit after intranasal administration has not been obviously reported. Herein, we presented a sensitive and rapid RP-HPLC method to determine the concentration of TMPH in plasma of rabbit after intranasal administration, and furthermore, we researched the intranasal absorption and pharmacokinetics of TMPH for better clinical actual needs. And the rationality of the compatibility of borneol and musk can promote the development of TMP in compound CMM.

2. Materials and methods

2.1 Chemicals and regents

Tetramethylpyrazine hydrochloride reference standard (TMPH, purity of 99%), borneol, and musk were purchased from Nanjing Zelang Medical Technology Co., Ltd. (China). HP-β-CD was purchased from Shandong Xinda Biological Technology Co., Ltd. (China). Coumarin (internal standard, purity of 99%) was purchased from Tianjin Chemical Reagent Factory. Methanol was from Tianjin Concord Science and Technology Co., Ltd. (China). Acetic acid was purchased from Tianjin Kaixin Chemical Industry Co., Ltd. (China). All other chemicals and reagents were of analytical grade.

2.2 Preparation of standard solutions and quality control samples

A standard stock solution of TMPH was prepared by dissolving 12.51 mg TMPH in 25 mL methanol and was further diluted into 1.0008, 5.004, 10.008, 25.02, 50.04, 100.08, and 150.12 μg/mL for the preparation of plasma calibration standards. The coumarin IS (internal standard) solution was prepared in methanol at the concentration of 14.864 μg/mL. All solutions were stored at −20 ^{o}C in dark glass. Pure standards prepared for the recovery calculations were diluted with methanol to appropriate concentration.

Calibration standards of TMPH with the concentration of 0.1112, 0.556, 1.112, 2.78, 5.56, 11.12, and 16.68 μg/mL were prepared by spiking appropriate amount of TMPH standard solutions in blank plasma. Quality control (QC) samples were prepared using the pooled plasma at low-, mid-, and high- concentration for TMPH, and we choose the concentration of 1.112, 5.56, and 11.12 μg/mL, respectively. The spiked samples were treated following the sample preparation procedure as indicated in section 2.5.

Combination A represented that TMPH was combined with borneol, and it was prepared as follows: borneol (200 mg) was dissolved with moderate anhydrous ethanol; HP-β-CD (2 g) was dissolved with water on magnetism miser at 40 ^{o}C; The borneol ethanol solution was added into HP-β-CD solution slowly and the mixture was stirred continuously for 6 h to obtain the borneol-HP-β-CD inclusion complex. And a certain amount of TMPH was dissolved in the mixture.

Combination B represented that TMPH was combined with borneol and musk, and it was prepared as follows: borneol (200 mg) and musk (100 mg) were dissolved with moderate anhydrous ethanol. HP-β-CD (2 g) was dissolved with water on magnetism miser at 40 ^{o}C. The ethanol solution was added into HP-β-CD solution slowly and the mixture was stirred continuously for 6 h to obtain the inclusion complex. And the same amount of TMPH was dissolved in the mixture.

2.3 Instruments and chromatographic conditions

Agilent 1100 HPLC System, XW−80A Turbine Mixer (Shanghai Qite Analytical Instrument Co., Ltd.), 1.5 mL

graduated centrifuge tubes, power centrifugal instrument (Beijing Jingli centrifuge Co., Ltd.), QL−901 Vortex Mixter (Qilinbeier Instrument Manufacturing Co., Ltd.), pipette (200 μL, Dragon Lab), AB204−N Analytical Balance (Metter Toledo Technology Co., Ltd.), and ultrasound cleaner (CBL photoelectron technology, 3860A) were used.

Separation was carried out using a Diamonsil C_{18} reverse phase column (250 mm × 4.6 mm, 5μm) at a flow rate of 1.0 mL/min. Chromatography was performed at 30 °C. TMPH was monitored at a wavelength of 280 nm with coumarin as IS, and injection volume was 20 μL. Mixture of methanol and 0.5% acetic acid solution (35:65) was used as mobile phase.

2.4 Animals

New Zealand rabbits [(2.5 ± 0.5) kg body weight] were obtained from Tianjin Chunle Experimental Animal Farms. In this study, 30 rabbits were used in the intranasal application with half males and half females. All the animals were clinically healthy and haematologically and biochemically normal throughout the experimental period. All rabbits were fasted for 12 h and with free access to water before drug administration. All the experiments were performed according to the guidelines for the care and use of animals as established by Tianjin Institute of Pharmaceutical Research.

2.5 Plasma sample preparation

Plasma sample was processed with the following steps: A 200 μL aliquot of blank plasma sample was transferred to a 1.5 mL polyethylene centrifuge tube. The standard solution (50 μL), IS solution (100 μL, 14.864 μg/mL) and methanol (100 μL) were added. And the mixture was vortexed for 2 min for fully extraction and deproteinization, followed by centrifugation for 15 min at 10 000 r/min. The supernatant (20 μL) was directly injected into the HPLC system for analysis.

2.6 Validation of RP-HPLC method

Validation runs were conducted on separate 5 d. Each validation run consisted of a series of the spiked standard samples at seven concentration over the concentration range (each in quintuplicate) and quality control (QC) samples at three concentration (five replicates of three different concentration). Standard samples were analyzed at the beginning of each validation run and other samples were distributed randomly throughout the run. The results from QC samples in three runs were used to evaluate the accuracy and precision of the method developed. To determine intra-day precision, samples were analyzed on the same day. To determine inter-day precision, samples (at three different concentration) were analyzed on days 1, 2, 3, 4, and 5 after using a daily calibration curve. The concentration of the analytes in plasma samples was determined by back-calculation of the observed peak area ratios of the analytes and IS from the best-fit calibration curve using a weighted ($1/x^2$) linear regression. During routine analysis, each analytical run included a set of standard samples, a set of QC samples in duplicate and plasma samples were determined.

The selectivity of the method was investigated by comparing chromatograms of blank plasma, plasma sample spiked with TMPH standard solution (50.04 μg/mL) and IS solution (14.864 μg/mL), and plasma sample at 10 min after intranasal administration of TMPH at a dose of 20 mg/kg.

The linearity of each calibration curve was determined by plotting the peak-area ratio (y) of the analyte to IS versus the nominal concentration (x) of TMPH. The calibration curves were obtained by weighted ($1/x^2$) linear regression analysis.

The extraction recoveries of TMPH were determined at low-, mid-, and high- concentration by comparing the peak areas of the standard samples prepared from plasma with those of the standard solutions.

The stability of TMPH was investigated after storing spiked plasma samples with the concentration of 1.112, 5.56, and 11.12 μg/mL for TMPH at 4 °C for 1, 3, and 7 d at −20 °C for one month.

2.7 Pharmacokinetic study of TMPH by intranasal administration

Thirty rabbits were randomly divided into five groups with half males and half females and fasted for 12 h, but allowed to take water freely. The rabbits in three groups were intranasally administered with TMPH at the doses of 10, 20, and 40 mg/kg dissolved in normal saline respectively. The rabbits in the other two groups were intranasally administered with the combination A (20 mg/kg TMPH) and combination B (20 mg/kg TMPH), separately. Blood samples were collected at the predetermined time points 1, 5, 10, 15, 30, 45, 60, 90, and 120 min. The blood sample of 0.5 mL was taken from marginal ear vein of rabbits after the intranasal administration. The plasma concentration of TMPH was determined in the same HPLC method.

2.8 Pharmacokinetic data analysis

Peak concentration (C_{max}) and peak time (t_{max}) of TMPH were derived from the experimental points directly. The other pharmacokinetic parameters were fitted by DAS.3.1.4 pharmacokinetic program. The plasma concentration of TMPH versus time profiles was described by the software origin 8.5.

3. Results

3.1 Selectivity

The selectivity of the method was investigated by comparing the chromatograms of blank plasma, standard plasma samples spiked with TMPH (5.56 μg/mL) and plasma sample at 10 min after intranasal administration of TMPH (20 mg/kg). The results showed that TMPH and coumarin were well separated without interference of endogenous substance in plasma. The retention time for TMPH and coumarin was 16.067 and 23.443 min, respectively (Figure 1). Coumarin

can be separated with other endogenous impurities completely, $R > 1.5$, without significant interference peaks.

3.2 Linearity, detection limit, and LOQ

The calibration for TMPH in plasma was linear in the range of 0.1112−16.68 μg/mL. The regression equation for calibration curve of TMPH was y = 0.172 9 x + 0.005 2 (correlation coefficient, r = 0.999 9). The detection limit for TMPH, defined as a signal-to-noise ratio of 3:1, was 11.12 ng/mL in plasma of rabbits. The limit of quantification (LOQ) was defined as the lowest drug concentration, which could be determined with a intra-day relative standard deviation (RSD) ≤ 20%. The LOQ was estimated as 55.6 ng/mL for TMPH in plasma of rabbits.

3.3 Precision and accuracy

The accuracy and precision of the method were evaluated by analyzing plasma samples spiked with three different concentration of TMPH (1.112, 5.56, and 11.12 μg/mL), added with 100 μL of IS solution (14.864 μg/mL) in three validation runs. The accuracy was determined by calculating the percentage deviation observed in the analysis of QC samples and expressed in the relative error (RE). The intra-day and inter-day precision was expressed as RSD. As shown in Table 1, the intra-day and inter-day precision (RSD) of TMPH was within 2.07%, and the accuracy (RE) of TMPH was within ± 4.32%, indicating the acceptable accuracy and precision of the method developed.

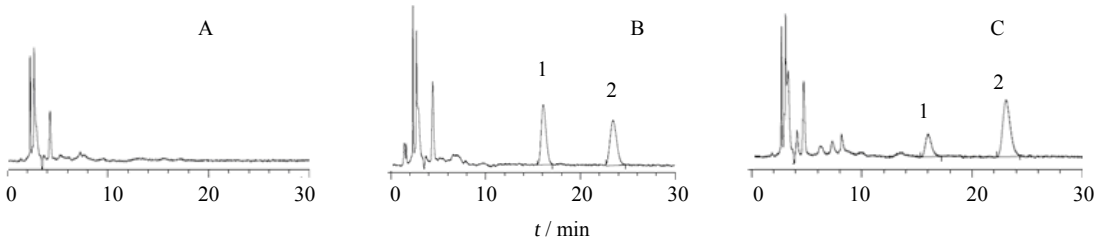

Figure 1 Representative chromatograms of blank rabbit plasma (A), blank rabbit plasma spiked with TMPH and its IS (B), and rabbit plasma sample at 10 min after intranasal administration of TMPH (C)

1: TMPH; 2: coumarin (IS)

Table 1 Precision and accuracy of TMPH in rabbit plasma (n = 5)

Parameters	Nominal concentration / (μg·mL^{-1})		
	1.112	5.56	11.12
Intra-day assay			
Mean	1.16	5.67	11.20
SD	0.022	0.041	0.163
RSD / %[a]	1.89	0.72	1.45
RE / %[b]	4.32	1.98	0.72
Inter-day assay			
Mean	1.16	5.49	11.06
SD	0.024	0.096	0.110
RSD / %[a]	2.07	1.75	0.99
RE / %[b]	4.32	−1.26	−0.54

[a] RSD = (standard deviation) / (mean concentration), same as Table 2.

[b] RE = (mean concentration − nominal concentration) / nominal concentration

3.4 Extraction recovery

The data showed that the extraction recoveries from plasma of rabbits were (88.85 ± 0.36)%, (90.75 ± 1.02)%, and (90.91 ± 0.67)% at the concentration of 1.112, 5.56, and 11.12 μg/mL for TMPH (Table 2).

3.5 Stability

The RSD values of concentration following this storage period for TMPH were 0.29%, 0.33%, and 0.39%, indicating that the plasma samples containing TMPH were stable during the period.

Table 2 Extraction recoveries of TMPH in rabbit plasma ($\bar{x} \pm s$, n = 5)

Concentration / (μg·mL^{-1})	Extraction recoveries / %	RSD / %[a]
1.112	88.85 ± 0.36	0.37
5.56	90.75 ± 1.02	0.96
11.12	90.91 ± 0.67	0.61

3.6 Absorption properties of TMPH at different doses by intranasal administration

To determine the pharmacokinetic parameters of TMPH, the concentration-time data were analyzed with a non-compartmental approach using DAS.3.1.4 procedure. The mean plasma concentration-time profiles of TMPH for each dosing regimen were shown in Figures 2 and 3. The pharmacokinetic parameters were presented in Table 3.

As shown in Table 3, TMPH was rapidly absorbed in rabbits when the drug was given by intranasal administration, with MRT of 32.031, 33.061, and 32.774 min, respectively. The C_{max} values after intranasal administration of single doses at 10, 20, and 40 mg/kg were 8.075, 16.537, and 33.115 μg/mL, respectively; The corresponding AUC_{0-t} values were 228.93, 399.273, and 728.917 mg/(L·min), respectively.

The linear regressions of ln(AUC_{0-t}) and ln(C_{max}) versus ln(dose) are shown in Figures 4 and 5. The equation for ln(AUC_{0-t}) versus ln(dose) was y = 3.497 + 0.836x (r = 0.991) ranging from 5.326 to 6.667 and the equation for ln(C_{max}) versus ln(dose) was y = −0.258 + 1.019x (r = 0.992) ranging from 1.938 to 3.609.

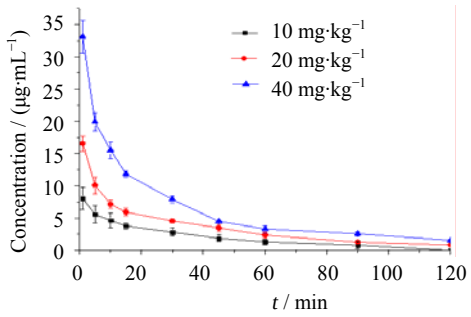

Figure 2 Plasma concentration profiles for TMPH in rabbits after intranasal administration (n = 6)

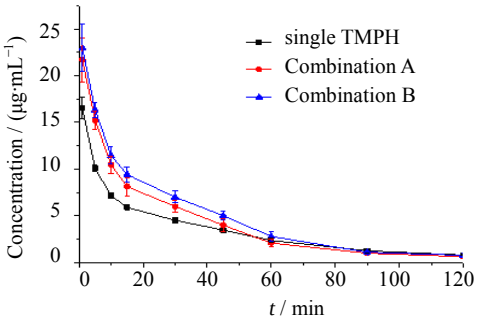

Figure 3 Plasma concentration profile in rabbits after intranasal administration of single TMPH, Combinations A and B (n = 6)

Table 3 Main pharmacokinetic parameters of TMPH in rabbits after intranasal administration at doses of 10, 20, and 40 mg/kg (n = 6)

Parameters	Units	Intranasal administration /(mg·kg^{-1})		
		10	20	40
C_{max}	mg/L	8.075 ± 0.308	16.537 ± 0.480	33.115 ± 1.043
t_{max}	min	1	1	1
AUC $_{(0-t)}$	mg/(L·min)	228.930 ± 6.384	399.273 ± 12.991	728.917 ± 16.065
AUMC$_{(0-t)}$		7333.888 ± 220.931	13 259.712 ± 794.661	23 881.796 ± 474.709
MRT$_{(0-t)}$	min	32.031 ± 0.238	33.061 ± 0.927	32.774 ± 0.198
VRT$_{(0-t)}$	min^2	727.604 ± 5.581	820.192 ± 54.461	997.942 ± 1.868
range	min	1−120	1−120	1−120

3.7 Pharmacokinetic properties of TMPH in Combination A and B

The mean plasma profile of TMPH in rabbits after intranasal administration was shown in Figure 5. The results showed that the average value of C_{max} was 16.537 μg/mL after intranasal administration of single TMPH solution with t_{max} at about 1 min. However, after intranasal administration of Combinations A and B, the mean values of C_{max} were 21.686 and 22.951 μg/mL with t_{max} at about 1 min. The mean values of AUC_{0-t} were 399.273, 485.465, and 561.511 mg/(L·min) after intranasal administration of single TMPH, Combinations A and B, respectively. The bioavailability (F) was calculated from the following equation: relative bioavailability = $AUC_t \times D_r / AUC_r \times D_t$, where t represents test formulation, r represents reference formulation and D represents dose of administration.

The other pharmacokinetic parameters were obtained by DAS.3.1.4 procedure (Table 4).

4. Discussion

Sample preparation plays a key role in the biological samples for the determination of drugs. At the beginning of this work, dichloromethane, ethyl acetate, and acetonitrile were tried and the extraction rate of TMPH was low. During the experiment, we tried to concentrate the organic phase under the protection of nitrogen in water bath at 40 ºC, but we found that the drying process in the extraction caused a significant loss of the TMPH due to its volatility. After several

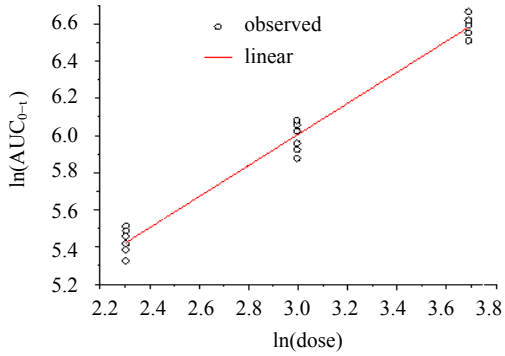

Figure 4 Linear regression profile of ln(AUC_{0-t}) versus ln(dose) in rabbits after intranasal administration of TMPH

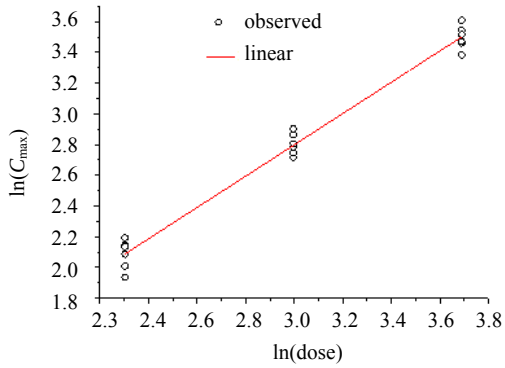

Figure 5 Linear regression profile of ln(C_{max}) versus ln(dose) in rabbits after intranasal administration of TMPH

Table 4 Pharmacokinetic parameters of plasma after intranasal administration of single TMPH, Combinations A and B with non-model in rabbits ($\bar{x} \pm s$)

Parameters	Unit	Single TMPH	Combination A	Combination B
$AUC_{(0-t)}$	$mg \cdot L^{-1} \cdot min^{-1}$	399.273 ± 12.991	485.465 ±25.492**	561.511 ± 22.907*
$AUMC_{(0-t)}$		13259.712 ± 794.661	13285.174 ± 1000.833	15999.788 ± 940.560
$MRT_{(0-t)}$	min	33.061 ± 0.927	27.210 ± 0.610*	28.399 ± 0.549*
C_{max}	$mg \cdot L^{-1}$	16.537 ± 0.480	21.686 ± 0.976*	22.951 ± 1.039*
t_{max}	min	1	1	1
$VRT_{(0-t)}$	min^2	820.192 ± 54.461	613.300 ± 28.95**	612.278 ± 29.409**
$t_{1/2z}$	min	31.958 ± 1.731	28.790 ± 0.932	20.707 ± 0.558*
V_z/F	$L \cdot kg^{-1}$	2136.118 ± 32.812	1377.303 ± 31.098*	1028.094 ± 29.202*
CL_z/F	$L \cdot min^{-1} \cdot kg^{-1}$	46.833 ± 1.951	41.507 ± 2.328	35.305 ± 1.528*
F	%	−	121.587	140.633

CL = clearance; AUC = area under the concentration curve; *$P < 0.05$, **$P < 0.01$ *vs* single TMPH

trials, a protein precipitation procedure was adopted and proved to be simple and reliable for the sample preparation in this work. Methanol was selected as extract and protein-precipitating agent to produce the expected peak shapes of analytes. IS is necessary for the determination of the analytes in biological samples and the determination of TMPH. Under the chromatographic conditions, ideal IS peak should close to the analyte peak as much as possible, and the IS with each component should separate sufficiently. In the initial stage of our work, several compounds were tried and finally coumarin was found to be the optimal IS. The plasma sample was directly injected for determination after precipitating protein with methanol, and the method was proved to be useful for the sample preparation in this work.

The analytical method described above was a rapid and sensitive HPLC assay for the quantification of TMPH in plasma of rabbits after intranasal administration of TMPH, and is useful for its pharmacokinetic studies. The method showed good overall linearity, detection limit, LOQ, recovery, accuracy, precision, and stability of TMPH.

C_{max} varied due to the different dosages of 10, 20, and 40 mg/kg by intranasal administration, but t_{max} of TMPH was almost the same, which peaked at 1 min, suggesting that TMPH was absorbed rapidly after the administration. MRT_{0-t} of TMPH had no significant difference after intranasal administration of single TMPH with different doses, which indicated that the dosage did not change the absorption of TMPH *in vivo*. Linear regression analysis of three TMPH doses showed that increase in absorption with increasing dose did not agree with that expected under the conditions of strict proportionality. The ratio of C_{max} (4.101:2.048:1) of TMPH at different doses was basic linearly related to the ratio of drug dosage (4:2:1). The ratio of AUC_{0-t} (3.171:1.95744:1) of TMPH at different doses was not obvious linearly related to the ratio of drug dosage (4:2:1) and we might speculate the AUC_{0-t} of TMPH is not linear at a range of 10−40 mg/kg. So we could conclude that a linear response to TMPH was not obvious at a higher dose.

C_{max} values of TMPH in plasma were increased by 31.136% and 38.786% than those without borneol and musk after intranasally administering Combinations A and B separately. The intranasal bio-availabilities were increased by 21.587% and 40.633% after intranasal administration of Combinations A and B more than single TMPH saline solution. The results demonstrated that both borneol and musk could enhance the absorption of TMPH. In this study, t_{max} of TMPH in plasma of rabbits remained the same, but MRT_{0-t} of TMPH were 33.061, 27.210, and 28.399 min after intranasal administration of single TMPH, Combinations A and B, respectively, which showed borneol and musk accelerated the absorption of TMPH *in vivo*.

5. Conclusion

The analytical method described above is validated to meet the requirement of pharmaceutical investigation of TMPH. The pharmacokinetic results show the relationship between absorption and dosage *in vivo*, providing the value information for studying borneol and musk used as compatibility agent in preparations containing TMPH and also indicates the potential use of borneol and musk in other drugs. The results show that TMPH used in the combination of borneol and musk is reasonable. It is also useful to provide a basis for evaluating the clinical effect of TMPH and for the development of TMP in compound CMM. Intranasal delivery is an available way, and TMPH used concomitantly with borneol and musk can achieve rapid drug absorption through nasal drug delivery. And it is suitable for the emergency treatment of acute diseases such as heart disease and cerebral thrombosi. So compatibility of these drugs has a good clinical significance.

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