Quantification of Torsemide in Rabbit Plasma by Liquid Chromatography/electrospray Mass Spectrometry and its Application

Zhiyi WANG 1, Zhibin WANG 2, Jinjin WANG 3, Chan CHEN 3, Xiaole CHEN 4 & Lufeng HU 3*

1 The Second Affiliated Hospital of Wenzhou Medical College, Wenzhou 325035, China;
2 School of Basic Medical Sciences of Wenzhou Medical College, Wenzhou 325035, China;
3 The First Affiliated Hospital of Wenzhou Medical College, Wenzhou 325035, China;
4 Analytical and Testing Center of Wenzhou Medical College, Wenzhou 325035, China.

SUMMARY. A sensitive and simple liquid chromatography/electrospray mass spectrometry (LC-ESI-MS) method for determination of torsemide in rabbit plasma using one-step protein precipitation was developed and validated. After addition of midazolam as internal standard (IS), protein precipitation by acetonitrile was used in sample preparation. Chromatographically separation was achieved on an SB-C18 (2.1 mm×150 mm, 5 μm) column with acetonitrile-0.1 % formic acid as the mobile phase with gradient elution. Electrospray ionization (ESI) source was applied and operated in positive ion mode; selected ion monitoring (SIM) mode was used to quantification using target fragment ions m/z 349 for torsemide and m/z 326 for the IS. Calibration plots were linear over the range of 5-1000 ng/mL for torsemide in rabbit plasma. Lower limit of quantification (LLOQ) for torsemide was 5 ng/mL. Mean recovery of torsemide from plasma was in the range of 82.7-88.2 %. CV of intra-day and inter-day precision were both less than 15 %. This method is simple and sensitive enough to be used in pharmacokinetic research for determination of torsemide in rabbit plasma.

INTRODUCTION

Torsemide, N-[(isopropylamino)carbonyl]-4-[(3-methylphenyl)amino]pyridine-3-sulfonamide, is a pyridine-sulfonylurea type loop diuretic acting in the ascending limb of the loop of Henle 1-3. Loop diuretics mainly inhibit the Na+/2Cl- / K+ carrier from the luminal side of the cell 4. It is mainly used in the management of edema associated with congestive heart failure, kidney or liver disease and mild to moderate essential hypertension, either alone or with the combination of other antihypertensive drugs 5. In this study, we developed and validated an analytical method to determine the torsemide in biological samples for therapeutic drug monitoring. There only two high-performance liquid chromatography method for determination of torsemide in biological samples was developed 6,8. In recent years, highly sensitive and selective mass spectrometry technologies had led to a growing trend of developing fast analytical methods. In this paper, a simple and sensitive LC-MS method for the determination of torsemide in rabbit plasma using one-step protein precipitation was developed and validated. The LC-MS method successfully applied to a pharmacokinetic study of torsemide after intravenous administration to rabbits.

MATERIAL AND METHODS

Chemicals and Reagents

Torsemide (purity > 98.0 %) was purchased from Nanjing Xingang Pharmaceutical Co., Ltd. (Nanjing, China) and Midazolam (purity > 98.0 %) was purchased from Institute of Forensic Science under the Ministry of Justice (Shanghai, China). LC-grade acetonitrile and methanol were from Merck Company (Darmstadt, Germany). While LC-grade formic acid was Tedia Company (Cincinnati, USA). Ultra-pure water was prepared by a Millipore Milli-Q purification system (Bedford, MA, USA).
**Instrumentation and Conditions**

All analysis was performed with a 1200 Series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump, a degasser, an autosampler, a thermostatted column compartment, and a Bruker Esquire HCT ion-trap mass spectrometer (Bruker Technologies, Bremen, Germany) equipped with an electrospray ion source and controlled by ChemStation software (Version B.01.03 [204], Agilent Technologies, Waldbronn, Germany).

Chromatographic separation was achieved on an Agilent Zorbax SB-C18 (2.1 mm×150 mm, 5 µm) column at 30 °C, with acetonitrile-0.1 % formic acid as mobile phase. The flow rate was 0.4 mL/min. The HPLC gradient profile can be seen in Table 1.

Drying gas flow and nebuliser pressure was set at 7 L/min and 30 psi. Dry gas temperature and capillary voltage of the system were adjusted at 350 °C and 3,500 V, respectively. SIM mode was applied to quantify analyte using target ions at $m/z$ 349 for torsemide and $m/z$ 326 for IS, Figure 1.

**Calibration Standards and Quality Control Samples**

Individual stock solutions of torsemide (1.0 mg/mL) and midazolam (internal standard, IS) (100 µg/mL) were prepared in methanol. Working solutions for calibration and controls were prepared from the stock solution by dilution using methanol. Two µg/mL working standard solution of IS was prepared by dilution of the IS stock solution with methanol. All of the solutions were stored at 4 °C and were brought to room temperature before use.

Torsemide calibration standards were prepared by spiking blank rabbit plasma with appropriate amounts of the working solutions. Calibration plots were constructed in the range 5-1000 ng/mL for torsemide in rabbit plasma (concentrations 5, 10, 20, 50, 100, 200, 500 and 1000 ng/mL). Quality-control (QC) samples were prepared by the same way as the calibration standards, three different plasma concentrations (10, 100, and 1000 ng/mL). The analytical standards and QC samples were stored at -20 °C before use.

**Sample Preparation**

Before analysis, the plasma sample was thawed to room temperature. In a 1.5 mL centrifuge tube, an aliquot of 10 µL of the internal standard working solution (2.0 µg/mL) was added to 100 µL of collected plasma sample followed by the addition of 200 µL acetonitrile. The tubes were vortex mixed for 0.5 min. After centrifugation at 14900 g for 10 min, the supernatant (20 µL) was injected into the LC-ESI-MS system for analysis.

**Method Validation**

The selectivity of the method was evaluated by analyzing blank rabbit plasma, blank plasma spiked with torsemide and IS, and a rabbit plasma sample. Calibration curves were constructed by analyzing spiked calibration samples on three separate days. Peak area ratios of torsemide to IS were plotted against analyte concentrations, and standard curves were well fitted to the equations by linear regression with a weighting factor of the reciprocal of the concentration ($1/x$) in the concentration range of 5-1000 ng/mL.

To evaluate the matrix effect, blank rabbit plasma were protein precipitated and then spiked with the analyte at 10, 100, and 1000 ng/mL. The corresponding peak areas were then compared to those of neat standard solutions at equivalent concentrations, and this peak area ratio is defined as the matrix effect (ME). The matrix effect of IS was evaluated at the working concentration (200 ng/mL) in the same manner.

<table>
<thead>
<tr>
<th>Load time (min)</th>
<th>Pump flow (µL/min)</th>
<th>Formic acid (%)</th>
<th>Acetonitrile (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>400</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>3.0</td>
<td>400</td>
<td>15</td>
<td>85</td>
</tr>
<tr>
<td>7.0</td>
<td>400</td>
<td>15</td>
<td>85</td>
</tr>
<tr>
<td>8.0</td>
<td>400</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>11.0</td>
<td>400</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 1. HPLC gradient for detection of torsemide in rabbit plasma.

![Figure 1. Chemical structure of torsemide (a) and IS (b).](image-url)
Accuracy and precision were assessed by the determination of QC samples at three concentration levels in six replicates (10, 100, and 1000 ng/mL) in three validation days. The precision was expressed by coefficient of variation (CV).

The recoveries of torsemide at three QC levels \( (n = 6) \) were determined by comparing peak-area of the analytes in QC samples to which the analytes were added post-protein precipitation at equivalent concentrations. The recovery of the IS was determined in a similar way.

The stabilities of torsemide in rabbit plasma were evaluated by analyzing three replicates of plasma samples at the concentrations of 10, 100, and 1000 ng/mL, which were exposed to different conditions. The short-term stability was determined after the exposure of the spiked samples at room temperature for 2 h, and the ready-to-inject samples (after protein precipitation) in the HPLC autosampler at room temperature for 24 h. The freeze/thaw stability was evaluated after three complete freeze/thaw cycles (-20 to 25 °C) on consecutive days. The long-term stability was assessed after storage of the standard spiked plasma samples at -20 °C for 30 days.

**Pharmacokinetic study**

Japanese male rabbits (2.1–2.3 kg) were raised from Laboratory Animal Center of Wenzhou Medical College (Wenzhou, China). Rabbits were intravenously given with torsemide via marginal ear vein at a dose of 5 mg/kg within 0.5 min. Blood samples (0.3 mL) were collected from the marginal ear vein into heparinized 1.5 mL polythene tubes at 0, 0.083, 0.167, 0.25, 0.5, 1, 2, 3, 4, 6, 12, and 24 h after dosing. The samples were immediately centrifuged at 3000 g for 5 min. The plasma obtained (100 µL) was stored at -20 °C until analysis.

**RESULTS AND DISCUSSION**

**Method Development**

The mobile phase played a critical role in achieving good chromatographic behavior (including peak symmetry and short analysis time) and appropriate ionization. Various combinations of acetonitrile, methanol, water and formic acid in water with changed content of each component were investigated and compared to identify the optimal mobile phase. Acetonitrile was chosen as the organic solvent because of its suitable sharper peak shape, compared to methanol. Formic acid added into the mobile phase could improve the sensitivity, therefore methanol-0.1 % formic acid was chosen as mobile phase. Gradient elution provided better peak symmetry, proper retention time, and avoided the matrix effects for the analyte and IS compared to isocratic elution \(^9\)-\(^11\). A flow rate of 0.4 mL/min produced good peak shapes and permitted a run time of 11 min.

An efficient clean-up for bio-samples to remove protein and potential interferences prior to LC-MS analysis was an important point in the studies. The simple and effective protein precipitation was employed in our work. Acetonitrile was chosen as the protein precipitation solvent because it exhibited better effect than methanol, trichloroacetic acid (10 %) or perchloric acid (6 %), which could provide acceptable recoveries.

**Selectivity and Matrix Effect**

Figure 2 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with torsemide and IS, and a plasma sample. No interfering endogenous substances were observed at the retention times of the analyte and IS. The ME for torsemide at concentrations of 10, 100, and 1000 ng/mL were measured to be 88.2 ± 8.1, 106.3 ± 7.4, and 91.7 ± 3.4 % \( (n = 6) \), respectively. The ME for IS (200 ng/mL) was 102.9 ± 4.8 %. As a result, ME from plasma was negligible in this method.

**Calibration Curve and Sensitivity**

The linear regressions of the peak area ratios versus concentrations were fitted over the concentration range 5-1000 ng/mL for torsemide in rabbit plasma. The typical equation of the calibration curve was: \( y = 0.0014x - 0.0041, r = 0.9989 \), where \( y \) represents the ratios of torsemide peak area to that of IS and \( x \) represents the plasma concentration. For torsemide, the present LC-ESI-MS method gave an LLOQ of 5 ng/mL with an accuracy of 115.6 % and a precision of 17.8 % in terms of CV.

**Precision, Accuracy and Recovery**

The precision of the method was determined by calculating CV for QCs at three concentration levels over three validation days. Intra-day precision was 13 % or less and the inter-day precision was 15 % or less at each QC level (10, 100, and 1000 ng/mL). The accuracy of the method ranged from 94.3 to 106.5 % at each QC level.

Assay performance data are presented in Table 2. The above results demonstrate that the values are within the acceptable range and the method is accurate and precise. Mean recoveries of torsemide were better than 82.7 %, Table 2. The recovery of the IS was 96.1 ± 6.5 % \( (n = 6) \).
The auto-sampler, room temperature, freeze-thaw and long-term (30 days) stability results indicated that the analyte was stable under the storage conditions described above since the bias in concentration was within ±15% of their nominal values, and the established method was suitable for the pharmacokinetic study.

### Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (ng/mL)</th>
<th>CV (%)</th>
<th>Accuracy (%)</th>
<th>Extraction Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Torsemide</td>
<td>10</td>
<td>12.7</td>
<td>14.3</td>
<td>Intra-day 103.0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>7.4</td>
<td>10.7</td>
<td>Inter-day 93.6</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>9.6</td>
<td>3.7</td>
<td>Intra-day 95.7</td>
</tr>
</tbody>
</table>

**Figure 2.** Representative LC-MS chromatograms of torsemide (1) and IS (2): (a) blank plasma; (b) blank plasma spiked with torsemide (20 ng/mL) and IS (200 ng/mL); (c) a rabbit plasma sample 4 h after Intravenous administration of torsemide (5 mg/kg).

### Stability

The auto-sampler, room temperature, freeze-thaw and long-term (30 days) stability results indicated that the analyte was stable under the storage conditions described above since the bias in concentration was within ±15% of their nominal values, and the established method was suitable for the pharmacokinetic study.

### Application of the Method

The method was applied to a pharmacokinetic study in rabbits. The plasma samples with analyte concentration above upper limit of quantitation were diluted with blank rabbit plasma. Pharmacokinetic analyses and plasma concentration versus time data were analyzed by DAS software (Version 2.0, Wenzhou Medical...
Pharmacokinetic parameters | Torsemide (Mean ± SD)  
--- | ---  
$t_{1/2}$ (h) | 2.60 ± 0.41  
CL (L/h) | 0.252 ± 0.022  
$C_{\text{max}}$ (ng/mL) | 42561.4 ± 2692.8  
$AUC(0-t)$ (h ng/mL) | 19930.89 ± 1685.98  
$AUC(0-\infty)$ (h ng/mL) | 19934.61 ± 1687.81  

Table 3. The main pharmacokinetic parameters after Intravenous administration of torsemide (5 mg/kg) in 6 rabbits. $t_{1/2}$, half-life; CL, plasma clearance; $C_{\text{max}}$, maximum plasma concentration; $t_{\text{max}}$, time to reach maximum plasma concentration; $AUC(0-t)$, the area under the plasma concentration-time curve from 0 to $t$; $AUC(0-\infty)$, the area under the plasma concentration-time curve from 0 to infinite.

CONCLUSION

A sensitive, simple and specific LC-ESI-MS method for the determination of torsemide in rabbit plasma was developed and validated over the concentration range of 5-1000 ng/mL. The method was validated to meet the requirements for pharmacokinetic determination of the torsemide in rabbit plasma.

REFERENCES