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Liquid chromatography–mass spectrometry method for determination of tetramethylpyrazine and its metabolite in dog plasma

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Abstract

A liquid chromatography–mass spectrometry method is described for the determination of tetramethylpyrazine (TMP) and its active metabolite, 2-hydroxymethyl-3,5,6-trimethylpyrazine (HTMP) in dog plasma. This method involves a plasma clean-up step using protein precipitation procedure followed by LC separation and positive electrospray ionization mass spectrometry detection (ESI-MS). Chromatographic separation of the analytes was achieved on a C18 column using a mobile phase of methanol, water and acetic acid (50:50:0.6, v/v/v) at a flow rate of 1.0 ml/min. Selected ion monitoring (SIM) mode was used for analyte quantitation at m/z 137.2 for TMP, m/z 153.2 for HTMP and m/z 195.2 for caffeine. The linearity was obtained over the concentration ranges of 20–6000 ng/ml for TMP and 20–4000 ng/ml for HTMP and the lower limit of quantitation was 20 ng/ml for both analytes. For each level of QC samples, both inter- and intra-day precisions (R.S.D.) were $\leq$7.4% for TMP and $\leq$6.0% for HTMP, and accuracy (R.E.) was $\pm$6.0% for TMP and $\leq$3.5% for HTMP. The proposed LC–MS method was successfully applied to the pharmacokinetic studies of a TMP formulation preparation after oral administration to beagle dogs.
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1. Introduction

Tetramethylpyrazine (TMP) is a biologically active ingredient isolated from the traditional herbal medicine *Ligusticum chuanxiong* Hort. TMP possesses anti-ulcers and antiplatelet activities and has been widely used in China for the treatment of patients with angina pectoris, ischemic vascular diseases and gastric lesion [1–3]. 2-Hydroxymethyl-3,5,6-tri-methyl pyrazine (HTMP) is one active metabolite of TMP and exhibiting similar effects. The chemical structures of TMP and HTMP are shown in Fig. 1.

For the pharmacokinetic study of a TMP formulation product in dogs, an analytical method with simplicity and high sensitivity was required in our laboratory. A recent survey revealed that few methods were available for the determination of TMP in pharmaceutical preparations, botanical or biological samples, which involved liquid chromatography with ultraviolet detection [4–8] and gas chromatography–mass spectrometry [9–11]. No analytical methods were available for the simultaneous determination of TMP and HTMP or individual determination of HTMP in biological samples. Taking into consideration the low levels of both analytes in plasma, a simple and sensitive method needed to be developed for the determination of TMP and HTMP in biological samples. Among the choices of analytical methods, liquid chromatography–mass spectrometry (LC–MS) became the first choice for our purpose.

This paper describes a simple and sensitive LC–ESI-MS method in positive selected ion monitoring (SIM) mode for the simultaneous determination of TMP and HTMP in dog plasma. The described method was validated in terms of se-

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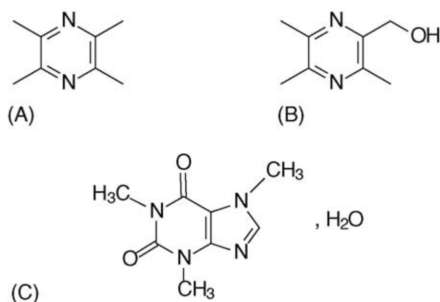

Fig. 1. The chemical structures of TMP (A), HTMP (B) and caffeine (C).

lectivity, linearity, limit of quantitation, accuracy, precision and stability of analyte at ambient temperature, and successfully applied to the pharmacokinetic studies of a TMP formulation preparation in beagle dogs.

2. Experimental

2.1. Chemicals and reagents

TMP phosphate reference standard (101.0% purity) was from Beijing Yanjing Pharmaceutical Factory. HTMP reference standard (99.3% purity), and aspirin and tetramethylpyrazine phosphate sustained release tablets (each tablet containing 75 mg of aspirin and 150 mg of tetramethylpyrazine, batch no. 030115) were from Shenyang Pharmtech Institute of Pharmaceuticals (Shenyang, China). Caffeine (99.6% purity, internal standard) was from Shandong Xinhua Pharmaceutical Company, Ltd. (Jinan, China). Methanol of liquid chromatographic grade was purchased from Tianjin Concord Tech Reagent Company (Tianjin, China). All the other reagents were of analytical grade.

2.2. Instrument and LC–MS conditions

HP 1100 series LC/MSD G1946D (Agilent, USA) was used for quantitative determination of TMP and HTMP in dog plasma.

Chromatographic separation was performed on a Diamonsil™ C_{18} column (150 mm × 4.6 mm I.D., 5 μm, Dikma, China). The mobile phase consisting of a mixture of methanol, water and acetic acid (50:50:0.6, v/v/v) was delivered at a flow rate of 1.0 ml/min. Chromatography was performed at ambient temperature. The injection volume was 50 μl.

Electrospray ionization (ESI) source in positive ion mode was used for the determination. The optimized ionization conditions were: nitrogen flow rate, 8.0 ml/min; gas temperature, 325 °C; nitrogen pressure, 30 p.s.i.g.; collision induced dissociation (CID), 70 V. Positive selected ion monitoring (SIM) mode was used for the quantitation at m/z 137.2 for TMP, m/z 153.2 for HTMP and m/z 195.2 for caffeine.

2.3. Preparation of calibration standards and quality control samples

Stock solutions of TMP and HTMP were individually prepared at 100 μg/ml in methanol. The stock solution of TMP was further diluted with methanol to give a series of standard solutions with concentration of 100, 200 ng/ml, 1, 3, 10 and 30 μg/ml. The stock solution of HTMP was further diluted with methanol to give a series of standard solutions with concentration of 100, 200 ng/ml, 1, 5, 10 and 20 μg/ml. The internal standard (caffeine) solution was prepared in methanol at a concentration of 500 μg/ml.

Calibration standards of TMP (20, 40, 200, 600, 2000 and 6000 ng/ml) and HTMP (20, 40, 200, 1000, 2000 and 4000 ng/ml) were prepared by spiking appropriate amount of the standard solutions of TMP and HTMP in blank plasma. Quality control (QC) samples were prepared using the pooled plasma at concentrations of 40, 600 and 6000 ng/ml for TMP and 40, 1000 and 4000 ng/ml for HTMP. The spiked samples were then treated following the sample preparation procedure as indicated in Section 2.4.

2.4. Sample preparation

A 0.5-ml aliquot of each plasma sample was transferred to a 5-ml polyethylene centrifuge tube. A 100 μl of caffeine solution in methanol (500 μg/ml), a 200 μl of methanol and 1.0 ml of methanol were added and the contents were mixed by vortexing for 1 min and allowed to stand still for 5 min and centrifuged for 10 min to separate the phases. An aliquot (50 μl) of the supernatant was directly injected onto the LC–MS system for analysis.

2.5. Method validation

Validation runs were conducted on three separate days. Each validation run consisted of a set of the spiked standard samples at six concentrations over the concentration range (each in triplicate) and QC samples at three concentrations ($n = 6$ at each 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. Concentrations of the analytes in plasma samples were determined by back-calculation of the observed peak area ratios of the analytes and internal standard 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 to be determined.

The selectivity of the method was investigated by comparing chromatograms of blank plasma, standard plasma sample spiked with TMP (200.0 ng/ml), HTMP (200.0 ng/ml) and caffeine (500 μg/ml) and plasma sample at 0.08 h after an oral dose of a TMP formulation preparation (300 mg).

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

The extraction recoveries of TMP and HTMP were determined at low, medium and high concentrations by comparing the responses from plasma samples spiked before extraction with those from standard solutions at the same levels.

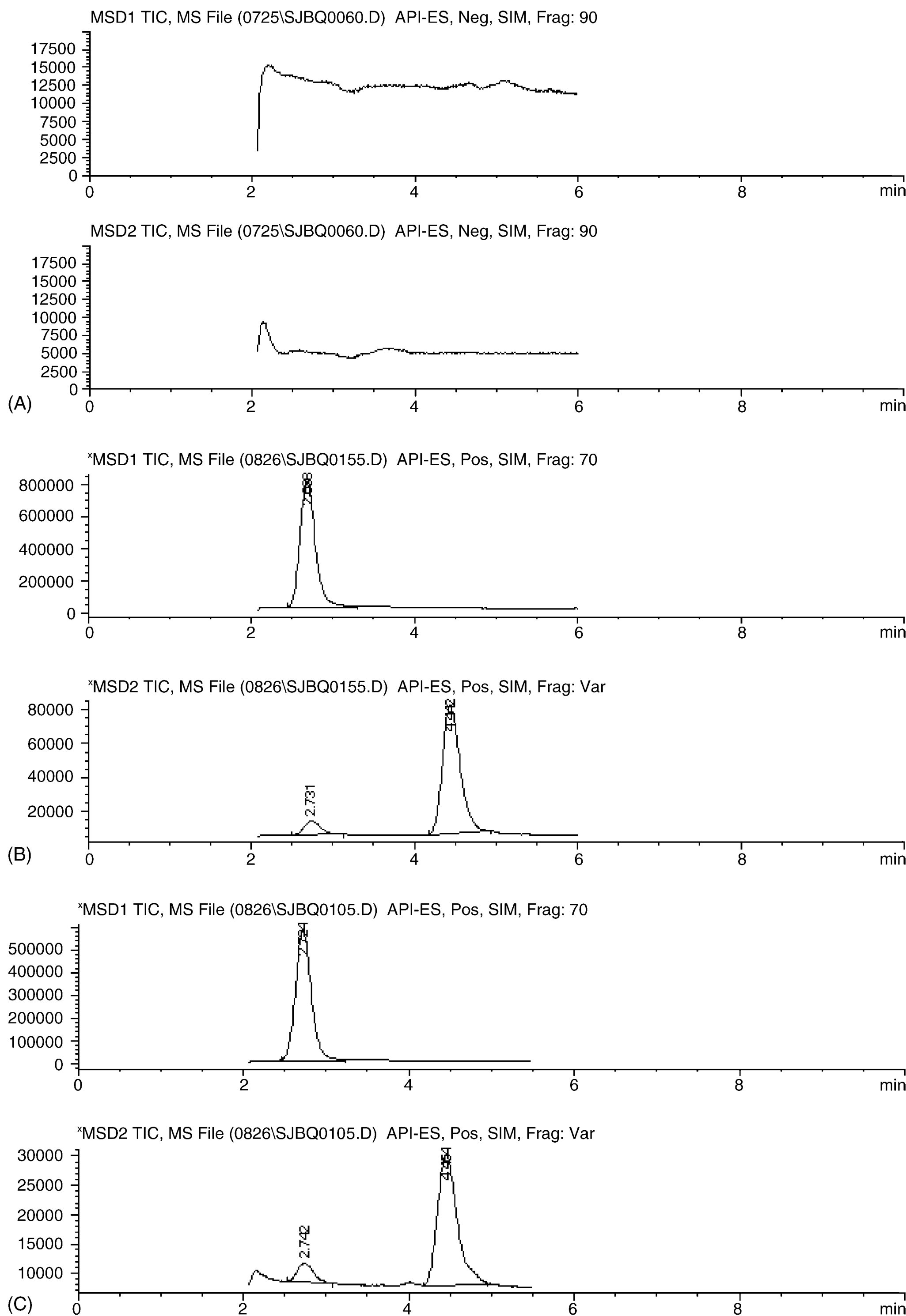

Fig. 2. Representative SIM chromatograms of: (A) blank dog plasma sample; (B) blank plasma sample spiked with TMP (200.0 ng/ml), HTMP (200.0 ng/ml) and caffeine (500 μg/ml); (C) plasma sample at 0.08 h after an oral dose of two aspirin and tetramethylpyrazine phosphate sustained release tablets (300 mg) to a beagle dog. Two channels were used for the quantitation, MSD1 for internal standard ($t_R = 2.7$ min) and MSD2 for TMP ($t_R = 4.4$ min) and HTMP ($t_R = 2.7$ min).

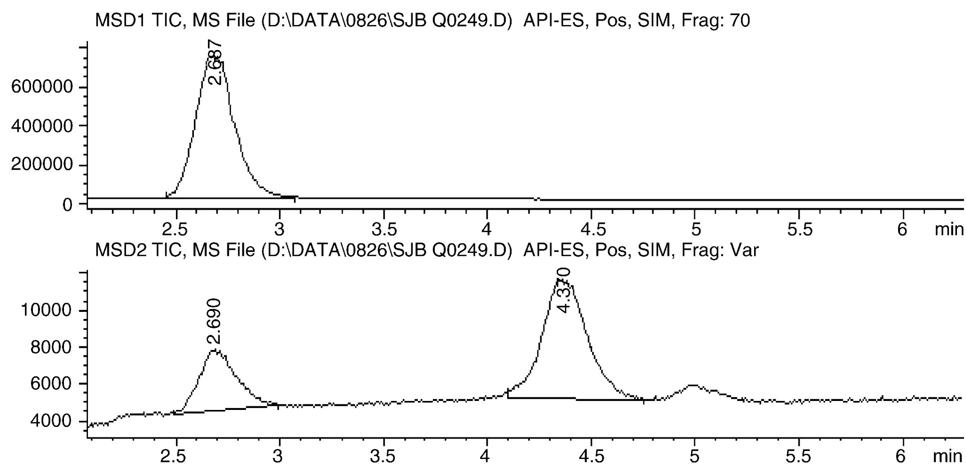

Fig. 3. Representative SIM chromatogram of blank plasma sample spiked with TMP (20.0 ng/ml), HTMP (20.0 ng/ml) and caffeine (500 μg/ml).

Sample stability was determined by analyzing QC samples containing TMP of 40, 600 and 6000 ng/ml and HTMP of 40, 1000 and 4000 ng/ml after protein precipitation and exposed to ambient temperature over a time period of 12 h.

2.6. Application of the LC–MS method

The LC–MS method was successfully applied to the pharmacokinetic studies of aspirin and tetramethylpyrazine phosphate sustained release tablets in beagle dogs. Six beagle dogs (3 male, 3 female, 12–13 kg) were from the Laboratory Animal Center in Shenyang Pharmaceutical University. After an overnight fast (12 h), the dogs were given single dose of two aspirin and tetramethylpyrazine phosphate sustained release tablets (equivalent to 300 mg of TMP). No food was allowed until 4 h after oral administration of the tablets while water intake was free. About 2 ml of blood samples were collected from a hind leg vein into heparinized tubes before (0 h) and at 0.08, 0.17, 0.33, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0, 12.0 and 24.0 h after dosing. Plasma was separated by centrifugation and kept frozen at −20 °C until analysis.

3. Results and discussion

3.1. Method development

Sample preparation plays a key role for the determination of drugs in biological samples. At the beginning of this work, liquid–liquid extraction was tried, but found unsuitable for the determination of TMP and HTMP in dog plasma because the drying process in liquid–liquid extraction caused a significant loss of the analytes due to their volatility. After several trials, a protein precipitation procedure was adopted and proved to be simple and reliable for the sample preparation in this work. Methanol other than acetonitrile was selected as protein-precipitating solvent to fit with the composition of mobile phase to produce the expected peak shapes of the analytes. In the procedure, the supernatant after centrifugation was directly injected onto the LC–MS system, which minimized the loss of the analytes in plasma and assured the accuracy of the developed method. This simple procedure produced a clean chromatogram for a blank plasma sample and yielded satisfactory recoveries for the analytes from the plasma.

A Diamonsil ™ C_{18} column (150 mm × 4.6 mm I.D., 5 μm) was used for the chromatographic separation. Other chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes of analytes as well as short run time. It was found that a mixture of methanol, water and acetic acid (50:50:0.6, v/v/v) could achieve our purpose and was finally adopted as the mobile phase for the chromatographic separation.

Internal standard is necessary for determination of analytes in biological samples. In initial stage of our work, several compounds were tried and finally caffeine was found to be optimal for our work.

For the quantitation of TMP and HTMP in dog plasma, some parameters related with mass spectrometric detection

Table 1
Accuracy and precision for the determination of TMP in dog plasma ($n = 3$ days, six replicates per day)

Added C (ng/ml)	Found C (ng/ml)	Intra-day R.S.D.[a] (%)	Inter-day R.S.D. (%)	Relative error (%)
40.0	38.54	6.1	7.4	−3.7
600.0	634.82	4.9	5.4	5.8
6000.0	5641.14	5.1	6.1	−6.0

Relative error: R.E. (%) = 100 × (mean concentration − nominal concentration)/nominal concentration.
[a] Relative standard deviation.

Table 2
Accuracy and precision for the determination of HTMP in dog plasma ($n = 3$ days, six replicates per day)

Added C (ng/ml)	Found C (ng/ml)	Intra-day R.S.D.[a] (%)	Inter-day R.S.D. (%)	Relative error (%)
40.0	39.98	5.8	6.0	0.0
1000.0	1034.93	2.4	5.0	3.5
4000.0	4059.65	5.4	5.5	1.5

Relative error: R.E. (%) = 100 × (mean concentration − nominal concentration)/nominal concentration.
[a] Relative standard deviation.

were investigated. ESI was adopted to quantify TMP and HTMP in dog plasma due to its lower levels of background noises. Parameters involving capillary temperature, vaporizer temperature and flow rate were optimized to obtain the protonated molecules of the analytes. The fragmentor energy was optimized to achieve maximum response of the fragment ion peaks. Selected ion monitoring (SIM) in positive mode was used for the quantitation of TMP, HTMP and caffeine at m/z 137.2, 153.2 and 195.2, respectively. Two detection channels were adopted, channel 1 (MSD1) for internal standard and channel 2 (MSD2) for TMP and HTMP.

3.2. Selectivity

The results for selectivity are shown in Fig. 2. The retention times were 4.4 min for TMP, 2.7 min for HTMP and 2.7 min for caffeine. The quasi-molecular ions for the quantitative determination of the analytes were m/z 137.2 for TMP, m/z 153.2 for HTMP and m/z 195.2 for caffeine. Fig. 2 indicated no interferences from endogenous substances in plasma with the analytes and internal standard.

3.3. Linearity

To evaluate the linearity of the LC–MS method, plasma calibration curves were determined in triplicate on three separate days. Representative regression equations for the calibration curve was $y = 5.10 \times 10^{-4}x + 4.97 \times 10^{-3}$ ($r = 0.9929$) for TMP and $y = 4.99 \times 10^{-5}x + 1.45 \times 10^{-3}$ ($r = 0.9957$) for HTMP. Good linearity was observed over the concentration ranges of 20–6000 ng/ml for TMP and 20–4000 ng/ml for HTMP.

3.4. Lower limit of quantitation

The lower limit of quantitation (LLOQ) is defined as the lowest concentration analyzed with an accuracy less than 20% and a precision less than 20%. LLOQ for both TMP and HTMP was found to be 20 ng/ml. At this level, the accuracy was 0.45% for TMP and 1.9% for HTMP, and the inter- and intra-precisions were 7.8% and 3.8% for TMP and 9.3% and 14.0% for HTMP. Representative SIM chromatogram of plasma sample with TMP and HTMP at LLOQ level is shown in Fig. 3.

3.5. Accuracy and precision

The accuracy and precision of the method were evaluated based on the data from QC plasma samples at three concentrations (40, 600 and 6000 ng/ml for TMP and 40, 1000 and 4000 ng/ml for HTMP) 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 (R.E.). The intra- and inter-day precision was expressed as the relative standard deviation (R.S.D.). As shown in Tables 1 and 2, for each QC level of TMP or HTMP, the inter- and intra-day precisions (R.S.D.) were ≤7.4% for TMP and ≤6.0% for HTMP, and accuracy (R.E.) was ±6.0% for TMP and ≤3.5% for HTMP, indicating the acceptable accuracy and precision of the method developed.

3.6. Extraction recovery

The extraction recoveries of TMP and HTMP from dog plasma were determined by comparing peak areas from plasma samples spiked before extraction with those from standard solutions at the same levels. The results showed that the extraction recoveries from dog plasma were 90.5 ± 11.4%, 108.0 ± 7.5% and 100.3 ± 4.9% at concentrations of 40, 600 and 6000 ng/ml for TMP, and 81.7 ± 1.8%, 86.3 ± 2.7% and 88.5 ± 3.4% at concentrations of 40, 1000 and 4000 ng/ml for HTMP.

3.7. Stability

The stability of TMP and HTMP in the supernatants after protein precipitation was determined. Both analytes were

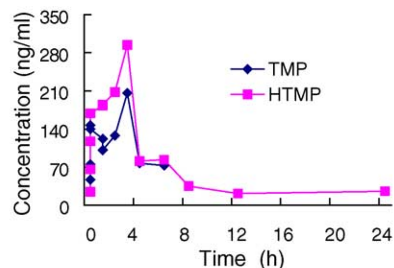

Fig. 4. Mean plasma concentration–time profiles of TMP and HTMP after oral administration of two aspirin and tetramethylpyrazine phosphate sustained release tablets (equivalent to 300 mg TMP) to six beagle dogs.

found to be stable for at least 12 h after sample preparation at ambient temperature with an accuracy (R.E.) ranging from −8.5% to −13.6% for TMP and from 5.8% to 10.9% for HTMP at three levels of QC samples.

3.8. Application of the developed LC–MS method

The LC–MS method achieved satisfactory results for the determination of TMP and HTMP in dog plasma and was successfully used for the pharmacokinetic study of a compound tetramethylpyrazine phosphate sustained release tablets following oral administration to six beagle dogs. The mean plasma concentration–time profiles for TMP and HTMP are shown in Fig. 4, which provides the bases for the further development of tetramethylpyrazine formulation products.

4. Conclusions

A simple and sensitive liquid chromatography–mass spectrometry method was developed for the simultaneous determination of TMP and HTMP in dog plasma. This method adopted a simple procedure for the sample preparation minimizing the losses of the analytes and facilitating its application in the pharmacokinetic studies of TMP formulated products. It also offered high sensitivity for both analytes with a lower limit of quantitation of 20 ng/ml, wide linearity and satisfactory selectivity. It can be used for the pharmacokinetic studies of tetramethylpyrazine formulation products.

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