Simultaneous Determination of 4-Methyl-Piperazine-1-Carbodithioc Acid 3-Cyano-3,3-Diphenylpropyl Ester Hydrochloride and its Major Metabolite in Rats by HPLC

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Abstract

A method for the simultaneous determination of TM208 and its major metabolite (sulfine 4-methyl-piperazine-1-carbodithioc acid 3-cyano-3,3-diphenylpropyl ester, TM208-SO) was developed and validated for the first time. The analytes were extracted from plasma samples by liquid–liquid extraction and analyzed using high-performance liquid chromatography. Flunarizine hydrochloride was used as the internal standard. Chromatographic separations were performed on a Diamonsil C_{18} analytical column. The mobile phases consisted of 20 mM ammonium acetate adjusted to pH 4.20 with acetic acid (solvent A) and acetonitrile (solvent B). The analytes were detected at 254 nm after linear gradient elution. The flow rate was 0.8 mL/min. Linearity was obtained over the concentration range of 0.104–5.20 µg/mL for TM208 and 0.145–5.80 µg/mL for TM208-SO in rat plasma. The limit of quantification was 0.104 µg/mL for TM208 and 0.145 µg/mL for TM208-SO, respectively. The inter- and intra-day precision was less than 12.8% for TM208 and 14.1% for TM208-SO. And the accuracy was 96.2–111.1% for TM208 and 95.5–108.6% for TM208-SO. This analytic procedure was applied to a pharmacokinetic study of TM208 and TM208-SO in rats, and the pharmacokinetic parameters were calculated.

Introduction

Besides being widely used as fungicides to protect crops from fungal diseases (1), dithiocarbamate derivatives have many other applications such as in photochemistry (2), catalysis in sulfur vulcanization of rubber (3) and polymerization (4,5), and detection and analysis of biological NO produced endogenously from NO synthases (6). Moreover, synthesized dithiocarbamates were found to both inhibit cell apoptosis (7–9) and induce cell apoptosis (10,11). Recently, we discovered several kinds of dithiocarbamates prepared in our laboratory possessing significant anticancer activity in vitro and in vivo (12,13), especially 4-methyl-piperazine-1-carbodithioic acid 3-cyano-3,3-diphenylpropyl ester hydrochloride (TM208) (Figure 1) with inhibition rates of 46–60% ($P < 0.01$–0.001), 39–52% ($P < 0.05$–0.001), and 18–59% against sarcoma 180 (S180), hepatocyte carcinoma 22 (H22), and implanted human gastric carcinoma in nude mice, respectively (14). Toxicological studies shows that in mice after intragastric administration of TM208, the maximal tolerance dose (MTD) was more than 1000 mg/kg, and the chronic toxicity was relatively low. Therefore, we consider that TM208 is a potential anticancer candidate drug and proceed to carry out its preclinical study. Metabolic study of TM208 in rats showed that TM208 is rapidly transformed into one main metabolite TM208-SO (15–17) (Figure 1). In this study, we established a method of measuring TM208 and its metabolite (TM208-SO) simultaneously and investigated the pharmacokinetics of them in rats for the first time.

Experimental

Chemicals and reagents

TM208 (purity > 99.6%) and TM208-SO (purity > 99.0%) were high-performance liquid chromatography (HPLC)-grade, syn-

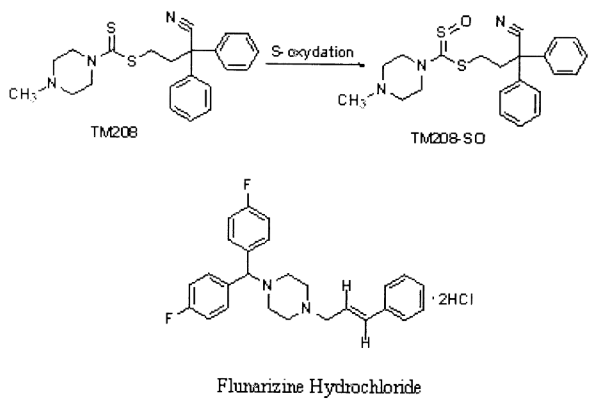

Figure 1. Chemical structures of TM208, its main metabolite (TM208-SO) in rat plasma, and flunarizine hydrochloride (internal standard).

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thesized by our research group, and the structure was confirmed by ^{1}H-NMR, ^{13}C-NMR, electrospray ionization-mass spectrometry (ESI-MS), and elemental analysis. Flunarizine hydrochloride was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and used as an internal standard (I.S.) in the assay. Methanol and acetonitrile were of HPLC-grade, and all other chemicals used were of analytical-grade. Distilled water, prepared from demineralized water, was used throughout the study.

Animals

Male Sprague-Dawley (S.D.) rats (Lab Animal Institute of Peking University Health Center, Beijing, China), weighing 255–270 g, were acclimated for at least one week under a standardized temperature (25–2°C), humidity (50–60%), and light (12:12 light–dark) conditions before being used in the study. Rats were allowed free access to standard food and tap water. Food was withheld the night before the study, but water was freely available. All care and handling of animals were performed with the approval of Institutional Authority for Laboratory Animal Care.

Equipment and chromatographic conditions

The Dionex Summit HPLC system consisted of P680 gradient pump, PDA-100 detector, TCC-100 column oven, ASI-100 autosampler, and Dionex Chromeleon chromatographic workstation (Dionex, Sunnyvale, CA). Sample separation was performed on a Diamonsil C_{18} column (250 mm × 4.6 mm i.d., 5 µm particle size, Dikma, Beijing, China) fitted with a C_{18} guard column (10 mm × 4.6 mm i.d., 5 µm, Tianjin, China). The analytes were detected at 254 nm after gradient elution. The optimized experimental conditions are as follows: the mobile phases consisted of 20 mM ammonium acetate adjusted to pH 4.20 with acetic acid (solvent A) and acetonitrile (solvent B). The mobile phases were delivered at using 45% B and 55% A between 0 and 10 min, followed by a linear gradient to 55% B and 45% A at 15 min, then to 60% B and 40% A at 30 min, and then isocratic for 5 min before returning to initial condition. The total run time was 35 min per sample. The flow rate was 0.8 mL/min, and the column temperature was maintained at 30ºC.

Sample preparation

To a 200 µL aliquot of rat plasma, 10 µL of the I.S. (10.0 µg/mL prepared in methanol) and 400 µL distilled water were added. This mixture was extracted with 2 mL redistilled ethyl acetate by vortex mixing for 1 min. After centrifugation at 1800 × g for 5 min, the upper organic layer was transferred to another tube and evaporated to dryness at 37°C under a gentle stream of nitrogen. The residue was dissolved in 200 µL of methanol, and 50 µL aliquot of the solution was injected into the HPLC system for analysis.

Methodological study for the determination of TM208 and TM208-SO in rat plasma

Preparation of standards and quality control samples

The stock standard solutions of TM208 and TM208-SO were prepared by dissolving the accurately weighted standard compounds in methanol to give final concentrations of 1040 µg/mL and 1160 µg/mL, respectively. The stock solutions were further diluted with methanol to achieve the spiking standard working solutions at concentrations of 0.104–5.20 µg/mL for TM208 and 0.116–5.80 µg/mL for TM208-SO. IS working solution (10.0 µg/mL) was prepared by diluting the 500 µg/mL stock solution of flunarizine hydrochloride with methanol. The spiking standard solutions (10 µL) were used to spike blank plasma samples (200 µL), either for calibration curves of both analytes or for quality control (QC) in the prestudy validation and during the pharmacokinetic study. All the working solutions were kept at 4°C and brought to room temperature before use.

Calibration curves

A series of standard working solutions of TM208 and TM208-SO (2.6.1) were added to 200 µL blank rat plasma, which enabled the concentrations of each standard series to be 0.104, 0.130, 0.208, 0.260, 0.520, 1.04, 2.60, and 5.20 µg/mL for TM208 and 0.116, 0.145, 0.232, 0.290, 0.580, 1.16, 2.90, and 5.80 µg/mL for TM208-SO. The calibration curves were constructed by performing a regression linear analysis of the peak area ratios of analytes to IS versus the concentrations of analytes. Concentrations of each analyte in QC samples or unknown samples were subsequently interpolated from these calibration curves.

Accuracy, precision, and extraction recovery

Accuracy and precision were assessed by determining QC samples at three concentration levels of 0.208, 0.520, and 1.04 µg/mL for TM208, 0.232, 0.580, and 1.16 µg/mL for TM208-SO. There were five QC samples for each concentration level, and each QC sample was analyzed two times on five different validation days. The accuracy of the method was determined by calculating the mean recovery of the target compounds by adding standards known concentrations to the samples. The mean recovery was obtained by the determined concentrations as a percentage of the nominal concentrations. The precision of the method obtained by QC samples was evaluated by relative standard deviation (RSD, $n = 5$).

The extraction recovery (absolute recovery, explaining the extraction efficiency) of TM208 and TM208-SO was evaluated by comparing the mean peak areas of five extracted low, medium, and high quality control samples to mean peak areas of five neat reference solutions (unprocessed). The recovery of I.S. was

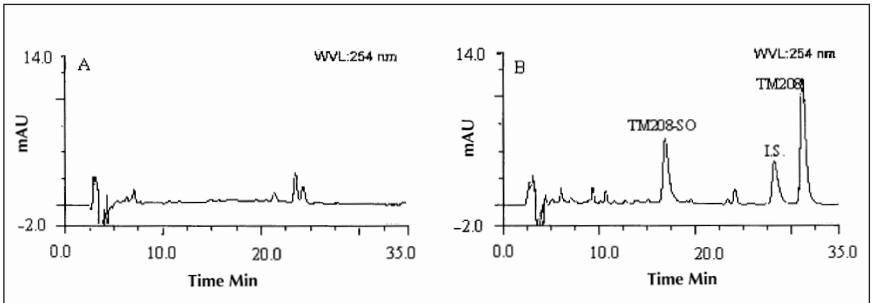

Figure 2. HPLC analysis of rat plasma samples: (A) chromatogram of a blank plasma sample and (B) chromatogram of a plasma sample at 4 h after oral administration of TM208 (250 mg/kg).

evaluated by comparing the mean peak areas of five extracted quality control samples to mean peak areas of five neat reference solutions (unprocessed) of the same concentration.

Selectivity

Selectivity was investigated by comparing chromatograms of blank plasma obtained from rats prior to dosing with those of corresponding standard plasma sample spiked with TM208, TM208-SO, and I.S. (10.0 µg/mL), and the plasma sample was obtained from rats after oral dose (250 mg/kg) of TM208 suspension.

Storage stability

The stability of TM208, TM208-SO, and I.S. stock solutions was evaluated after storage at refrigeration (4°C) for 1 day, 4 days, and 7 days. The stability of TM208 and TM208-SO in rat plasma was assessed in QC samples after storage in a freezer (-20ºC) for 7 and 10 days. The short-term stability was analyzed after the storage at room temperature for 4 h and 24 h. And the freeze-thaw stability of the samples was obtained over three freeze-thaw cycles by thawing in a water bath (37°C for 2–3 min) and refrozen in a frozer (–20°C for 12–24 h). For each concentration and each storage condition, three replicates were analyzed in one analytical batch. The concentration of TM208 and TM208-SO after each storage period was compared to the initial concentration as determined for the samples that were freshly prepared and processed immediately.

Application to pharmacokinetic study

The developed HPLC procedure was used to investigate the plasma profile of TM208 and TM208-SO in rats. A single oral dose (250 mg/kg) of TM208 suspension with distilled water was given to rats by gavage from 8:00 to 9:00 a.m. to avoid chronopharmacokinetic effects. At predetermined time intervals (0, 1, 2, 4, 6, 8, 12, 16, 24, 48, and 72 h), blood samples (about 0.5 mL each) were drawn from the ocular vein into heparinized tubes and separated by centrifugation at 1000 × g for 15 min, and subsequently the plasma was obtained and stored at –20°C before analysis.

Results and Discussion

Optimization of method

In this study, a simple and sensitive HPLC–UV method was developed and validated for the simultaneous determination of TM208 and its major metabolite (TM208-SO) for the first time. The method was applied to spiked biological matrices. Moreover, the method was applied to real samples of plasma after an oral administration. This kind of analytical method was previously reported in many relevant studies (18,19).

Separation was achieved on a C_{18} analytical column. In preparing the mobile phase, several combinations of buffer and organic phase have been tested. It was found that the mixture of acetonitrile–ammonium acetate (pH 4.2; 20 mM) (gradient) produced the best chromatographic separation for TM208, TM208-SO, I.S., and endogenous substances. When the mobile phase composition was in ratio of ACN–ammonium acetate (45:55, v/v), the plasma samples were separated very well, but retention times of TM208 was more than 50 min. When the ACN concentration in mobile phase was increased, the retention times decreased, TM208/I.S. and TM208-SO/endogenous substances were not separated well. Therefore, the gradient elute was optimized in our experiments. The used gradient is already mentioned in the Equipment and chromatographic conditions section. Retention times of TM208, TM208-SO, and I.S. were shorter, and satisfactory resolution and symmetry values were achieved. LLE is the commonly used extraction techniques and usually offers much cleaner sample that in turn makes the method more robust and scalable. According to properties of TM208, TM208-SO, and I.S., ethyl acetate was chosen as solvent of LLE in our experiments because it does not produce emulsification, makes experiments to be operated easily, can satisfy requirements of analysis samples, and is less harmful compared to some other organic solvents.

Method validation

Assay selectivity

Typical chromatograms of a blank rat plasma sample and plasma samples after oral administration of TM208 are illustrated in Figure 2. TM208, TM208-SO, and IS were well-separated, and no endogenous interference in rat plasma components was observed at the retention times corresponding to both analytes and IS. The nominal retention times for TM208, TM208-SO, and IS were 31.24, 16.88, and 28.26 min, respectively.

Linearity of calibration curves, limit of detection, and limit of quantification

Excellent linearity was obtained over the concentration range of 0.104–5.20 µg/mL for TM208 and 0.116–5.80 µg/mL for TM208-SO in rat plasma. Typical equation of calibration curves were as follows, TM208: $y = 2.0825x - 0.0934$ ($r = 0.9993$) and TM208-SO: $y = 0.9877x - 0.0103$ ($r = 0.9998$). The limit of detection (LOD), defined as the lowest sample concentration which can be detected (signal-to-noise ratio = 3), was 0.052 µg/mL for TM208 and 0.116 µg/mL for TM208-SO in rat plasma. The lower limit of quantification (LOQ), defined as the lowest sample concentration which can be quantitatively determined with suitable precision (≤ 15%) and accuracy (within ± 15%), was 0.104 µg/mL

Table I. Accuracy, Precisions, and Extraction Recovery of TM208 and TM208-SO in Rat Plasma*

Analyte	Nominal conc. (µg/mL)	Mean conc. determined (µg/mL)	Intra-run RSD (%)	Inter-run RSD (%)	Accuracy (%)	Extraction recovery (%)
TM208	0.208	0.231	12.8	10.8	111.1	81.2
	0.520	0.510	5.2	9.1	98.1	78.8
	1.04	1.00	4.2	12.4	96.2	87.0
TM208-SO	0.232	0.252	10.4	11.1	108.6	103.3
	0.580	0.554	9.2	6.0	95.5	85.3
	1.16	1.16	3.6	14.1	100.0	100.2

*n = 5 day, five replicates per day.

(RSD = 6.8%, n = 5) for TM208 and 0.145 µg/mL (RSD = 12.5%, n = 5) for TM208-SO.

Precision, accuracy, and extraction recovery

The data from QC samples in validation were examined by Excel to estimate the intra- and inter-run precision and accuracy of the method. The results are presented in Table I. The intra-run precision was less than 12.8% for each QC level of TM208 and less than 10.4% for each QC level of TM208-SO. The inter-run precision was less than 12.4% and 14.1% for TM208 and TM208-SO at each QC level, respectively. The accuracy was 96.2–111.1% for TM208 and 95.5–108.6 % for TM208-SO at three different concentrations. The precision and accuracy of the method met the acceptable criteria (precision: RSD < 15%; accuracy: recovery within 85–115%).

The extraction recoveries of analytes under the LLE conditions were 81.2%, 78.8%, and 87.0% (n = 5) for TM208 and 103.3%, 85.3%, and 100.2% (n = 5) for TM208-SO at each QC level, respectively. The recovery of I.S. averaged 64.8% (n = 5) in rat plasma.

All of the results indicated that the method was within the accepted range, and this method has good precision, accuracy, and extraction recovery.

Storage stability

The stock solutions were discovered to be stable for at least seven days (< 5% difference between fresh and test solution) when stored at 4°C. The stability experiments of TM208 and TM208-SO in rat plasma were designed to test effects of short-term storage at room temperature, storage in freezer (–20°C), and freeze–thaw cycles. These experiments were performed as described in the Storage stability section, and the stability data are summarized in Table II. It indicated that TM208 was relatively stable compared to TM208-SO under these storage conditions for its stability data is within the acceptable range (85–115%). TM208-SO was stable in a freezer (–20°C) for seven and 20 days but not adequately stable under room temperature and three freeze-thaw conditions because some stability data (116.5% and 136.0%) were beyond or very close (112.8% and 114.4%) to the acceptable criteria. The unstable property of TM208-SO coincided with some conclusion reported in previous studies (15). Therefore, the stability experiments suggest that plasma samples should be extracted and analyzed as soon as possible; otherwise, it needs to be refrigerated (–20°C) when detecting TM208-SO in plasma samples.

Application of analytical method in pharmacokinetic studies

The developed HPLC method was applied to determine concentrations of TM208 and its major metabolite TM208-SO in plasma samples obtained after oral administration of 250 mg/kg to five S.D. rats, and the results are presented in Figure 3, which shows mean plasma concentration–time curves of TM208 and TM208-SO after an oral administration (n = 5). The corresponding pharmacokinetic parameters of TM208 were calculated from the curve. The elimination rate constant (k) values were TM208: k = 0.0378 ± 0.0134/h and TM208-SO: k = 0.0139 ± 0.0068/h. The mean values of T_{max} were 4.741 ± 0.708 h for TM208 and 2.581 ± 0.265 h for TM208-SO, and the mean values of C_{max} were 0.860 ± 0.380 µg/mL for TM208 and 1.096 ± 0.387µg/mL for TM208SO, respectively. The elimination half-life time ($t_{1/2}$) of TM208 and TM208-SO were 20.404 ± 7.432 h and 60.987 ± 30.132 h, respectively. The areas under curve (AUC_{0-72}) values were 16.006 ± 3.454 h·µg/mL for TM208 and 15.880 ± 3.093 h × µg/mL for TM208-SO, respectively. Further, it is very interesting that mean plasma concentration–time curve of TM208-SO has two peak concentrations at 2 h and 6 h, which indicates that TM208-SO possibly experienced the process of hepatic metabolism and enterohepatic circulation because it is also the main metabolite in rat feces in previous studies (16).

Conclusion

A simple, accurate, and reliable HPLC method was established for the simultaneous determination of TM208 and its main metabolite TM208-SO in rat plasma samples. The method has good linearity, accuracy, precision, selectivity, and stability over the relevant concentration range. The described method is suitable for monitoring plasma level of TM208 and TM208-SO in pharmacokinetic studies.

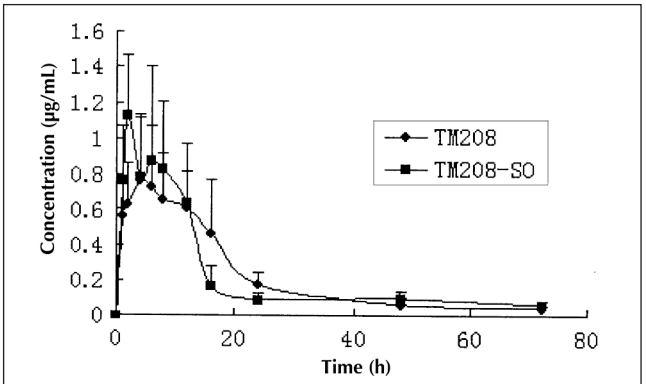

Figure 3. Mean plasma concentration–time profiles of TM208 and its metabolite TM208-SO after an oral administration of TM208 (250 mg/kg).

Table II. Stability of TM208 and TM208-SO In a Rat Plasma Sample*

Analyte	Nominal conc. (µg/mL)	Room temp. (× 4 h)	Room temp. (× 24 h)	–20°C × 7 days	–20°C × 20 days	Three freeze–thaw
TM208	0.208	91.4	106.4	94.7	96.1	93.4
	0.520	99.2	96.3	93.4	95.7	92.2
	1.04	97.9	96.4	89.1	88.4	97.0
TM208-SO	0.232	99.6	136.0	91.3	92.0	114.4
	0.580	116.5	107.7	94.5	88.0	112.8
	1.16	89.8	96.9	91.6	88.4	85.2

* Unit: percent of initial values, n = 3.

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