Rapid and Sensitive Liquid Chromatography–Electrospray Ionization-Mass Spectrometry Method for the Determination of Eperisone in Human Plasma: Method and Clinical Applications

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Abstract

A sensitive liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS) method for determination of eperisone in human plasma using buflomedil as the internal standard (IS) is established. After being made alkaline with saturated sodium bicarbonate solution, plasma samples are extracted with a mixture of diethyl ether–cyclohexane (1:1, v/v) and separated by high-performance liquid chromatography on a reversed-phase C_{18} column with a mobile phase of 10mM ammonium acetate buffer (adjusted the pH to 3.9 with acetic acid)–methanol (20:80, v/v). Eperisone is determined using ESI in a single-quadrupole MS. LC–ESI-MS is performed in the selected ion monitoring mode using target ions at m/z 260 for eperisone and m/z 308 for the IS. Calibration curves are linear over the ranges 0.02–20 ng/mL for eperisone. The intra- and interassay variability values are less than 9.0% and 11.5%, respectively. The mean plasma extraction recovery of eperisone is 91.7 ± 6.6%. The method has been successfully applied to study the pharmacokinetics of eperisone in healthy, male, Chinese volunteers. Pharmacokinetic parameters of the reference and test tablets have been compared.

Introduction

Eperisone (Figure 1) hydrochloride, 4'-ethyl-2-methyl-3-piperidinopropiopenone hydrochloride, is an antispastic agent (1–3) that has been shown to have very low bioavailability after oral administration and extensive first-pass metabolism (4,5). Recently, a new tablet formulation of eperisone hydrochloride was developed by Eisai (Suzhou, China). As entrusted by Eisai, we carried out the study to determine the relative bioavailability of this new tablet formulation compared with Myonal tablet (the commercial tablet formulation of eperision hydrochloride, 50 mg) (Eisai Pharmaceutical, Tokyo, Japan) and to assess the bioequivalence of the two products based on the plasma concentration data.

As a result of extensive first-pass metabolism, the human plasma concentration of eperisone is very low. To evaluate the pharmacokinetics of eperisone, an extremely sensitive method for determination of eperisone in human plasma is required. Fu et al. (6) developed a high-performance liquid chromatography (HPLC) method for determination of eperisone hydrochloride in tablets. A packed-column gas chromatographic (GC)–mass spectrometric (MS) method was mentioned (7), in which the limit of quantitation (LOQ) was 1.0 ng/mL. Takamatsu et al. (7) reported a more sensitive GC–MS method for determination of eperisione in human plasma, in which the LOQ was 0.2 ng/mL, the eperisone plasma concentrations attained 6 h following a single dose of 50–100 mg in most of volunteers. In fact, the LOQ of 0.2 ng/mL was not sensitive enough for pharmacokinetic research because the maximum plasma concentrations (C_{max}) of eperisone in some volunteers were only approximately 0.5 ng/mL, and
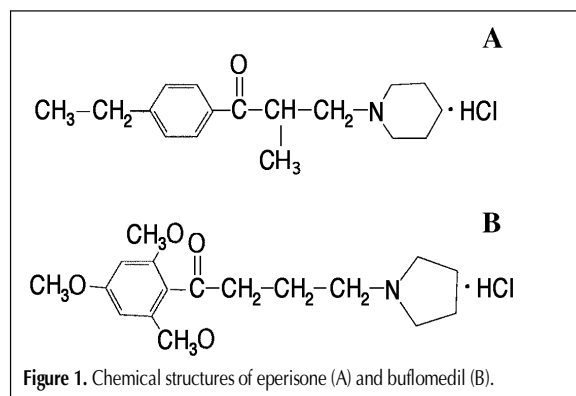
Figure 1. Chemical structures of eperisone (A) and buflomedil (B).

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many of the plasma levels on the terminal elimination phase were below 0.2 ng/mL.

This paper reports a rapid and sensitive liquid chromatography (LC)–electrospray ionization(ESI)-MS method that can determine eperisone plasma concentration as low as 0.02 ng/mL and allows the determination of an eperisone pharmacokinetic profile for 12 h, at least. This high-throughput method was used to quantitate 250 samples per day and was successfully applied to study pharmacokinetics of eperisone tablets in healthy, male, Chinese volunteers.

Experimental

Chemicals

Eperisone hydrochloride was supplied by Eisai. Buflomedil (Figure 1) hydrochloride was a gift from Jiangsu Institute of Drug Control (Nanjing, China). The test formulation was a new tablet formulation of eperisone hydrochloride (containing 50 mg of eperisone hydrochloride per tablet) (Eisai). The reference formulation was Myonal tablet (containing 50 mg eperisone hydrochloride per tablet (Eisai Pharmaceutical). Methanol was gradient grade for LC (Merck, Darmstadt, Germany). Diethyl ether, cyclohexane, and ethyl acetate were analytical-grade purity and were purchased from Nanjing Chemical Regent (Nanjing, China).

Instrumental conditions

HPLC analyses were performed using an Agilent 1100 LC–ESI-MS system (Agilent Technologies, Palo Alto, CA) with a Lichrospher ODS column (4.6 × 150 mm, 5 µm) (Hanbang Science, Huaiyin, China). The mobile phase was 10mM ammonium acetate buffer (adjusted the pH to 3.9 with acetic acid)–methanol (20:80, v/v), and the column temperature was maintained at 20ºC. A constant flow rate of 1.0 mL/min was used in the LC separation, and the eluent from the LC column was split into the MS ion source at a flow rate of 0.3 mL/min. LC–ESI-MS was carried out using nitrogen to assist nebulization. A quadrupole MS equipped with an ESI source was set with a drying gas (N_2) flow of 10 L/min, nebulizer pressure of 40 psi, drying gas temperature of 350ºC, capillary voltage of 4 kV, and the positive ion mode. The fragmentor voltage was 70 V. LC–ESI-MS was performed in selected-ion monitoring (SIM) mode using target ions at m/z 260 for eperisone and m/z 308 for the internal standard (IS).

Preparation of standard solutions, calibration standards, and quality control samples

Stock solutions of eperisone and the IS were prepared at 1 mg/mL in methanol and stored at –20ºC. Standard solutions of eperisone were prepared at concentrations of 1 µg/mL, 100 ng/mL, 10 ng/mL, and 1 ng/mL by diluting the stock solution with methanol. A solution containing 99.7 ng/mL IS was also prepared in methanol.

Calibration standards of eperisone were prepared by spiking appropriate amounts of the standard solutions in blank plasma obtained from healthy, nonsmoking volunteers. Quality control (QC) samples were prepared in blank plasma at concentrations of

0.02, 0.1, 0.5, 3, and 10 ng/mL for eperisone and stored at –20ºC.

Sample preparation

One-millileter plasma samples were extracted with a 5-mL mixture of diethyl ether–cyclohexane (1:1, v/v) after addition of 50 µL IS solution (99.7 ng/mL) and 1 mL saturated sodium bicarbonate solution. Following centrifugation and separation, the organic phase was evaporated in a water bath of 37ºC to dryness under a stream of nitrogen, the residue was reconstituted in 100 µL mobile phase, and a 30-µL aliquot was injected into the LC–ESI-MS system.

Assay validation

Linearity and limits of detection and quantitation

Calibration standards of ten concentrations of eperisone (0.02, 0.05, 0.1, 0.3, 0.5, 1, 3, 5, 10, and 20 ng/mL) were extracted and assayed. Peak-area ratios of eperisone to the IS obtained from selected-ion chromatograms were utilized for construction of calibration curves, using weighted (1/C) linear least-square regression of the plasma concentrations and the measured ratios. The linearity of the calibration curve was confirmed by plotting the peak-area ratios versus the concentrations of eperisone. The calibration curve was prepared and assayed daily with QC and clinical plasma samples.

The limit of detection (LOD) was defined as the plasma sample concentration of eperisone resulting in a peak height of three times the noise [signal-to-noise ratio (s/n) = 3]. The limit of quantitation (LOQ) was defined as the plasma sample concentration of eperisone resulting in a peak height of ten times the noise (s/n = 10).

Precision, accuracy, and specificity

The precision of the assay was determined at low, medium, and high concentrations of eperisone by replicate analyses of the QC samples. Intraday precision and accuracy were determined by repeated analysis of QC samples on one day ($n = 5$), and interday precision and accuracy were determined by repeated analysis of QC samples on five consecutive days ($n = 1$ series/day). The concentration of each sample was determined using calibration curves prepared on the same day. Accuracy is defined as the relative deviation in the calculated value (E) of a standard from that of its true value (T) expressed as a percentage (%RE). It was calculated using the following formula:

$$\%RE = (E - T)/T \times 100\% \qquad \text{Eq. 1}$$

Assay precision was defined as the relative standard deviation (RSD) from the mean (M), calculated using the equation:

$$\%RSD = SD/M \times 100\% \qquad \text{Eq. 2}$$

The specificity of the assay was checked by analyzing blank and subjects' predose plasma samples. The accuracy of the assays was checked by preparation of QC samples at the start of the clinical study. These QC samples were assayed, along with clinical samples, each day when the analyses were performed. The calculated concentrations of QC samples were compared on a day-to-day basis.

Extraction recovery

The extraction recovery of eperisone was determined at low, medium, and high concentrations, respectively. Recovery was calculated by comparison of the peak areas of eperisone extracted from plasma samples with those of injected standards.

Clinical study design and pharmacokinetic analysis

Twenty healthy, young, male Chinese volunteers participated in this study. After an overnight fast, each volunteer received tablets containing 100 mg of eperisone hydrochloride. Standard meals were provided at 3 and 9 h post dose. Blood was sampled predose and at 0.33, 0.66, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 10.0, and 12.0 h postdose for determination of plasma concentration of eperisone. Model-independent pharmacokinetic parameters were calculated for eperisone. The maximum plasma concentrations (C_{max}) and the time to those (T_{max}) were noted directly. The elimination rate constant (k_{el}) was calculated by linear regression of the terminal points of the semi-log plot of plasma concentration against time. Elimination halflife ($t_{1/2}$) was calculated from the formula:

Figure 2. Typical SIM mass chromatograms of blank plasma.

Figure 3. Typical SIM mass chromatograms of blank plasma spiked with eperisone (3.06 ng/mL) (A) and the IS (4.99 ng/mL) (B).

$$t_{1/2} = 0.693/\, k_{el} \quad \text{Eq. 3}$$

Area under the plasma concentration-time curve AUC_{0-12} to the last measurable plasma concentration (C_p) was calculated by the linear trapezoidal rule.

Results and Discussion

Conditions of chromatography

When selecting the mobile phase for LC–MS, attention should be paid to the influence of the mobile phase on the MS sensitivity. Because there is a tertiary amine in its structure, eperisone is a weak base and has proton affinity. To achieve a short turn-around time and high positive ionization efficiency, an acidic buffer solution was utilized in the mobile phase. Further experiment results showed that an ammonium acetate buffer solution could not only improve peak shapes of eperisone and IS but also increased the MS sensitivity to eperisone and IS. Thus, a 10mM ammonium acetate buffer (adjusted the pH to 3.9 with acetic acid) was finally adopted in the mobile phase. The ratio of aqueous solution in the mobile phase could significantly affect the ionization efficiency and MS sensitivity. The experiment results showed that the MS sensitivity increased, along with the decreasing of the ratio of ammonium acetate buffer solution in the mobile phase, until the ratio decreased to 10% (v/v). Finally, high sensitivity, good separation of eperisone, and short run time were obtained by using an elution system of 10mM ammonium acetate buffer (adjusted the pH to 3.9 with acetic acid)–methanol (20:80, v/v) as the mobile phase. Representative selected-ion chromatograms are shown in Figures 2–4, in which the retention times were 2.5 min for eperisone and 1.9 min for IS.

Conditions for ESI-MS

Because eperisone is a weak base and has proton affinity, the positive ion mode was adopted in the LC–MS. Both atmospheric pressure chemical ionization (APCI) and ESI sources were evalu-
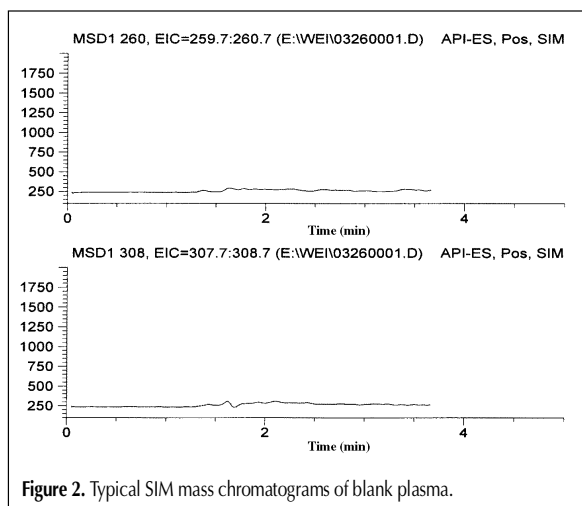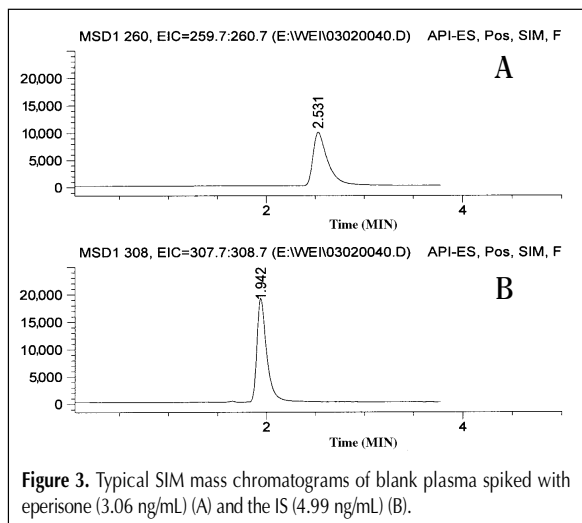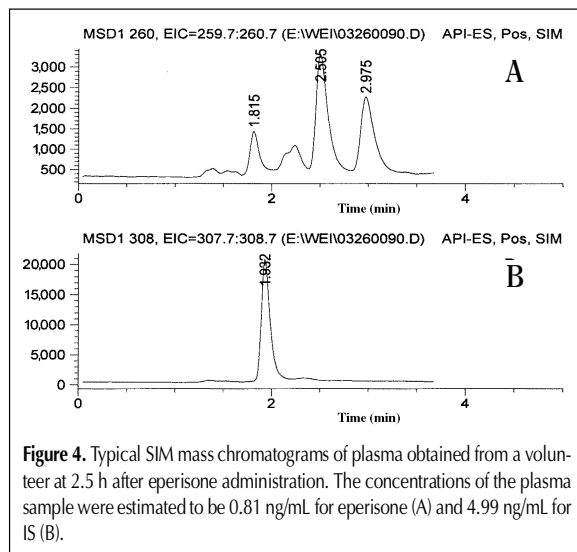

Figure 4. Typical SIM mass chromatograms of plasma obtained from a volunteer at 2.5 h after eperisone administration. The concentrations of the plasma sample were estimated to be 0.81 ng/mL for eperisone (A) and 4.99 ng/mL for IS (B).

ated for assay development in the positive ion mode. ESI produced greater sensitivity and exhibited less interference than we were able to achieve with APCI; thus, ESI was selected for this assay. In order to minimize undesirable fragmentation of eperisone, the fragmentor voltage was set at a lower value. Figure 5 shows a typical full-scan ESI-positive mass spectrum of eperisone at a 70 V fragmentor voltage. At lower fragmentor voltages, ESI produced abundant protonated molecular ion ([M+H]$^+$) m/z 260 for eperisone, with little or no fragmentation. By monitoring protonated molecular ion m/z 260 in the SIM mode, a highly sensitive assay for eperisone was achieved. In order to determine the optimal fragmentor voltage, the intensities of protonated molecular ion ([M+H]$^+$) m/z 260 were compared at fragmentor voltages of 50, 60, 70, 80, 100, 120, and 150 V in the SIM mode. The results showed that the highest sensitivities could be obtained by using a 70 V fragmentor voltage. Therefore, a 70 V fragmentor voltage was used to carry out LC–ESI-MS in the SIM mode. At this fragmentor voltage, the most intensive ion in the mass spectrum of IS was also its protonated molecular ion ([M+H]$^+$) m/z 308 (Figure 5). Therefore, protonated molecular ion ([M+H]$^+$) m/z 308 of IS was selected as the target ion of IS in the SIM.
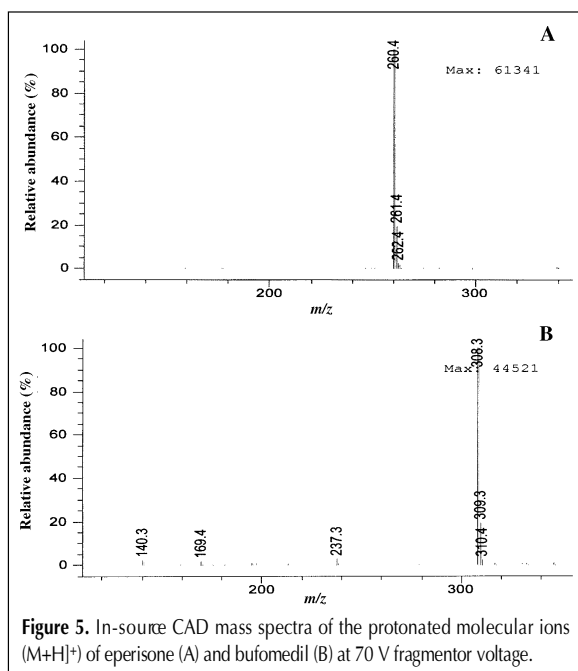
Method validation

Calibration curve and sensitivity

The calibration curves of eperisone showed good linearity in the range 0.02–20 ng/mL. The linear regression data are presented in Table I. LOD (defined at s/n = 3:1) and LOQ (defined at s/n = 10:1) for eperisone in plasma were 0.007 and 0.02 ng/mL, respectively. Those data show that this assay is sensitive enough for pharmacokinetic study of eperisone. Calibration curves were prepared with each batch of clinical samples.

Precision and accuracy

The intra- and interday (n = 5) precision and accuracy are summarized in Table II. Those results in Table II demonstrate that the precision and accuracy of this assay are acceptable.

Figure 5. In-source CAD mass spectra of the protonated molecular ions (M+H]$^+$) of eperisone (A) and bufomedil (B) at 70 V fragmentor voltage.

Table I. Regression Parameters for the Calibration Curves of Eperisone in Human Plasma

Batch	Slope	Intercept	Correlation coefficient
1	0.2230	0.0013	0.9991
2	0.2140	0.0032	0.9994
3	0.2153	0.0017	0.9993
4	0.2196	0.0015	0.9999
5	0.2214	–0.0007	0.9998

Table II. Precision and Accuracy of the Assay for Determination of Eperisone in Plasma (n = 5)

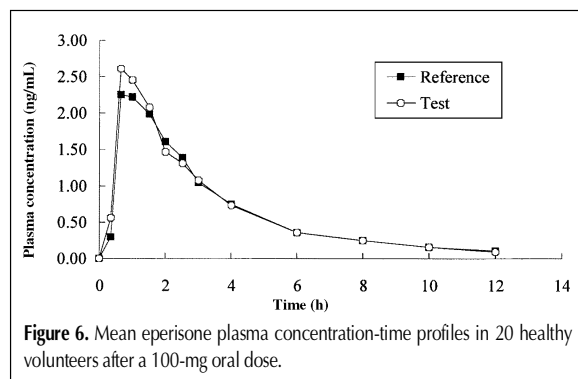|	Intra-assay			Interassay		
Added to plasma (ng/mL)	Measured concentration (mean ± SD) (ng/mL)	%RE	%RSD	Measured concentration (mean ± SD) (ng/mL)	%RE	%RSD
0.02	0.0211 ± 0.00189	5.50	8.96	0.0218 ± 0.00251	8.95	11.5
0.1	0.0986 ± 0.00601	–1.39	6.10	0.106 ± 0.0110	6.00	10.4
0.5	0.517 ± 0.0291	3.40	5.63	0.529 ± 0.0301	5.80	5.69
3	2.98 ± 0.109	–0.667	3.66	2.95 ± 0.131	–1.67	4.44
10	10.1 ± 0.240	1.20	2.38	10.1 ± 0.318	0.90	3.15

Figure 6. Mean eperisone plasma concentration-time profiles in 20 healthy volunteers after a 100-mg oral dose.

Table III. Pharmacokinetic Parameters for 20 Volunteers after Administration of a Single Dose of 100 mg Eperisone Hydrochloride (Mean ± SD)

Parameters	Test tablet	Reference tablet
$t_{1/2}$ (h)	2.81 ± 0.470	2.66 ± 0.416
C_{max} (ng/mL)	3.08 ± 3.58	2.75 ± 2.75
T_{max} (h)	1.1 ± 0.39	1.1 ± 0.51
MRT (h)	4.05 ± 0.827	4.00 ± 0.716
$AUC_{0-12 \times h \times ng/mL}$	8.03 ± 10.5	7.86 ± 9.34
150.82		

Extraction recovery

Ethyl acetate, diethyl ether and cyclohexane were evaluated as extraction solvents. The mixture of diethyl ether–cyclohexane (1:1, v/v) was chosen as the extraction solvent for its higher extraction efficiency of eperisone. The extraction recoveries of eperisone and IS can be promoted by alkalizing the plasma samples with saturated sodium bicarbonate solution; thus, 1 mL saturated sodium bicarbonate solution was added to 1 mL plasma sample before extraction. In this assay, the mean plasma extraction recovery of eperisone was $91.7 \pm 6.6\%$.

Pharmacokinetic study

The method previously described was successfully applied in the pharmacokinetic study in which plasma concentrations of eperisone in 20 healthy, male Chinese volunteers were determined up to 12 h after administration of test or reference tablets containing 100 mg eperisone hydrochloride. The mean plasma concentration-time curves of eperisone are shown in Figure 6. The mean pharmacokinetic parameters of two formulations for 20 volunteers are calculated and summarized in Table III. Eperisone was rapidly absorbed from both formulations. Plasma eperisone concentrations showed a very large interindividual variation for both formulations at all sampling times, which was also reflected in the calculated pharmacokinetic parameters. The relative bioavailability of test formulation was $101 \pm 13.2\%$, based on the test–reference ratios of AUC. Results of variance analysis and two one-sided t-tests showed that there was no statistical significant difference between the two formulations in the AUC and C_{max}. In the case of T_{max}, comparison between the two formulations was carried out by the Wilcoxon ranked sign test for matched pairs. No significant difference was observed in this parameter between the two formulations. Thus, the test formulation was bioequivalent to the reference formulation.

Conclusion

The method achieved good sensitivity and specificity for the determination of eperisone in human plasma. This simple and rapid assay is suitable for pharmacokinetic study of eperisone in human subjects. The two formulations of eperisone hydrochloride were bioequivalent.

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