Simple High-Performance Liquid Chromatographic Method for the Determination of Tetramethylpyrazine Phosphate in Very Small Volumes of Dog Plasma: Application to a Pharmacokinetic Study

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Abstract

A rapid and sensitive high-performance liquid chromatographic (HPLC) method is developed for the determination of tetramethylpyrazine phosphate, an antiplatelet aggregation agent, in 100 µL of dog plasma. Sample preparations are carried out by deproteinization with an internal standard (carbamazepine) solution in acetonitrile. An aliquot of the supernatant (20 µL) is directly injected into an HPLC apparatus with methanol–phosphate buffer (0.01M, pH 3.0) (62:38, v/v) as the mobile phase at a flow rate of 1.0 mL/min. Separation is performed with a C_{18} column at 30°C. The peak is detected using a UV detector set at 279 nm. The capacity factors are 1.48 for tetramethylpyrazine phosphate and 2.09 for carbamazepine, with a total run time of 10 min. The calibration curve is linear in the 0.2–50-µg/mL range. The limit of detection is 0.05 µg/mL. Mean recoveries are 92.6–98.1%. The within- and between-day variation coefficients are less than 4.9% and 7.5%, respectively. The present method has been successfully used to provide pharmacokinetic data after oral administration of tetramethylpyrazine phosphate pulsincap capsules and immediate-release tablets to dogs.

Introduction

Tetramethylpyrazine phosphate (TMPP) (Figure 1A), an antiplatelet aggregation agent, has been widely used in China for the treatment of angina pectoris (1,2). Because angina is a typical cardiovascular disease that has the highest incidence at approximately 6 h after falling asleep (3), the TMPP pulsincap capsules with a delayed release to match the timing of TMPP's effect on the intrinsic circadian rhythm of angina were developed. This pulsincap system was composed of a water-insoluble body with a TMPP tablet contained within the capsule and sealed with an erodible plug (EP) at the mouth of the capsule body. A soluble gelatin cap was placed on the capsule body. When the pulsincap formulation was swallowed, the cap dissolved in the gastrointestinal fluids, exposing the plug that was eroded at a constant rate. At a predetermined time after ingestion, the EP erosion was almost complete, and a few small holes appeared on the EP, allowing entry of water. Then the TMPP tablet would absorb water and release drug (4). Therefore, with the pulsincap system, the drug was released after a programmed lag time. This time-delayed drug-delivery system had an advantage over conventional immediate-release tablets to address emerging chronotherapeutic requirements. Thus pharmacokinetic and pharmacodynamic studies were of great value in the evaluation of this novel pulsatile drug delivery system.

Several studies on the determination of tetramethylpyrazine (TMP) in biological fluids have been performed using gas chromatography–mass spectrometry (MS) (5–8) or liquid chromatography (LC)–MS (9), but it requires MS equipment that may not be available in many laboratories. Some workers have also employed the high-performance liquid chromatography (HPLC) method with UV detection for the assay of TMP (10–12). Most of these methods (11,12) require complicated sample preparation procedures involving basification of plasma sample, extraction, acidification, preconcentration, and reconstitution of the analytes. These procedures are rather time-consuming and are more complex than the procedure described in this paper. With this method, samples could be applied to HPLC with simple deproteinization without multiple pretreatment steps, and the drug

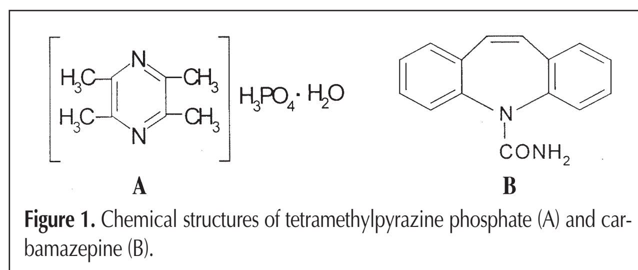

Figure 1. Chemical structures of tetramethylpyrazine phosphate (A) and carbamazepine (B).

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level in plasma could be easily determined with sufficient sensitivity. Furthermore, none of the previously described methods measured TMPP in dog plasma. Until now, the pharmacokinetics of TMPP have not been investigated in combined pharmacokinetic and pharmacodynamic studies, in which more blood samples are needed for both the HPLC assay and platelet aggregation test. To minimize the volume of the collected sample and lessen the possible physiological influence on animals, it was necessary to develop an HPLC method for the determination of TMPP in small volumes of dog plasma.

However, most of the previous HPLC methods used 200–700 µL of plasma for the determination of TMPP (11,12); this was not satisfactory for the assay of TMPP in plasma during the combined pharmacokinetic and pharmacodynamic study. The objective of the present work was to develop a simple, rapid, and sensitive HPLC method with UV detection for the analysis of TMPP in dog plasma using only 100 µL of plasma for detection. On the basis of previous work (13), a method in which plasma samples could be applied to HPLC with simple deproteinization without multiple pretreatment steps was developed. The method enables precise and accurate quantitation of TMPP in small sample volumes over a wide concentration range. The application of this method to a combined pharmacokinetic and pharmacodynamic study in beagle dogs after oral administration of TMPP pulsincap capsules and immediate-release tablets as a reference formulation is presented.

Experimental

Chemicals, reagents, and standards

All solvents and reagents used were of analytical or HPLC grade. Methanol and acetonitrile (HPLC grade) were purchased from Sanli Chemical Factory (Zhejiang, China). Ultrapure water was prepared using a Milli-Q system (Millipore, Milford, MA). Drug-free dog plasma was from the Department of Pharmacology, Sichuan University (Sichuan, China). TMPP (purity of 99.7%) was obtained from the Fine Chemical Industry Branch of Shanghai Hua Mei Auxiliaries (Shanghai, China). Carbamazepine (Figure 1B) used as the internal standard was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Adenosine diphosphate (ADP) was purchased from Biopool International (Ventura, CA) and used as a platelet aggregation agent.

The reference formulation was immediate-release tablets (Double-crane, Beijing, China) containing 50 mg of TMPP per tablet. TMPP pulsincap capsules with lag time of 5 to 6 h were prepared in our laboratory. Each capsule contains 50 mg of TMPP.

Preparation of standards and controls

A concentrated stock solution of TMPP was prepared at a concentration of 1000 µg/mL in ultrapure water and was further diluted with water into 2–500 µg/mL for the preparation of plasma calibration standards. The internal standard (carbamazepine) solution was prepared in acetonitrile at a concentration of 2 µg/mL. All solutions were stored at –20°C in dark glass. Different standard solutions (10 µL each) were transferred into tubes containing 90 µL of drug-free dog plasma to obtain plasma calibration standards. A plasma calibration curve was constructed with seven different standards covering the expected concentration range (0.2–50 µg/mL). The calibration curve was produced by linear regression of peak-area ratios (TMPP to carbamazepine) against their respective concentrations. The regression line was used to calculate concentrations of TMPP in the unknown plasma samples based on the peak-area ratio.

Quality control (QC) samples to determine accuracy and precision of the method were independently prepared at low (1.0 µg/mL), medium (5.0 µg/mL), and high (20.0 µg/mL) concentrations in the same manner as the calibration standards and stored at –20°C before use.

Sample preparation

To 100 µL of a plasma sample in a centrifuge tube, 150 µL of the carbamazepine internal standard solution in acetonitrile was added. The solution was vortex-mixed for 1 min and centrifuged at 12,000 × *g* for 10 min. A 20-µL volume of the supernatant was subjected to HPLC analysis.

Apparatus and operating conditions

HPLC determinations were performed with a Shimadzu model 10ATvp LC system (Chiyoda-Ku, Tokyo, Japan), consisting of two pumps (LC-10AT), a 20-µL injection loop, column oven (CTO-10A), and UV detector (SPD-10A). The system was controlled through a system controller (SCL-10A) and a personal computer using a CLASS-VP 5.0 workstation with a data processing system (Shimazu, Kyoto, Japan) installed on it. The separation was performed on a Shimadzu Shim-Pack C_{18} reversed-phase column (5-µm particle size, 150- × 6-mm i.d.) (Chiyoda-Ku) protected by a Shimadzu Shim-Pack guard column (10- × 2.5-mm i.d., 5-µm particle size). A mobile phase consisting of a mixture of methanol–phosphate buffer (0.01M, pH 3.0) (62:38, v/v) was filtered, degassed by sonication, and used at a column temperature of 30°C and a flow rate of 1.0 mL/min. The column effluent was monitored at 279 nm with UV detection.

Only one column has been devoted to this method, and so far approximately 270 injections have been made using this method. The column still has good performance. To maintain the column in good condition, after analytical use the column is flushed with approximately 10 column-volumes of HPLC-grade water. A gradient is then run (approximately 20 column volumes) from 100% water to 100% methanol to remove the adsorbed contaminants on the column during the analytical procedure. Finally, the column is equilibrated with methanol for five column volumes and stored in pure methanol.

Animal experiment

Three male and three female beagle dogs (weighing 10–12 kg) were divided into a test group and a control group at random. The dogs were made to fast overnight and had free access to water before drug administration. The dogs were administered, in a crossover design, either the immediate-release tablets containing TMPP 50 mg (control group) or the pulsincap capsules with a lag time of 5 to 6 h (test group). The formulations were swallowed with 200 mL water. The dogs were provided a standard meal 7 h after drug intake. At 30 min before administration, a control blood sample was obtained. After oral administration, 5-mL blood samples were collected at the following times for each treatment,

respectively: (control group) 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, and 8 h and (test group) 2, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8, and 10 h. Every time after the samples were drawn, 3.6 mL of the 5 mL blood sample was transferred into a plastic test tube containing 0.9 mL of 3.8% sodium citrate as an anticoagulant for the platelet aggregation test. The rest of the blood was put into a heparinized tube and was centrifuged for 10 min at $2000 \times g$ to obtain the plasma sample. Plasma samples were immediately frozen and kept at –20°C until assay. There was a 2-week washing-out time between each treatment period.

Platelet aggregation study was performed using a TYXN-91 Intelligent Blood Aggregometer (Shanghai Institute of General Machinery and Electronic Technology, Shanghai, China). Platelet-rich plasma (PRP) was prepared by centrifuging blood sample at $100 \times g$ for 10 min. ADP, a platelet aggregation agent, was added to 0.2 mL of PRP to yield a final concentration of 5 µmol/L, and a percentage aggregation profile from 0 to 5 min was recorded. The maximum aggregation rate (MAR) was the primary outcome measured.

Results and Discussion

Optimization of sample treatment and HPLC technique

In this study, a simple deproteinization method with internal standard solution in acetonitrile was used for sample preparation. Initially, a liquid–liquid extraction process was performed using methylene dichloride, ethyl acetate, or ether after a spiked TMPP plasma sample was basified with 0.5 mol/L of NaOH. However, the method was not reproducible, and the extraction recovery was less than 50%. Several deproteinization solvents such as acetonitrile, methanol, or acetone were also tested for sample preparation. The results showed that deproteinization by acetonitrile gave good resolution and high recovery. Thus, acetonitrile was selected to precipitate proteins, as already described by other authors (14,15). In the present study, carbamazepine was chosen as the internal standard because it is commercially available and less toxic than coumarin used in the previous report (10). Most importantly, the separation using carbamazepine offered baseline resolution between peaks of carbamazepine and TMPP with a faster analysis compared with that using coumarin. Therefore, carbamazepine was chosen rather than coumarin as the internal standard. It should be noted that the temperature should be held at 20°C when adding carbamazepine internal standard solution to plasma samples, in order to prevent the possible change in the volume of the internal standard solution because of variation of temperature.

From the spectophotometry, TMPP was found to absorb strongly at 213 and 279 nm. If the detector was set at 213 nm, TMPP and the internal standard were not well separated from endogenous interference peaks. A detection wavelength of 279 nm proved to be suitable for the assay. During the method development, the percentage of the mobile phase organic solvents was varied using different combinations of methanol–phosphate buffer (0.01M, pH 3.0) (65:35, 62:38, 60:40, and 55:45). It demonstrated longer retention time of TMPP with a decrease in the organic composition. Here, the 62% methanol and 38% 0.01M phosphate buffer (pH 3.0) combination was chosen to achieve a good peak shape, satisfactory resolution, and relatively short analysis time.

Selectivity and chromatography

The degree of interference by endogeneous plasma constituents with TMPP and carbamazepine (internal standard) was assessed by inspection of chromatograms derived from processed blank plasma sample. Typical chromatograms obtained from blank dog plasma, blank dog plasma spiked with TMPP and carbamazepine, and dog plasma sample spiked with carbamazepine are presented (Figure 2). TMPP and carbamazepine were eluted at 5.7 and 7.1 min, respectively. The capacity factors were 1.48 for TMPP and 2.09 for carbamazepine. The total run time was only 10 min. A good separation of TMPP and carbamazepine was obtained under the chromatographic conditions described previously. No interfering peaks were found at the retention time of TMPP and the internal standard carbamazepine.

Linearity and sensitivity

The calibration curve for the determination of TMPP in dog plasma was linear with a correlation coefficient of 0.9999 over the range of 0.2–50 µg/mL. This linear correlation obtained over five independent runs had a slope of 0.1928 ± 0.0134 and an intercept of 0.0194 ± 0.0083. The limit of detection for this method, defined as the concentration at which the signal-to-noise ratio was 3:1 (16,17), was 0.05 µg/mL. The limit of quantitation (LOQ) was defined as the lowest drug concentration that can be determined reproducibly (coefficient of variation < 20%) and accurately (percent error < 20%) (18,19). The LOQ of this method was 0.15 µg/mL.

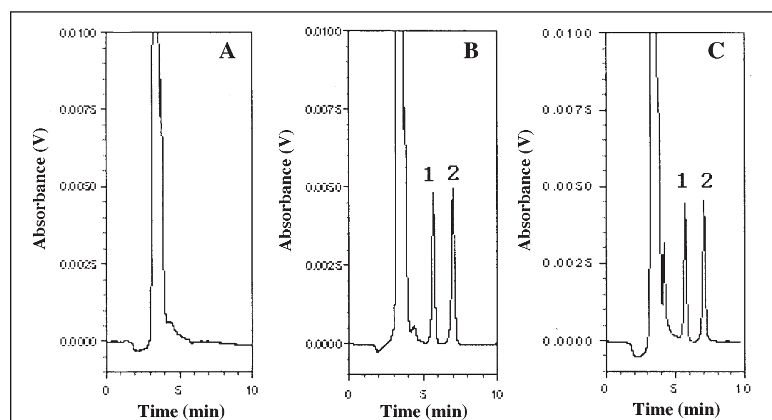

Figure 2. Typical chromatograms of blank dog plasma (A), blank dog plasma spiked with 5 µg/mL TMPP and carbamazepine (internal standard) (B), and dog plasma sample containing 5.8 µg/mL of TMPP spiked with carbamazepine (internal standard) (C). TMPP, 1, and internal standard (carbamazepine), 2.

Precision and accuracy

Within- and between-day precision and accuracy were evaluated by analyzing five replicates of quality control samples at three different concentrations of TMPP (Table I). Precision was expressed as the coefficient of variation, though accuracy was presented as a percent error (relative error), [(observed concentration – nominal concentration)/nominal concentration] × 100(%)] (20,21).

Within- and between-day relative standard deviations were less than 4.9% and 7.5%, respectively. Accuracy was within 6.2% when compared with nominal concentrations. The results indicate that the method is reliable, reproducible, and accurate.

Recovery and stability

Recovery was calculated by comparing the peak areas obtained from the quality control (QC) samples to those from the standard solutions containing the same amount of TMPP (21). The mean recoveries of TMPP from plasma performed at three representative concentrations of 1.0, 5.0, and 20.0 µg/mL were 92.6% [relative standard deviation (RSD) = 1.8%], 97.7% (RSD = 1.4%), and 98.1% (RSD = 1.3%), respectively.

The stability of TMPP in dog plasma was investigated through three freeze–thaw cycles of the QC samples during the storing period of 1 month at –20°C. TMPP was considered to be stable in dog plasma after three freez–thaw cycles at concentration of 1.0, 5.0, and 20.0 µg/mL. The mean concentrations following this storage period were 100.6% (RSD = 1.2%), 97.5% (RSD = 2.2%), and 97.9% (RSD = 3.6%) of TMPP concentrations in freshly prepared samples, respectively. Results of the stability experiments indicated that TMPP in the plasma samples was stable for at least 1 month when stored at –20°C.

Method application

The validated method was applied to the measurement of TMPP in dog plasma samples in support of a combined pharmacokinetic and pharmacodynamic study of TMPP pulsincap capsules (test group) and reference tablets (control group). The results of determination in combination with the MAR results of the platelet aggregation test are shown in Table II. Pharmacokinetic parameters were estimated according to a two-compartment model with a lag time (T_{lag}) using the 3p87 program (The Chinese Society of Mathematical Pharmacology, Beijing, China). The maximum drug plasma concentration (C_{max}) and the time required to reach the C_{max} (T_{max}) were obtained directly from individual plasma concentration-time results for TMPP. The area under the plasma concentration-time curve (AUC), up to the last measured time, was calculated using a linear trapezoidal method. The mean residence time (MRT) was computed by statistic moment analysis. Differences between the two formulations in each parameter were statistically evaluated by a one-way analysis of variance test. The main pharmacokinetic parameters are listed in Table III.

The results revealed that the mean in vivo T_{lag} and T_{max} value of the test group were 3.23 and 5.00 h, respectively, which were significantly different from those of the control group (0.21 and 0.79 h) ($P < 0.05$), suggesting a remarkably delayed release of the drug in vivo. The C_{max} of the test group were 1.71 ± 1.00 µg/mL, which was lower than that of the control group, which was 11.10 ± 3.33 µg/mL. The MRTs of the test group and the control group were 5.73 ± 0.52 h and 1.83 ± 0.69 h, respectively. All these results implied that TMPP pulsincap capsules took a significantly longer time for drug absorption in the gastrointestinal tract, and this was probably brought about from the delayed release of drug. Also, TMPP pulsincap capsules were thought to release TMPP at the lower part of the gastrointestinal tract because the onset time of drug release was delayed. It was often pointed out that drug absorption from the lower intestine tended to decrease because of the lower water content (22). Therefore, the developed HPLC method has been successfully applied in the pharmacokinetic study of TMPP. The obtained parameters showed that delayed release could be fulfilled using TMPP pulsincap capsules to satisfy the necessary time concentration profile for the treatment of angina. The MAR results of the pharmacodynamic study indicated that the ADP-induced maximum platelet aggregation rate was significantly reduced from 4–7 h for the test group ($P \times 0.01$, compared with the MAR value at 0 h), and the time period

Table I. Within- and Between-Day Precision and Accuracy for Determination of TMPP in Spiked Plasma

	Within-day			Between-day		
Nominal concentration (µg/mL)	Observed concentration* (µg/mL)	CV† (%)	Relative error (%)	Observed concentration* (µg/mL)	CV (%)	Relative error (%)
1.00	1.02 (0.05)	4.9	2.0	1.06 (0.08)	7.5	6.2
5.00	4.90 (0.03)	0.6	–2.0	4.88 (0.10)	2.1	–2.4
20.00	20.01 (0.08)	0.4	0.1	20.14 (0.21)	1.0	0.7

* Mean (standard deviation), *n* = 5.
† CV = coefficient of variation.

Table II. Mean Concentration-Time Data of TMPP and Maximum Aggregation Rate in Dog Plasma Samples Induced by ADP after Administration of TMPP Pulsincap Capsules or Immediate-Release Tablets

Pulsincap capsules			Immediate-release tablets		
Time (h)	Plasma concentration* (µg/mL)	MAR (%)*,†	Time (h)	Plasma concentration* (µg/mL)	MAR (%)*,†
0	0	82.9 (7.6)	0	0	85.3 (2.9)
2	0.15 (0.15)	84.2 (6.1)	0.25	2.21 (3.01)	69.8 (12.4)‡
4	0.45 (0.33)	58.4 (25.8)§	0.5	6.10 (3.86)	24.8 (6.1)§
4.5	1.34 (1.18)	45.1 (22.7)§	0.75	11.10 (3.34)	19.3 (2.0)§
5	1.44 (0.53)	61.7 (11.3)§	1	7.51 (2.56)	73.3 (1.4)§
5.5	0.75 (0.32)	63.7 (19.4)§	1.5	4.19 (1.73)	79.8 (20.2)
6	0.65 (0.26)	66.0 (9.3)§	2	2.57 (1.36)	80.0 (20.1)
6.5	0.54 (0.30)	70.9 (7.7)§	3	0.96 (0.46)	83.5 (16.6)
7	0.45 (0.19)	71.3 (5.7)§	4	0.44 (0.16)	84.4 (2.5)
8	0.35 (0.14)	82.0 (8.2)	6	0.15 (0.12)	85.0 (7.6)
10	0.28 (0.07)	83.7 (3.4)	8	ND**	88.4 (5.8)

* Mean (standard deviation), *n* = 6.
† MAR = maximum aggregation rate.
‡ $P < 0.05$, compared with the MAR value at 0 h.
§ $P < 0.01$, compared with the MAR value at 0 h.
** ND = not determined.

Table III. Mean Pharmacokinetic Parameters after Oral Administration of TMPP Pulsincap Capsules or Immediate-Release Tablets to Dogs*

Parameters	Pulsincap capsules	Immediate-release tablets	ANOVA† test
T_{max} (h)‡	5.00 (0.55)	0.79 (0.10)	$P < 0.05$
C_{max} (μg/mL)§	1.71 (1.00)	11.10 (3.33)	$P < 0.05$
T_{lag} (h)**	3.23 (1.05)	0.21 (0.04)	$P < 0.05$
AUC_{0-tn} (h · μg/mL)††	4.37 (1.91)	14.05 (3.74)	$P < 0.05$
MRT (h)‡‡	5.73 (0.52)	1.83 (0.69)	$P < 0.05$

* Mean (standard deviation), $n = 6$.
† ANOVA = analysis of variance.
‡ T_{max} = Time required to reach the C_{max}.
§ C_{max} = maximum drug plasma concentration.
** T_{lag} = lag time.
†† AUC = area under the plasma concentration-time curve.
‡‡ MRT = mean residence time; mean (standard deviation), $n = 6$.

was 0.25 to 1 h ($P < 0.01$ or $P < 0.05$) for the control group. These pharmacodynamic data revealed that TMPP took significant action from 4–7 h after TMPP pulsincap capsules were administered, just covering the period with highest incidence of angina (6 h after falling asleep). Both pharmacokinetic and pharmacodynamic results indicated that the delayed release of the drug and chronotherapeutic effect could be achieved with TMPP pulsincap capsules for treating angina.

Conclusion

The described HPLC method is simple, specific, and sufficiently sensitive for the analysis of TMPP in dog plasma. The simple deproteinization process for sample preparation and HPLC analyses with only a 10-min run time enhanced the efficiency of the procedure. The method required only 100 μL of plasma, making it suitable for the combined pharmacokinetic and pharmacodynamic studies of TMPP, in which small volumes of plasma samples for HPLC assay are required. This assay has been successfully applied to a pharmacokinetic study of TMPP after oral administration of TMPP pulsincap capsules or immediate-release tablets to dogs, demonstrating the delayed release characteristics of this novel drug release system.

Acknowledgments

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