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Short communication

Quantitative determination of pimozide in human plasma by liquid chromatography–mass spectrometry and its application in a bioequivalence study

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ABSTRACT

A simple, sensitive and specific LC–ESI/MS method was developed for the determination of pimozide in human plasma. Pimozide and cinnarizine (internal standard) were isolated from plasma samples by liquid–liquid extraction. The chromatographic separation was accomplished on a Thermo Hypersil-HyPURITY C18 reversed-phase column (150 mm × 2.1 mm, i.d., 5 μm) with the mobile phase consisting of 5 mM ammonium acetate (pH 3.5, adjusted with acetic acid)–methanol–acetonitrile (39:5:56, v/v/v). The lower limit of quantification was 0.02 ng/mL, and the assay exhibited a linear range of 0.025–12.800 ng/mL. The established method has been successfully applied to a bioequivalence study of 2 pimozide formulations in 32 healthy male Chinese volunteers.

1. Introduction

Pimozide (3-[1-[4,4-bis(4-fluorophenyl)butyl]piperidin-4-yl]-1H-benzimidazol-2-one) is a drug with similar efficacy to other commonly used antipsychotic drugs such as chlorpromazine for schizophrenic patients [1]. It has special neurologic indications for Tourette syndrome and resistant tics [2]. A recent research suggested that pimozide, as a therapy, may be suitable for intracellular bacterial infections [3].

At present, several analytical methods for the determination of pimozide in biological matrix are described, including GC [4], HPLC [5], and capillary electrophoresis [6]. Most of them show low sensitivity and long retention time (RT). Kratzsch et al. [7] developed a sensitive (LOQ = 2 ng/mL) LC–APCI/MS method but with long RT (about 7.4 min). LC–MS/MS method was applied by Alderman [8] but it was lacking in analytical details. Recently, Kirchherr and Kuhn-Velten [9], and Kumazawa et al. [10] developed more sensitive (e.g. LOQ = 0.45 ng/mL) LC–MS/MS methods with shorter RT (e.g. 4.1 min) to determine many analytes simultaneously. These methods are competent for therapeutic drug monitoring (TDM) but not suitable for most laboratories to carry out studies involving low oral doses for current formulations and samples in high-throughput. According to the report, after a single oral dose of 4 mg pimozide tablet in 144 h, the mean plasma concentration of pimozide was about 0.1 ng/mL. In addition, plasma levels of pimozide can vary widely between individuals. It was essential to establish an assay capable of quantifying pimozide at concentrations down to 0.025 ng/mL for evaluation and interpretation of bioequivalence data. In our study, a simple, rapid (RT = 2.3 min) and sensitive LC–ESI/MS method was developed, and it has been successfully applied to a study with more than 900 plasma samples from healthy male volunteers.

2. Experimental

2.1. Materials and reagents

Pimozide standard (purity >99.2%) was kindly supplied by Vickmans Laboratories Ltd., Hong Kong. Cinnarizine standard (purity >99.5%) was purchased from Chengdu Great Southwest Pharmaceutical Company Ltd.; ultra-pure water prepared by a Millipore Milli-Q purification system (USA) was used as the mobile phase for LC–MS. All other chemicals and solvents were of analytical grade.

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2.2. Instrumentation

A Shimadzu LC–MS 2010 system (Japan) was used, equipped with LC-10AD *VP* low pressure gradient pump, CTO-10A *VP* column temperature oven, SCL-10AD *VP* system controller, and LCMSsolution chemstation (version 2.04). Separation was achieved on a Thermo Hypersil-Hypurity C18 column (150 mm × 2.1 mm, i.d., 5 μm, USA) at 45 °C. Compounds were eluted up to a total retention time of 4.5 min using an isocratic mobile phase consisting of 5 mM ammonium acetate (pH 3.5, adjusted with acetic acid)–methanol–acetonitrile (39:5:56, v/v/v) at 0.22 mL/min, and the injection volume was 5 μL. The ESI–MS spectrometry was as follows: capillary voltage was 4.5 kV; nebulizer nitrogen gas flow-rate was 1.5 L/min; drying N_2 flow was 10 L/min; curved desolvation line temperature was 250 °C; the gas used was of high purity and system control and data evaluation were carried out using LCMSsolution chemstation. The mass selective detector was operated in the ESI positive ionization mode with selected-ion monitoring at *m*/*z* 462 for pimozide and *m*/*z* 369 for cinnarizine.

2.3. Preparation of stock solutions and calibration standards

Primary stock solutions of pimozide (80 μg/mL) and cinnarizine (internal standard, I.S. 250 μg/mL) were prepared in methanol. Calibration standards of pimozide were prepared by spiking the appropriate amount of the stock solution into the blank plasma at 0.025, 0.050, 0.100, 0.200, 0.400, 0.800, 1.600, 3.200, 6.400 and 12.800 ng/mL. Quality control (QC) samples were prepared separately according to the same manner as described above and the final concentrations were 0.050, 0.800 and 6.400 ng/mL.

2.4. Sample preparation and extraction procedures

A 50 μL aliquot of I.S. (10 ng/mL) standard solution was added to 500 μL of each plasma sample and vortex-mixed. After a thorough vortex mixing for 30 s, the mixture was extracted with 1.2 mL ethyl acetate, vortex-mixed for 3 min, and centrifuged at 14,000 rpm for 5 min. The organic layer was removed and dried under a gentle stream nitrogen gas at 45 °C. The dry residue was dissolved with 50 μL mobile phase. After centrifugation, 5 μL of the clear supernatant was injected into the LC–MS system.

2.5. Method validation

The recovery and matrix effect were determined as described by Matuszewski et al. [11]. Briefly, recovery was determined by comparing the peak response between standards spiked in the plasma before or after the extraction, while the matrix effect was determined by comparing the peak areas between standards spiked in the plasma after the extraction or in solution. Intra-day and accuracy were evaluated by analyzing QC samples 5 times over 1 day, while inter-day precision and accuracy were estimated by analyzing QC samples 5 times on 3 different days. The precision was defined as the intra- and inter-day relative standard deviation (R.S.D., %). Accuracy was defined as the ratio of the mean computed value (*E*) to the true value (*T*) expressed as a percentage (accuracy, %). All the stability studies were conducted QC samples at 3 concentration levels with 5 determinations for each.

3. Results and discussion

3.1. Optimization of MS and separation conditions

The choice of ionization mode was guided by base peak with higher intensity in the LC–MS analysis. The mass spectra of pimozide and I.S. obtained from scan mode were characterized by a protonated molecular ion $[M+H]^+$ as the base peak. Fig. 1 shows the positive ion mass spectra of pimozide (A) and internal standard (B) by ESI selective ion monitoring. So, selective ion monitoring (SIM) mode ($[M+H]^+$ at *m*/*z* 462 and 369) was used for quantitative analysis of pimozide and I.S., respectively.

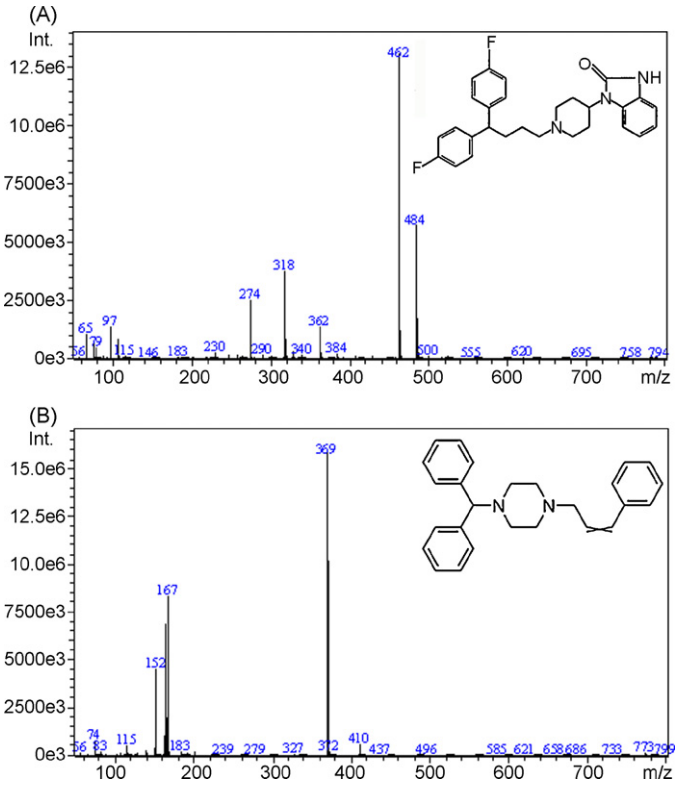

Fig. 1. ESI-MS positive ion scanning spectra and chemical structures of pimozide (A) and cinnarizine (B).

3.2. Method validation

The matrix effect for pimozide and cinnarizine was 92.2% and 87.6%, respectively. The ionization suppression of pimozide was negligible and the matrix effect of I.S. did not affect the quantification of pimozide. Six lots of blank plasma extracts from different sources were analyzed. Interference peaks from endogenous substances in free-drug human plasma at the retention time of pimozide and cinnarizine were not observed in any of the plasma lots. In addition, pimozide and I.S. were separately injected and selective ions were monitored. Fig. 2(A) shows 1 of the representative chromatogram of 6 lots of blank plasma extracts. Fig. 2(B) shows the selective ion chromatogram of the plasma sample at LOQ (0.025 ng/mL). No cross-talk was observed. The ten-point linear regression equation from calibration curve samples was obtained as follows: $y = 0.9387x + 0.0485$ with correlation coefficient $r^2 = 0.9984$. A good linearity between *y* and *x* was attainable over 0.025–12.800 ng/mL. The LOQ was 0.025 ng/mL. The method is precise and accurate (Table 1). Table 1 shows the stability data of pimozide kept at various storage conditions and freeze–thaw cycles.

3.3. Application to bioequivalence study

The bioequivalence study was approved by the Ethical Committee of Second Xiangya Hospital of Central South University, and

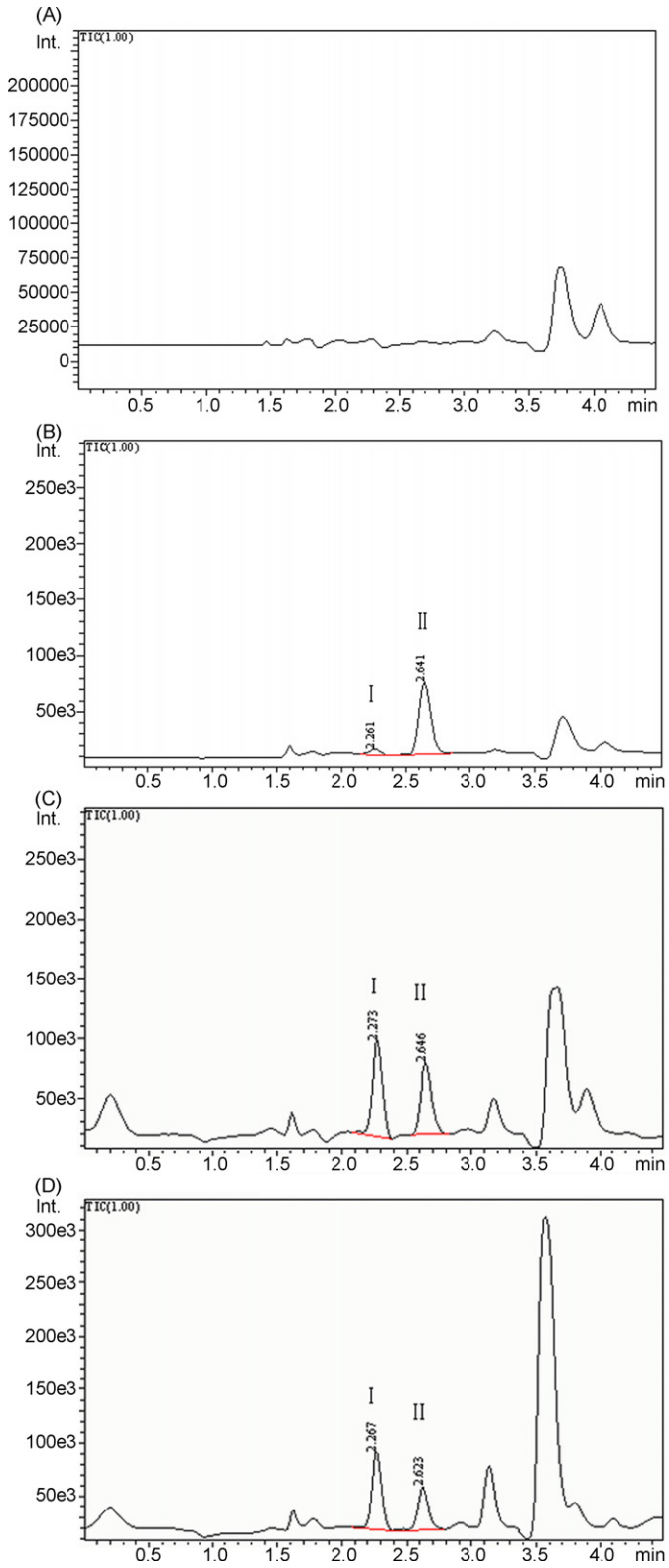

Fig. 2. Selective ion chromatograms of pimozide (I) and cinnarizine (I.S., II) (A) blank plasma, (B) blank plasma spiked with 0.025 ng/mL (LOQ) of pimozide and I.S., (C) blank plasma spiked with standard (1.10 ng/mL) and I.S., and (D) human plasma sample (1.80 ng/mL) after administration of pimozide and spiked with I.S.

all subjects signed the informed consent before participation. The study was based on a single dose, randomized, 2-treatment, and 2-period cross-over design. Thirty-two adult healthy male volunteers took 4 mg pimozide with 250 mL water. Blood samples were extracted from the forearm vein at 0, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120 and 144 h after the administration of preparation. The blood samples were immediately centrifugated at 3000 rpm for 10 min and the supernatants were transferred and stored frozen at −20 °C until analysis. The method was applied to determine the plasma concentration of pimozide. Mean plasma concentration time profiles of pimozide were presented in Fig. 3. Table 2 shows the pharmacokinetic parameters of pimozide. The means and standard deviations of AUC_{0-t}, $AUC_{0-\infty}$, and C_{max} for the test and reference formulation were similar, indicating similar pharmacokinetics of

Table 1
Precision, accuracy and stability data for pimozide in human plasma ($n = 5$).

Nominal concentration (ng/mL)	Precision R.S.D (%)	Accuracy (%)
Intra-day		
0.025 (LLOQ)	12.0	102.3
0.050	10.9	100.0
0.800	5.2	97.5
6.400	7.1	96.2
Inter-day ($n = 3$ days, five replicates per day)		
0.025 (LLOQ)	11.4	98.8
0.050	9.9	100.1
0.800	7.6	98.3
6.400	7.2	98.3
Short-term stability for 12 h in plasma at room temperature		
0.050	8.83	104.0
0.800	6.22	100.3
6.400	6.42	99.8
Long-term storage at −20 °C for 30 days		
0.050	9.55	100.0
0.800	6.93	100.9
6.400	6.52	99.7
Four freeze/thaw cycles		
0.050	9.34	104.0
0.800	6.47	100.1
6.400	7.16	98.7

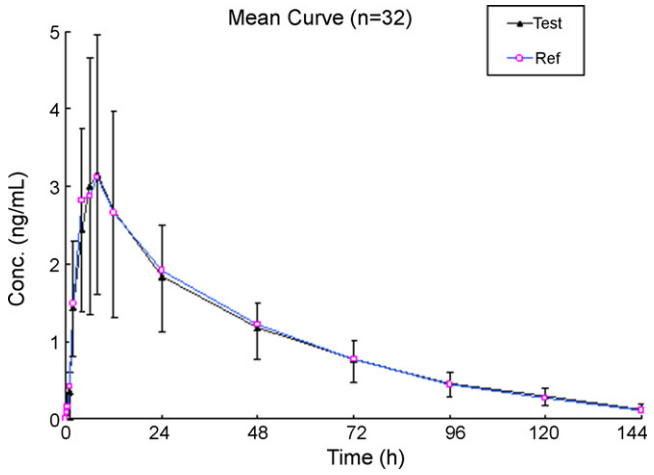

Fig. 3. Mean plasma concentration–time profile of pimozide from 32 healthy volunteers after a single oral dose of 4 mg test or reference tablets. *Test*—test formulation; *Ref*—reference formulation.

Table 2
Pharmacokinetic parameters (mean ± S.D.) of two oral formulations of single dose 4 mg pimozide in healthy subjects ($n = 32$).

Parameters	Test formulation	Reference formulation
t_{max} (h)	7.0 ± 2.6	6.4 ± 1.9
C_{max} (ng/mL)	3.532 ± 1.727	3.521 ± 1.611
AUC_{0-144} (ng h/mL)	144.091 ± 43.985	145.283 ± 51.273
$AUC_{0-\infty}$ (ng h/mL)	150.011 ± 44.679	150.654 ± 51.448
$t_{1/2}$ (h)	29.7 ± 8.2	29.3 ± 10.6

pimozide in the 2 formulations. The 90.0% confidence intervals for the ratio of test drug to reference drug in terms of AUC_{0-t} and C_{max} were within 80.0–125.0%, which is the range accepted by the State Food and Drug Administration of China.

4. Conclusion

The purpose of the present study was to develop a standard protocol for the bioequivalence testing of pimozide tablet. We devised and validated, a rapid and convenient HPLC–ESI/MS method using a simple liquid–liquid extraction procedure and isocratic chromatography, to determine pimozide levels in human plasma, and used this method to conduct a bioequivalence study by administering 4 mg of pimozide tablets to 32 healthy Chinese male volunteers. The assay is reproducible.

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