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Short communication

Determination of tolperisone in human plasma by liquid chromatography/tandem mass spectrometry for clinical application

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ABSTRACT

We have developed and validated a simple, rapid, and sensitive liquid chromatography analytical method employing tandem mass spectrometry (LC–MS/MS) for the determination of tolperisone, a centrally acting muscle relaxant, in human plasma. After liquid–liquid extraction with methyl *t*-butyl ether, chromatographic separation of tolperisone was performed using a reversed-phase Luna C_{18} column (2.0 mm × 50 mm, 5 μm particles) with a mobile phase of 10 mM ammonium formate buffer (pH 3.5) – methanol (12:88, v/v) and quantified by tandem mass detection in ESI positive ion mode. The flow rate of the mobile phase was 250 μL/min and the retention times of tolperisone and the internal standard (IS, dibucaine) were both 0.6 min. The calibration curves were linear over a range of 0.5–300 ng/mL ($r > 0.999$). The lower limit of quantification, using 200 μL human plasma, was 0.5 ng/mL. The mean accuracy and precision for intra- and inter-day validation of tolperisone were within acceptable limits. The LC–MS/MS method reported here showed improved sensitivity for quantification of tolperisone in human plasma compared with previously described analytical methods. Lastly, the validated method was successfully applied to a pharmacokinetic study in humans.

1. Introduction

Tolperisone (2-methyl-1-(4-methylphenyl)-3-(1-piperidyl) propan-1-one) is a centrally acting muscle relaxant that is used for relieving spasticity of neurological origin and muscle spasms associated with painful locomotor diseases [1–3]. There is little information about the pharmacokinetic behavior of tolperisone in humans, in which rapid oral absorption, very low oral bioavailability, and short elimination half-life have been observed [4,5].

Only a few reports on the analytical methods for the determination of tolperisone in human plasma have been described, using potentiometry [6], spectrophotometry [7], high performance thin layer chromatography [8], gas–liquid chromatography [4], and high performance liquid chromatography (HPLC) with UV detection [9,10] or tandem mass spectrometry [11]. Importantly, past studies have employed a relatively large volume of plasma (>500 μL) for the determination of tolperisone in humans. In addition, longer retention times and insufficient sensitivity are obstacles for executing successful and reliable pharmacokinetic studies of tolperisone. Furthermore, the plasma concentrations of tolperisone can be measured only up to 5–7 h after the drug intake due to the relatively higher lower limit of quantification (LLOQ) of previous assay methods.

In this study, a simple, rapid, and sensitive method using a liquid chromatography–tandem mass spectrometry (LC–MS/MS) system for the determination of tolperisone in human plasma is described. Evaluation of the time-profile of plasma concentrations and pharmacokinetic parameters of tolperisone in humans validated this method.

2. Materials and methods

2.1. Reagents and chemicals

Tolperisone hydrochloride was purchased from Quantum Chemicals Ltd. (Auckland, New Zealand). Dibucaine hydrochloride and ammonium formate were purchased from Sigma–Aldrich (MO, USA). HPLC grade methanol was purchased from Honeywell Burdick & Jackson (NJ, USA). All other chemicals were of analytical grade and used without further purification.

2.2. Chromatographic instruments and conditions

Chromatography was performed using an Agilent 1200 series HPLC system (Agilent Technologies Inc., Santa Clara, CA,

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USA). Separation was carried out on a 30 °C Luna C_{18} column (2.0 mm × 50 mm, 5 μm, Phenomenex Inc., Torrance, CA, USA). The mobile phase consisted of 10 mM ammonium formate buffer (pH 3.5) and methanol (12:88, v/v) at a flow rate of 250 μL/min. The autosampler was maintained at 4 °C. The total run time for each sample analysis was 2.0 min.

Mass spectrometric detection was performed on an API 3200 tandem mass spectrometer (Applied Biosystems/MDS SCIEX, Toronto, ON, Canada) equipped with an electrospray ionization (ESI) source. The mass spectrometer was operated in positive ion mode. The tandem mass spectrometry conditions for tolperisone and the internal standard (IS, dibucaine) were optimized by carrying out full scans in positive ion detection mode. The detection and quantification of tolperisone and IS were performed in multiple reaction monitoring (MRM) mode. Quadrupoles Q1 and Q3 were set to unit resolution. Data acquisition and quantitation were carried out using Analyst software version 1.4.2 (Applied Biosystems/MDS SCIEX).

2.3. Standard solution and quality control sample preparation

Stock solutions of 0.1 mg/mL tolperisone and dibucaine (IS) were prepared in deionized water. Standard working solutions (0.01, 0.04, 0.2, 1, 2, 4, and 6 μg/mL) were prepared by serial dilution of the stock solutions with deionized water. For preparation of calibration standards and quality control samples, appropriate aliquots of the stock solution or working solutions were added to drug-free human plasma. The final concentrations were 0.5, 2, 10, 50, 100, 200, and 300 ng/mL for the calibration standards and 0.5, 1.5, 25, and 250 ng/mL for quality control (QC) samples. The working IS solution (dibucaine 50 μg/mL) was prepared in deionized water. All stock, standard working and QC working solutions were stored at 4 °C.

2.4. Sample preparation

All samples were stored in a freezer at −70 °C and allowed to thaw at room temperature before processing. Briefly, 200 μL of each plasma sample and 10 μL of IS solution (dibucaine, 50 μg/mL) were added to a glass tube. After brief vortexing, 2 mL of methyl *t*-butyl ether (MTBE) was added and the mixture was vortexed for 45 s. After centrifugation at 2200 × *g* for 10 min, the organic layer was transferred to a new glass tube and evaporated to dryness under a gentle stream of nitrogen gas at 50 °C. The residue was reconstituted with 450 μL mobile phase and a 5 μL aliquot was injected into the HPLC system.

2.5. Method validation

Validation was performed based on 'Guidance for Industry: Bioanalytical Method Validation' from the US FDA (http://www.fda.gov/downloads/Drugs/Guidance ComplianceRegulatory Information/Guidances/ucm070107.pdf).

2.5.1. Selectivity and linearity

Selectivity was assessed by comparing the chromatograms of six different batches of plasma obtained from six subjects. The plasma samples were spiked with tolperisone and IS. The linearity of the method was evaluated using five different calibration curves (ranges 0.5–300 ng/mL). The calibration curves were obtained by plotting the area ratios of analyte and IS *vs.* the concentration of analyte by least-squares linear regression with $1/x$ (where x represents the concentration of each analyte in ng/mL) as the weighting factor. The LLOQ was defined as the lowest concentration yielding a signal to noise ratio of at least 10 with a coefficient of variation (CV) < 20% and accuracy of 80–120%. The LLOQ was analyzed five times for confirmation.

2.5.2. Matrix effect and recovery

The matrix effect and recovery tests were performed in triplicate at three different QC sample concentrations (1.5, 25, and 250 ng/mL). The matrix effect was determined by extracting blank human plasma from six different sources and then reconstituting the final extract in the injection solvent, which contained known amounts of the analyte and IS. Absolute recovery of the analyte in normal plasma was determined by extraction from blank human plasma samples spiked with the analyte. After extraction, recovery was calculated by comparing the responses of plasma QC samples that were spiked with analyte prior to extraction with the response of those that were spiked with blank plasma.

2.5.3. Accuracy and precision

Intra- and inter-day precision was determined by replicate analysis of five sets of QC samples that were spiked with four different concentrations of tolperisone within one day or on five consecutive days, respectively. The precision was determined to be the CV and the accuracy was expressed as the relative standard error (RSE (%) = measured concentration/targeted concentration × 100).

2.5.4. Stability

The stability of tolperisone in human plasma was tested in triplicate with three different concentrations of QC sample (1.5, 25, and 250 ng/mL). For short-term stability, frozen plasma samples (−70 °C) were kept at room temperature for 4 h before sample preparation. The freeze–thaw stability of the tolperisone was determined over three freeze-thaw cycles over a period of three days. In each freeze-thaw cycle, the spiked plasma samples were frozen for 24 h at −70 °C and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 12–24 h at −70 °C. Long-term stability was evaluated after storing the frozen plasma samples at −70 °C for 60 days. The stability of the prepared plasma samples was tested after keeping the samples in an autosampler at 4 °C for 24 h. Stability samples were processed and extracted along with freshly spiked calibration curve standards. Samples were considered stable if assay values were within the acceptable limits of accuracy (85–115%) and precision (±15%).

2.6. Pharmacokinetic application

Fourteen healthy Korean male volunteers were enrolled in this study. All subjects provided informed consent both verbally and in writing. The study was performed according to the Declaration of Helsinki and was approved by the Institutional Ethics Committee of the School of Pharmacy at Sungkyunkwan University (Suwon, Korea).

Each subject received a single 150 mg oral dose of tolperisone (Mydocalm® tablet, Han Lim Pharm. Co., Seoul, Korea) with 240 mL water. Venous blood samples were obtained at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, and 12 h after the administration of tolperisone. Blood samples were centrifuged immediately and the plasma fractions were stored at −70 °C until needed.

Pharmacokinetic parameters of tolperisone were estimated using non-compartmental methods and BA Calc 2007 (KFDA, Seoul, Korea). Actual blood sampling times were used and observed values were maximum plasma concentrations (C_{max}) and times to reach C_{max} (T_{max}). The area under the plasma concentration–time curve (AUC) was calculated using the linear trapezoidal rule. The half-life ($t_{1/2}$) was calculated from the following equation: $\ln 2/k_e$.

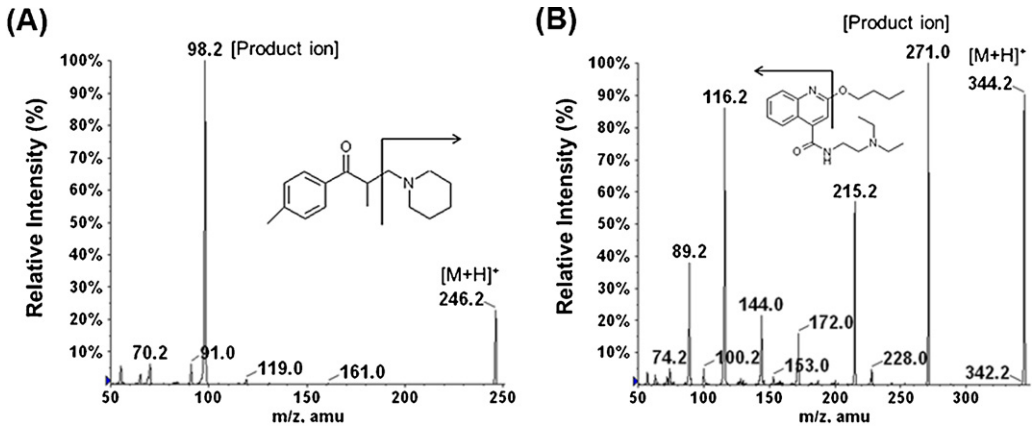

Fig. 1. Product ion mass spectra of (A) tolperisone and (B) dibucaine (IS).

3. Results and discussion

3.1. Method development

The tandem mass ESI conditions for tolperisone and IS were first optimized by carrying out full scans in positive ion detection mode. During a direct infusion experiment, the mass spectra for tolperisone and IS showed peaks as protonated molecular ions $[M+H]^+$ at 246.2 and 343.7 *m*/*z*, respectively. The major fragment ions observed in each product spectrum were at 98.1 *m*/*z* and 271.2 *m*/*z* for tolperisone and IS, respectively (Fig. 1). The mass parameters were optimized by observing the maximum responses of the product ions. The adjusted values of declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) for tolperisone were 36 V, 10 V, 21 V and 4 V, while those for IS (dibucaine) were 51 V, 10 V, 27 V and 6 V. The optimized curtain gas (CUR), collision gas (CAD), nebulizer gas (GS1), and turbo gas (GS2) parameters were 25 psi, 7 psi, 50 psi, and 50 psi, respectively.

Chromatographic conditions, particularly the composition of the mobile phase, were optimized by several trials to increase the signal of the analyte and minimize running times. The mobile phase consisted of a mixture of 10 mM ammonium formate buffer (pH 3.5) and methanol. Ammonium formate is primarily used to improve the peak shape and promote source ionization. The optimal proportion of ammonium formate buffer and methanol was tested from 5:95 to 20:80 (v/v), and we selected a ratio of 12:88 (v/v) as the final condition of the mobile phase. The flow rate of the mobile phase was set to 250 μL/min. The chromatographic sensitivity using a Luna C_{18} column (2.0 mm × 50 mm, 5 μm) was considered good.

3.2. Method validation

3.2.1. Selectivity and linearity

No endogenous interference was found at the retention times of tolperisone and IS. Fig. 2 shows representative chromatograms for blank human plasma (Fig. 2A); human plasma spiked with tolperisone (0.5 ng/mL) and IS (dibucaine 50 μg/mL; Fig. 2B); and

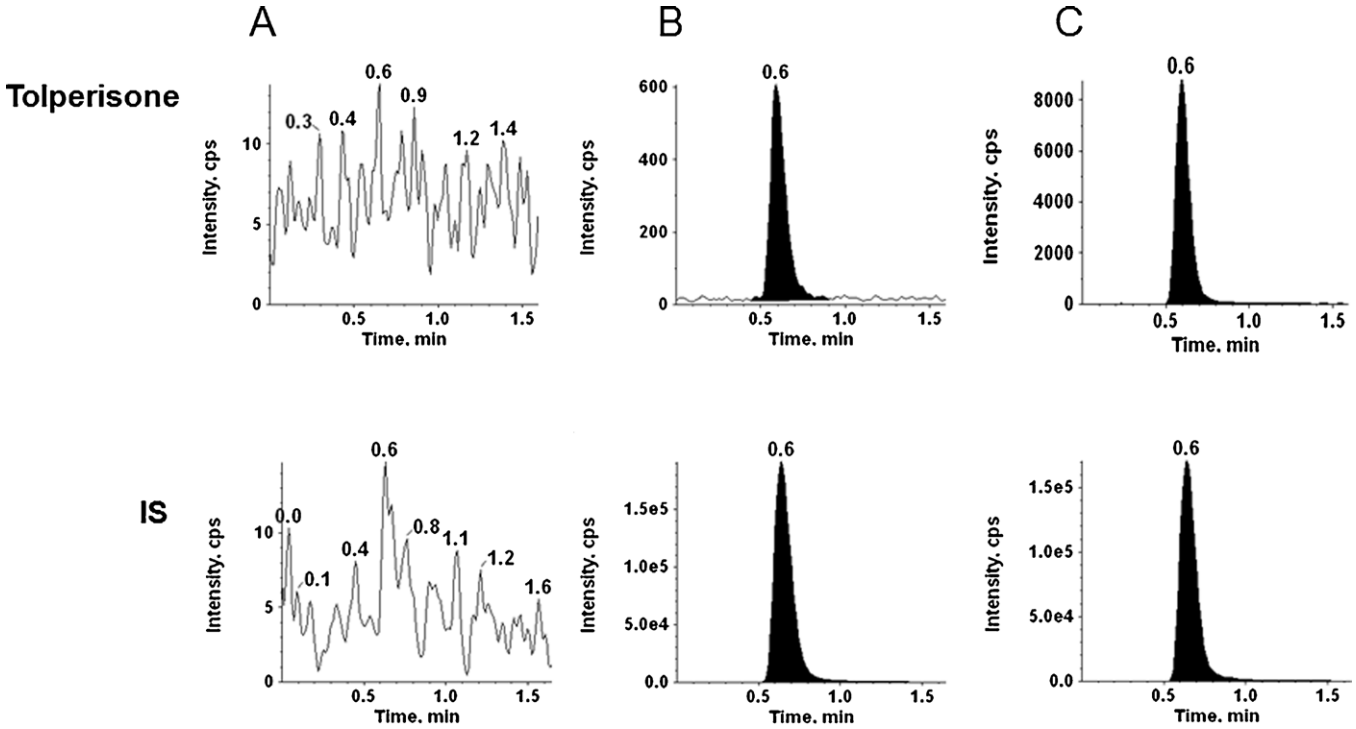

Fig. 2. Chromatograms of tolperisone and IS (dibucaine) in human plasma. (A) Blank human plasma; (B) blank human plasma spiked with tolperisone (0.5 ng/mL) and IS (50 μg/mL); (C) human plasma sample 3 h after administration of a single 150 mg oral dose of tolperisone.

Table 1
Precision and accuracy of the LC–MS/MS assay method for plasma tolperisone.

Targeted concentration (ng/mL)	Intra-day (n = 5)			Inter-day (n = 5)		
	Measured concentration (ng/mL)	Accuracy (%)	Precision (CV%)	Measured concentration (ng/mL)	Accuracy (%)	Precision (CV%)
0.5	0.582 ± 0.007	116.4	1.2	0.572 ± 0.009	114.3	1.5
1.5	1.49 ± 0.07	99.3	4.9	1.45 ± 0.02	96.5	1.7
25	24.4 ± 1.3	97.7	5.3	23.1 ± 0.9	92.4	4.0
250	244.4 ± 2.4	97.8	1.0	249.1 ± 5.2	99.6	2.1

a plasma sample obtained from a healthy volunteer at 3 h after oral administration of 150 mg tolperisone (Fig. 2C). The LLOQ was 0.5 ng/mL, and the signal-to-noise ratio of LLOQ was >10. The intra- and inter-day CV was not more than 5.3% (Table 1). The standard calibration curves were linear over tolperisone concentration ranges in human plasma of 0.5–300 ng/mL with mean correlation coefficients (r) of >0.999 (n = 5). The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/x$ weighting factor.

3.2.2. Recovery and matrix effect

Ethyl ether, ethyl acetate, dichloromethane, and MTBE were tested as solvents for extraction of tolperisone from human plasma. Among these, MTBE was found to be the best solvent, producing a clean chromatogram for blank human plasma samples with the best recovery and least matrix effects. The recovery of analyte from a 200 μL plasma sample using the liquid–liquid extraction procedure with MTBE was measured at three different concentrations of QC sample. Regardless of sample concentration, mean recoveries for tolperisone and IS were 81.7 ± 4.3% and 85.9 ± 1.8%, respectively.

Matrix effects, which are a result of ion suppression or enhancement of the analyte of interest, should be evaluated during method development because the assay accuracy and precision of the LC–MS/MS method can be significantly affected [12,13]. Observed matrix effects for tolperisone and IS ranged from 89.5 to 98.3% and the respective CV values in each concentration from six lots of plasma were less than 4.8%, indicating that no co-eluting substances influenced the ionization of either the analyte or IS [14]. This result indicates that the extraction efficiency for the analyte using liquid–liquid extraction was satisfactory, consistent, and concentration-independent. In addition, these results showed that ion suppression or enhancement from the plasma matrix was consistent under the current conditions.

3.2.3. Accuracy and precision

Table 1 provides a summary of the accuracy and precision for four concentrations of tolperisone (0.5, 1.5, 25 and 250 ng/mL). The intra-day and inter-day accuracies for tolperisone were 97.7–116.4% and 92.4–114.3%, respectively. The intra- and inter-day precisions for tolperisone were 1.0–5.3% and 1.5–4.0%, respectively. These results suggest that the method assessed in this study has satisfactory accuracy, precision and reproducibility.

3.2.4. Stability

Stability was assessed under a variety of conditions and the results are summarized in Table 2. Three freeze-thaw cycles of the QC samples did not appear to affect the quantification of the tolperisone. In addition, QC samples stored in a freezer at −70 °C remained stable for at least 60 days. Thawing the frozen samples and maintaining them at room temperature for 4 h had no effect on quantification. The extracted samples were also analyzed after at least 24 h at 4 °C. These results suggest that human plasma samples containing tolperisone can be handled under normal laboratory conditions without incurring any significant loss of detection.

Table 2
Stability of tolpersione in human plasma (n = 3).

Targeted concentration (ng/mL)	Measured concentration (ng/mL)	Accuracy (%)	Precision (CV%)
Post-preparative stability			
1.5	1.47 ± 0.04	98.0	2.5
25	24.6 ± 0.6	98.5	2.2
250	249.7 ± 0.6	99.9	0.2
Short-term stability			
1.5	1.49 ± 0.09	99.1	6.0
25	24.4 ± 0.6	97.6	2.4
250	249.3 ± 6.0	99.7	2.4
Freeze-thaw stability			
1.5	1.48 ± 0.05	98.9	3.2
25	24.2 ± 1.7	96.9	7.2
250	245.7 ± 6.4	98.3	2.6
Long-term stability			
1.5	1.43 ± 0.02	95.3	1.4
25	22.4 ± 0.3	89.7	1.1
250	246.0 ± 1.0	98.4	0.4

3.3. Incurred sample reanalysis

Incurred sample reanalysis (ISR) was performed for tolperisone. Of the 156 analyzed human plasma samples, 19 (12.1%) were chosen and reanalyzed to evaluate the reproducibility of the analytical method. The selection of samples covered the whole range of concentrations from the C_{max} to the terminal elimination phase in the pharmacokinetic profile. The original concentrations of tolperisone were confirmed to be within ±20% limits for 18 (94.7%) of the reanalyzed samples (data not shown). These ISR results met the acceptance recommendation on reproducibility for incurred samples from the third AAPS/FDA Bioanalytical Workshop (Crystal City III) [15].

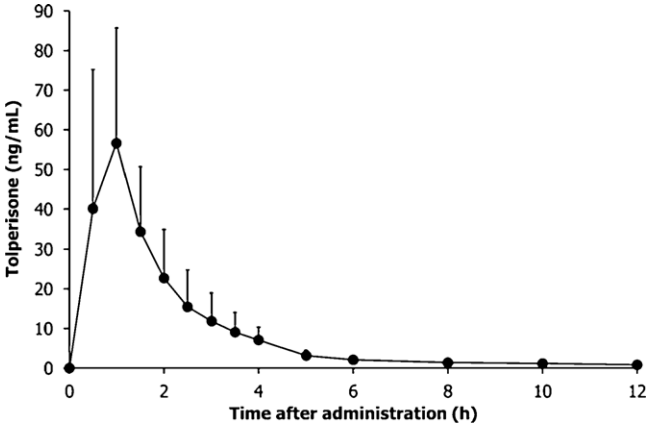

Fig. 3. Plasma concentration–time profile of tolperisone after administration of a single 150 mg oral dose of tolperisone in healthy male subjects (n = 14).

3.4. Pharmacokinetic application

Our method was successfully applied to a pharmacokinetic study of tolperisone after the administration of a single oral dose of 150 mg tolperisone. Fig. 3 shows the mean plasma concentration–time profile of tolperisone. The mean C_{max}, T_{max}, $t_{1/2}$, and AUC_{0-12} for tolperisone in fourteen healthy, male volunteers were 64.1 ± 30.0 ng/mL, 0.8 ± 0.2 h, 3.3 ± 1.3 h, and 111.2 ± 51.4 ng h/mL, respectively.

4. Conclusions

We have developed and validated a simple, rapid, and sensitive analytical LC–MS/MS method for the determination of tolperisone in plasma samples with LLOQ values of 0.5 ng/mL. This method was sufficiently sensitive for analyzing tolperisone in human plasma for up to 12 h after the administration of a single oral dose of 150 mg of tolperisone. The lower LLOQ and shorter run time compared with previous determinant methods make our new method particularly suitable for use in routine assays.

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