Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 253 (2003) 177–183
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Physicochemical characterization and percutaneous delivery of 2,3,5,6-tetramethylpyrazine

Xiaohong Qi [a,*], Chrisita Ackermann [b], Duxin Sun [c], Minli Sheng [d], Huimin Hou [e]

[a] *Global Product Development, Bristol-Myers Squibb Company, Worldwide Consumer Medicines, 1350 Liberty Avenue, Hillside, NJ 07111, USA*
[b] *College of Pharmacy, University of Michigan, 428 Church Street, Ann Arbor, MI 48109-1065, USA*
[c] *Pharmaceutical Research Institute, Bristol-Myers Squibb Company, New Brunswick, NJ 08903, USA*
[d] *Medical Radiology Research Institute, School of Medicine, Fudan University, Yixueyuan Road, Shanghai 200032, China*
[e] *National Pharmaceutical Engineering Research Center, Shanghai Institute of Industrial Pharmaceutics, Zhongshanbeiyi Road, No. 1111, Shanghai 200437, China*

Received 12 July 2002; received in revised form 18 December 2002; accepted 19 December 2002

Abstract

The objective of this work is to investigate the percutaneous permeability of 2,3,5,6-tetramethylpyrazine (TMP), an active ingredient originally isolated from *Ligusticum wallichii* Franch. Certain physicochemical properties of TMP, including its partition coefficient and pH–solubility profile, were studied. The influence of pH on the percutaneous permeation of TMP was studied in vitro using hairless mouse skin. Comparative in vitro permeability of TMP through hairless mouse, rat, rabbit, and human cadaver skin was also investigated. The results indicate that hairless mouse skin and rat skin were about three to four times more permeable to TMP than human cadaver skin. The permeability of TMP through rabbit skin was not significantly different from that of human cadaver skin. The observed lag times for all skin membranes were about 1–2 h. Although pharmacokinetic data are not currently available to permit precise calculation of a clinically effective patch size, the data from this study indicate that the transdermal delivery of TMP should nevertheless be possible.

Keywords: Transdermal delivery; Tetramethylpyrazine; *Ligusticum wallichii* Franch; Skin permeability; Traditional Chinese herbal medicines

1. Introduction

2,3,5,6-tetramethylpyrazine (TMP) is a biologically active ingredient originally isolated from *Ligusticum wallichii* Franch in 1957 and currently used in China for the treatment of cardiovascular disease (Guo et al., 1983). It was found to have significant therapeutic activity, including improving brain microcirculation, inhibiting thrombus formation, decreasing platelet aggregation, and improving blood viscosity (Watanabe, 1997; Chang et al., 1999). It was reported recently that TMP has appreciable blood–brain barrier (BBB) penetrability (Liang et al., 1999; Tsai and Liang, 2001).

After oral administration, TMP reached peak plasma concentrations within about 0.25–0.5 h. It possesses low oral bioavailibility (10–30%) due to hepatic first pass metabolism and has a short biological half-life of 0.5–2 h. (Cai et al., 1989). TMP

* Corresponding author. Tel.: +1-908-851-6279; fax: +1-908-851-6280.
E-mail address: xiaohong.qi@bms.com (X. Qi).

0378-5173/03/$ – see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0378-5173(03)00002-4

can be given orally 100 mg three times daily. In order to maintain therapeutic blood levels for long periods, intravenous infusion is needed, e.g. TMP hydrochloride injection (40 mg/2 ml) or TMP phosphate injection (50 mg/2 ml) is diluted with 250–500 ml of isotonic saline or glucose solution and intravenously infused for 4–6 h. For maintenance therapy, controlled release by way of an alternative route may be more suitable since frequent oral dosing virtually guarantees poor patient compliance and intravenous infusion is invasive and physically constraining.

Ligusticum wallichii Franch, the herbal medicine from which TMP was originally isolated, is a primary component in several Chinese medicated plasters for local and systematic pharmacological actions (Jia, 1957; Wang, 1981). The healing power of *L. wallichii* Franch contained in Chinese medicated plasters lead to this study of the potential percutaneous permeability of TMP. While there is no doubt about the effectiveness of these plasters, little support from basic scientific data is available relative to their use. This contrasts sharply with Western medicine, which emphasizes a therapeutic system based on defined scientific rules and technology (Bensky and Gamble, 1993; Huang and Williams, 1993). Western successes in medicinal chemistry, structure-activity studies, pharmacokinetics, and pharmaceutical technology have opened an avenue to the development of better and more effective drugs or drug delivery devices for Chinese medicines (Chen, 1991).

As a first step in a series of investigations on the percutaneous permeability of *L. wallichii* Franch, this study is focused on TMP, one of its active ingredients, due to its reported penetrability through BBB and seemingly suitable pharmacokinetic properties for transdermal delivery. The results of physicochemical characterization and in vitro skin permeation studies and the comparison of TMP permeation through hairless mouse, rat, rabbit, and human cadaver skin are reported in this study.

2. Materials and methods

2.1. Materials

TMP phosphate was obtained from Liming Pharmaceutical Company (Guangzhou, China). All other chemicals used were of reagent grade. Hairless mice (Kuming Strain) (10–20 g), Sprague–Dawley (SD) rats (250–300 g), and New Zealand rabbits (2.5–3.0 kg) were obtained from the Animal Center of Shanghai Institute of Industrial Pharmaceutics (Shanghai, China). Human breast skin was provided by Ruijin Hospital, Shanghai Second Medical University (Shanghai, China).

Modified horizontal diffusion systems with a 2.5-ml capacity receiver compartment and a 2.54-cm^2 diffusion surface area were made by the Technical Support Department, Shanghai Institute of Industrial Pharmaceutics (Shanghai, China).

2.2. Preparation of TMP base

TMP base was prepared in our laboratory as follows. 2N KOH was added to an aqueous solution of TMP phosphate (approximately 0.5 g/ml) until reaching a pH of 11. The aqueous solution was saturated with sodium chloride and the free base was extracted into diethyl ether. The ether extract was dried over anhydrous sodium sulfate and then evaporated to dryness under reduced pressure using a rotary evaporator. The residue was dried overnight under reduced pressure. The purity of TMP was >99.8% as determined by thin layer chromatography and HPLC with UV detector, and by the sharpness of the endothermic peak in differential scanning calorimetry (DSC).

2.3. HPLC assay method

The assay of TMP was performed using a Shimazdu LC-10A liquid chromatography system equipped with a SPD-10A UV detector, and a Hewlett-Packard data module. A reversed phase YMG-C18 column (20 cm × 4.6 mm I.D.; Changchun Applied Chemistry Research Institute, China) was utilized as the analytical column and the temperature was maintained at 35 °C. The mobile phase consisted of 35% methanol and 65% of water adjusted to pH 5 with phosphoric acid. The flow rate was 1.0 ml/min. An injection volume of 10 μl was used; the detector wavelength was set at 280 nm. The chromatographic peak for TMP was detected at a retention time of 5.8 min. Calibration curves established excellent linearity over the concentration range of 0.01–100 μg/ml.

2.4. Determination of pH–solubility profiles of TMP

Solubility studies were conducted by adding excess amounts of TMP into screw capped vials containing 10 ml of aqueous solution or 0.05 M phosphate buffer-saline solutions of various pH (4.0–9.0) values. The pH was adjusted by adding either 0.1N HCl or 0.1N NaOH solution. The tightly sealed vials were shaken in a water bath set at 37 °C. Forty-eight hours were sufficient to ensure that the solutions were saturated.

The suspensions were allowed to settle for 24–48 h, then each suspension was filtered through a pre-warmed 0.8 μm filter (Shanghai membrane & Filter Company, Shanghai, China). The concentrations of TMP in the filtrates were determined by HPLC after appropriate dilution. The filtered saturated solutions at various pH conditions were used in permeation experiments to investigate the effect of pH on the flux of TMP through hairless mouse skin.

2.5. Determination of apparent partition coefficient ($k_{oct/water}$)

The apparent partition coefficient of TMP was determined using *n*-octanol and double distilled water. A known weight of the compound was allowed to partition between equal volumes of *n*-octanol and water placed in a screw-capped vial. The two-phase system was equilibrated in a constant temperature bath maintained at 37 °C. Under these conditions, equilibrium was reached within 24 h. After equilibration, the aqueous and oil phases were separated by centrifugation at 4000 rpm for 10 min. The concentrations of TMP in each phase were determined by HPLC. The apparent partition coefficient ($k_{\mathrm{oct/water}}$) was the ratio of C_{oct} to C_{water}.

2.6. Preparation of skin membranes

Animals were sacrificed either in a CO_2 chamber or by cervical dislocation. The abdominal skins were removed by blunt dissection. For rat and rabbit, the abdominal fur was removed using clippers. Human breast skin, obtained from Shanghai Second Medical University, was also used in the permeation experiments. All skin samples were cut and washed with water. Fat and connective tissue were carefully removed with a scalpel. Each skin specimen was inspected for damage using a magnifying lens. Freshly excised abdominal mouse skin was used in the pH–permeability experiments. Otherwise, the skin specimens were wrapped in plastic film and stored in a freezer at −20 °C until they were needed. The skin samples were thawed to room temperature before mounting them within the diffusion apparatus. All frozen skin samples were used within 1 month of their preparation.

2.7. In vitro skin permeation experiments

Permeation experiments were carried out using a modified side-by-side horizontal diffusion apparatus with a 2.54-cm^2 diffusion surface area. A piece of skin was mounted between the two half-cells of the horizontal diffusion system. The temperature of the cells and their contents was kept constant at 37 ± 0.5 °C throughout each experiment. One milliliter of drug solution was placed in the donor compartment, and 2.50 ml of water was then filled into the receiver half-cell. All of the receiver solution was withdrawn at predetermined time, and replaced with an equivalent volume of drug free medium. The samples were assayed for TMP using HPLC. Cumulative corrections were made to determine the total amount of TMP permeated at each time interval. The steady-state flux of TMP was then determined from the linear portion of the amount permeated versus time profile for each run, using the following equation:

$$J_{\mathrm{ss}} = \frac{\mathrm{d}Q/\mathrm{d}t}{A} = V\frac{\mathrm{d}C/\mathrm{d}t}{A} = K_{\mathrm{p}}\Delta C$$

where Q and C are the drug mass (mg) and concentration gradient (mg/cm^2), respectively, in the receiver phase. A is the diffusion area (cm^2), V is the receiver solution volume (ml), and t is time (min). The best-fit slopes of the apparent linear portions represent the steady-state flux values (J_{ss}) (mg/cm^2/h). K_{p} is the apparent permeability coefficient (cm/h). Lag time was determined by extrapolating the straight-line portion of each steady-state permeation curve to the time axis.

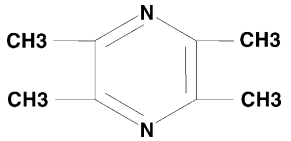

Fig. 1. Structure of 2,3,5,6,-tetramethylpyrazine (TMP).

3. Results and discussion

3.1. Physicochemical characterization of TMP

TMP, the structure of which appears in Fig. 1, has a molecular weight (MW) of 136.2. Its melting point is 76–78 °C and its solubility in water is 11 ± 2.4 mg/ml. The partition coefficient ($k_{oct/water}$) of TMP was found to be 123.5 ± 6.5, the $\log k_{oct/water}$ thus being 2.3. The compound is a highly symmetrical, aromatic base, having no active (H-bonding) hydrogen atoms.

3.2. Effect of pH on the skin permeation of TMP

Freshly excised abdominal mouse skin was utilized in the pH–permeability experiments.

Fig. 2 illustrates how the pH of aqueous buffers affected the solubility and skin permeability of TMP. TMP is a relatively weak base with a pK_{a_1} of 7 and pK_{a_2} of 10 in aqueous solution (Pilarski and Osmialowski, 1985). As expected for a weak base, the aqueous solubility decreased with increasing pH over the pH range of 4–9. It was found that at pH 4 and 9, there is about a 250% increase in concentration, while there was only a 26% increase in flux. These ratios are inconsistent with a permeability that is simply dependent on total aqueous solubility. Rather, they seem to speak to the stabilization of the free bioactivity at the partitioning interface as the result of the limit equilibrium. This means that the high concentration of TMP at lower pH values could functionally eliminate the boundary layer (hydrodynamic layer) that fronts the membrane. The more than 300 μg/cm^2/h permeation rate also made a sink condition difficult to maintain as pH decreased. We are suggested here that the pseudo steady-states were more and more affected by the applied concentration as the pH was raised, which could also add to an effect of 26% magnitude (Flynn, 2002, personal communication).

3.3. Comparative permeability of TMP through skin membranes

Fig. 3 shows the cumulative amounts of TMP as a function of time through four different skins; mouse, rat, rabbit, and human. In a previous study, using the horizontal diffusion system with 0.95 cm^2 of diffusion area and 5.0 ml of receiver phase, the steady-state flux

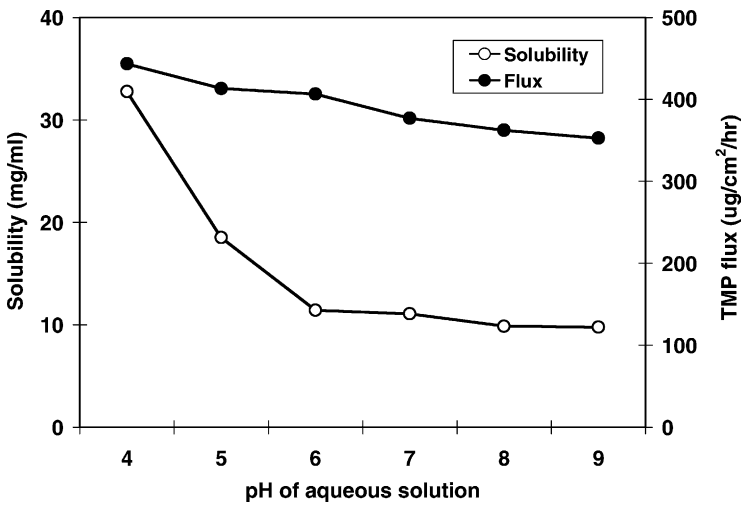

Fig. 2. pH dependence of the solubility and TMP's flux across hairless mouse skin from its saturated solutions. Each point represents the average of three measurements; the RSD did not exceed 5%.

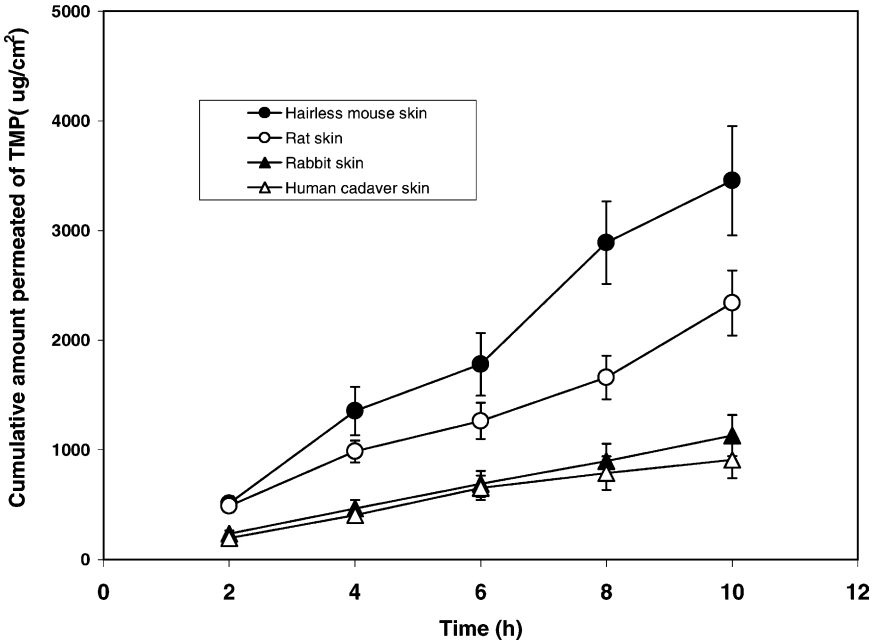

Fig. 3. Comparative permeation of TMP through mouse, rat, rabbit, and human skin. Each point represents the average of three measurements.

of TMP through hairless mouse skin was found to be 369.8 μg/cm^2/h with a 1.0 h lag time (Qi and Hou, 1997). The results from this study, 398.4 μg/cm^2/h permeation rate with 1.0 h lag time, using a different horizontal diffusion system with 2.54 cm^2 of diffusion area and 2.5 ml of receiver phase are in good agreement with the original values. In this study, water was used as a receiver phase solution due to the relative high water solubility of TMP (∼10 mg/ml). In order to maintain a sink condition, all of the receiver solution was withdrawn at each time point and replaced with an equivalent volume of drug free medium.

The generally small error bars (Fig. 3) are indicative of good experimental reproducibility, even with different tissue donors.

Table 1 shows steady-state flux and permeability coefficients with their standard deviations (S.D.). The lag times were determined by extrapolating the linear portion of the plot, representing steady-state flux to the *x*-axis. The lag times seen are relatively short, indicating that permeation steady-states were reached rapidly.

It is instructive to compare the experimental permeability for human skin with that obtained from the correlation of Potts and Guy (1992).

$$\log K_p\,(\mathrm{cm/h}) = -2.7 + 0.71\log(k_{\mathrm{oct/water}}) - 0.0061\,\mathrm{MW}.$$

The calculated value of skin permeability coefficient is 8.9×10^{-3} cm/h, which is very close to the measured value of skin permeability coefficient through human cadaver skin of 7.8×10^{-3} cm/h. The experimentally determined permeability coefficient also appears to be in good agreement with values estimated from the empirical algorithm reported by Flynn and Stewart (1988) in which an upper limit

Table 1
Comparative transdermal fluxes of TMP across various skin membranes (each value represents the average of three measurements, $X \pm$ S.D.)

Skin	J_{ss} (μg/cm^2/h)	K_p ($\times 10^3$, cm/h)	T_{lag} (h)
Hairless mouse	398.4 ± 42.5	34.8 ± 4.4	1.0 ± 0.1
Rat	243.2 ± 30.7	21.3 ± 3.1	1.2 ± 0.2
Rabbit	115.6 ± 8.9	10.1 ± 0.9	1.6 ± 0.2
Human cadaver	89.5 ± 12.4	7.8 ± 1.1	2.1 ± 0.3

permeability coefficient of 10^{-2} cm/h was assigned to compounds with $\log k_{oct/water} > 2.000$.

Tsai and Liang (2001) reported that TMP was found within the brain within 10 min after giving an intravenous injection of 10 mg/kg TMP to rats. The brain/blood ratio progressively increased from 10 to 120 min after administration. Their findings are evidence for the BBB penetrability of TMP and support its clinical use in ischemic cerebral disease. Nie et al. (1994) found that TMP affects the membrane fluidity of liposomes. The similarity in membrane properties between BBB, skin, and liposome explains why TMP so easily penetrates through skin with a relative short lag time. Despite its high water solubility, the compound is more lipophylic than hydrophilic and thus readily dissolves in lipoidal phases.

3.4. Estimation of patch size

Liu et al. (1991b) investigated the relationship between the pharmacokinetic and pharmacodynamic attributes (PK-PD) of TMP using the rat as a model. Unfortunately, the therapeutic plasma levels of TMP were not established and thus the determination of the approximate dimensions of a clinically effective patch is not possible at the present time. However, we still can make a rough estimate of the patch size based on the limited pharmacokinetic parameters and its clinical observations available. The target transdermal flux (K_0) resulting in steady-state therapeutic plasma drug concentration can be estimated using the following equation (Shah and Maibach, 1993):

$$K_0 = C_{min} \times CL_T$$

where C_{min} is the minimum effective blood concentration and CL_T is total body clearance. The therapeutic plasma concentration can be estimated from pharmacokinetic study (Cai et al., 1989) and clinical observations (Guo et al., 1983; Liu et al., 1991a,b; Sun, 1994). TMP is administrated in a dose of 58 mg (base form) three times daily. The peak effect is observed within 1–3 h after oral administration. The plasma concentration at 1–2 h is ~200 ng/ml. CL_T is around 15.7 l/h.

Assuming the 200 ng/ml plasma level to be clinically effective, K_0 would be on the order of 3.2 mg/h. Based on a steady-state flux of TMP, J_{ss}, through human cadaver skins of 89.5 μg/cm^2/h, the patch size (A) that would maintain a 200 ng/ml plasma level would be around 40 cm^2.

4. Conclusions

On the basis of this in vitro percutaneous permeation study, it is concluded that TMP can be delivered through skin at rates consistent with transdermal therapy. Hairless mouse skin and rat skin were about three to four times more permeable to TMP than human cadaver skin. The permeability of TMP through rabbit skin was not significantly different to that of human cadaver skin. TMP quickly reached steady-state, which is evident from the short lag times observed for all skin membranes of about 1–2 h. Although pharmacokinetic data are not currently available to permit precise calculation of a patch size adequate for clinical therapy with TMP, the data from this study indicate that the transdermal delivery of TMP should be feasable.

Acknowledgements

This study was a part of Dr. Xiaohong Qi's Ph.D. disseration (Shanghai Institute of Industrial Pharmaceutics, 1999), supported by research grants from Shanghai Science Foundation (Shanghai, China) and Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences (Beijing, China). The authors would like to thank Dr. Gordon Flynn (University of Michigan) for his critical review and valuable suggestions.

References

Bensky, D., Gamble, A., 1993. Chinese Herbal Medicine: Materia Medica, revised ed. Eastland Press, Seattle, WA, pp. 1–22.

Cai, W., Dong, S.N., Lou, Y.Q., 1989. HPLC determination of tetramethylpyrazine in human serum and its pharmacokinetic parameters. Acta Pharmaceutica. Sinica. 24, 881–886.

Chang, F.C., Huang, Y.T., Lin, H.C., Hong, C.Y., Lin, J.G., Chen, K.J., 1999. Beneficial effects of combined terlipressin and tetramethylpyrazine administration on portal hypertensive rats. Can. J. Physiol. Pharmacol. 77, 618–624.

Chen, K.J., 1991. Move Towards the 21st Century Integration of Traditional Chinese Medicines and Western Medicines.

China Medicine Pharmaceutics Science-Technology Publisher, Beijing, pp. 207–216 (in Chinese).

Guo, S.K., Chen, K.J., Qian, Z.H., Weng, W.L., Qian, M.Y., 1983. Tetramethylpyrazine in the treatment of cardiovascular and cerebrovascular diseases. Planta Med. 47, 89.

Huang, K.J., Williams, W.M. 1993. The Pharmacology of Chinese Herbs. CRC Press, Boca Raton, FL, pp.1–10.

Jia, W.C. 1957. GaoYaoFangJi. People's Public Health Publisher, Beijing (in Chinese).

Flynn, G.L., Stewart, B., 1988. Percutaneous drug penetration choosing candidates for transdermal development. Drug Dev. Res. 13, 169–185.

Liang, C.C., Hong, C.Y., Chen, C.F., Tsai, T.H., 1999. Measurement and pharmacokinetic study of tetramethylpyrazine in rat blood and its regional brain tissue by high-performance liquid chromatography. J. Chromatogr. B Biomed. Sci. Appl. 724, 303–309.

Liu, X.Q., Lou, Y.C., Chen, Q.T., 1991a. The clinical pharmacokinetics studies of tetramethylpyrazine hydrochloride in normal volunteers and patients with acute cerebral ischemia disease (CID). Chin. J. Clin. Pharmacol. 7, 32–36 (in Chinese with English abstract).

Liu, X.Q., Lou, Y.C., Shi, W.Z., 1991b. Study on the relationship between pharmacokinetics and pharmacodynamics of tetramethylpyrazine and effects of acute hepatic poisoning on its pharmacokinetics in rats. J. Beijing Med. Univ. 23, 185–189 (in Chinese with English abstract).

Nie, S.Q., Kwan, C.Y., Epand, R.M., 1994. Analogue of tetramethylpyrazine affect membrane fluidity of liposome: relationship to their biological activities. Eur. J. Pharmacol. Mol. Pharmacol. 266, 11–18.

Pilarski, B., Osmialowski, K., 1985. The relationship between electron densities and the pK_a values in s series of methylpyazines. Int. J. Quant. Chem. XXVIII, 239–244.

Potts, R.O., Guy, R.H., 1992. Predicting skin permeability. Pharm. Res. 9, 663–669.

Qi, X.H, Hou, H.M., 1997. Transdermal delivery system of tetramethylpyrzaine: the influence of varied EVA copolymer membrane on skin permeation of TMP. In: CRS-CPA Joint Workshop on Recent Advances in Drug Delivery Science and Technology, Beijing, September 20–22, p. 48.

Shah, V.P., Maibach, H.I., 1993. Topical Drug Bioavailablity, Bioequivalence, and Penetration. Plenum Press, New York, p. 27.

Sun, B.L., 1994. Clinical use of tetramethylpyrazine: an overview. Chin. J. Integr. Trad. West. Med. 14, 465–468 (in Chinese).

Tsai, T.H., Liang, C.C., 2001. Pharmacokinetics of tetramethylpyrazine in rat blood and brain using microdialysis. Int. J. Pharm. 216, 61–66.

Wang, G.Q., 1981. ZhongGuoGaoYaoXue. Science and Technology Publisher, Xi'an, China, pp. 111–154 (in Chinese).

Watanabe, H., 1997. Candidates for cognitive enhancer extracted from medicinal plants: paeoniflorin and tetramethylpyrazine. Behav. Brain Res. 83, 138–141.