Determination of Sulpiride in Rabbit Plasma by LC-ESI-MS and its Application to a Pharmacokinetic Study

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SUMMARY. A sensitive and selective liquid chromatography-mass spectrometry (LC–MS) method for determination of sulpiride in rabbit plasma was developed and validated. The analyte and internal standard (IS) were extracted from plasma by liquid-liquid extraction using ethyl acetate, and chromatography involved Agilent Extend-C18 column (2.1 mm x 50 mm, 3.5 μm) using 0.2 % formic acid in water and acetonitrile (60: 40, v/v) as a mobile phase. Detection involved positive ion mode electrospray ionization (ESI), and selective ion monitoring (SIM) mode was used for quantification of target fragment ions m/z 342.0 for sulpiride and m/z 294.8 for estazolam (internal standard, IS). The assay was linear over the range of 10–2000 ng/mL for sulpiride, with a lower limit of quantitation (LLOQ) of 10 ng/mL for sulpiride. Intra- and inter-day precisions were less than 12 % and the accuracies were in the range of 94.1-108.7 % for sulpiride. This developed method was successfully applied for the determination of sulpiride in rabbit plasma for pharmacokinetic study.

INTRODUCTION
Sulpiride, (±)-5-(aminosulfonyl)-N-[(1-ethyl-pyrrolidin-2-yl)methyl]-2-methoxybenzamide, is a typical antipsychotic drug of the benzamide class which is reported to be a selective antagonist of central dopamine (D2, D3 and D4) receptors. It is mainly used in the treatment of psychosis associated with schizophrenia and major depressive disorder. It is also claimed to have mood-elevating properties. Nevertheless, some cases of serious side effects have also been reported, i.e., sulpiride could induced torsade de points and long QT syndrome 1,2. Neuroleptic malignant syndrome caused by sulpiride 3. Taking into account its relatively extensive practicability and possible health effects, it is thus vital to develop an analytical method to quantify sulpiride in biological fluids.

There are several peer-reviewed analytical methods for determination of sulpiride in biological samples, such as synchronous fluorescence spectroscopy 4,5, excitation-emission matrix fluorescence 6, capillary electrophoresis 7,8, linear sweep cathodic stripping voltammetry 9, high-performance liquid chromatography 10-14 and spectrofluorometry 15-17. In recent years, highly sensitive and selective mass spectrometry technologies had led to a growing trend of developing fast analytical methods. The mass spectrometry detection method has a much higher selectivity than fluorescence and ultra violet detector, and can separate analytes from co-eluents based on their mass-to-charge ratios.

In this paper, a fast and sensitive LC-MS method for the determination of sulpiride in rabbit plasma after a simple liquid-liquid extraction procedure was developed and validated.

EXPERIMENTAL
Chemicals and Reagents
Sulpiride (purity > 98 %) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Estazolam (1.0 mg/mL in methanol, IS) was obtained from Institute of Forensic Science under the Ministry of Justice (Shanghai, China).

KEY WORDS: LC-MS, Sulpiride, Pharmacokinetics, Rabbit plasma.

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LC-grade acetonitrile and methanol were from Merck Company (Darmstadt, Germany), and LC-grade formic acid was from Tedia Company (Cincinnati, USA). Ultra-pure water was obtained by a Milli-Q purification system from Millipore (Bedford, USA). All other chemicals were analytical grade and used without further purification.

**Preparation of Standard Solutions**

Stock solutions of sulpiride (1.0 mg/mL) and estazolam (100 µg/mL) were separately prepared in 10-mL volumetric flasks with methanol–water (50:50, v/v) and stored at 4 °C. Working solutions for calibration and controls were prepared from the stock solution by dilution using methanol–water (50:50, v/v). The IS working solution (1.0 µg/mL) was prepared by diluting its stock solution with methanol–water (50:50, v/v). Calibration curves were prepared using blank rabbit plasma spiked at concentrations of 10, 20, 50, 100, 200, 500, 1000 and 2000 ng/mL for sulpiride. Low, medium, and high quality control (QC) samples at concentrations of 20, 200, and 2000 ng/mL for sulpiride were prepared in a same way as the calibration standards.

**Instrumentation and Conditions**

The LC–MS system consisted of a 1200 Series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) 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 a Agilent Zorbax Extend-C18 column (50 mm × 2.1 mm, 3.5 µm) at 30 °C with acetonitrile (A) and 0.2% formic acid in water (B) (40: 60, v/v) as mobile phase. The flow rate was 0.3 mL/min.

Analyses were performed with the ESI source operated in positive mode with a drying gas (N2) at a flow rate of 6 L/min, nebulizer pressure of 20 psi, drying gas temperature of 350 °C, capillary voltage of 3.0 kV. SIM mode was applied to quantify analyte using target ions at m/z 342.0 for sulpiride and m/z 294.9 for IS (Fig. 1).

**Sample preparation**

The plasma samples were extracted using liquid-liquid extraction technique. One hundred µL aliquot of the plasma sample and IS working solution (10 µL) were taken into a 5 mL glass test tube, and 50 µL of 4% sodium hydroxide solution was added. Then 2 mL of extraction solvent (ethyl acetate) was added and vortex-mixed on a multiple vortexer for 1.0 min, followed by centrifugation at 1660 g for 5 min. The supernatant organic layer was transferred into a 5 mL glass test tube and dried under a stream of nitrogen at 40 °C. The dried eluent was dissolved in 200 µL of methanol–water (50:50, v/v) and 5 µL aliquot was injected into LC-MS.

**Method Validation**

Specificity was determined by analysis of blank rabbit plasma, without addition of sulpiride and IS. To evaluate the linearity, the calibration curves were generated using the analyte to IS peak area ratios by weighted (1/x²) least-squares linear regression on three consecutive days. The LLOQ was estimated in the process of calibration curve construction and defined as the lowest concentration for which precision was less than 20 % and accuracy within 80-120 %.

To evaluate the matrix effect, blank rabbit plasma samples were extracted and then spiked with the sulpiride at concentrations of 20, 200, and 2000 ng/mL. The corresponding peak areas were then compared to those of neat standard solutions at equivalent concentrations, and this peak area ratio was defined as the matrix effect (ME). The matrix effect of IS (100 ng/mL) was evaluated in the same manner.
QC samples at three concentration levels were analyzed to assess the accuracy and precision of the method. Again, the assays were performed on three separate days, and on each day, six replicates of the QC samples at each concentration level were analyzed. The assay precision for each QC level was determined as the relative standard deviation (RSD) of the measured concentrations. The intra- and inter-day precisions were required to be less than 15%, and the accuracy to be within 85-115%.

The extraction efficiency of sulpiride was evaluated by comparing peak area ratios of extracted QC samples with those of reference QC solutions reconstituted in blank plasma extracts. Stability in plasma was assessed in the autosampler at room temperature for 12 h and on storage at -20 °C for 30 days. The effect of three freeze–thaw cycles was also investigated.

Pharmacokinetics

Japanese male rabbits (2.1-2.3 kg) raised by Laboratory Animal Center of Wenzhou Medical College (Wenzhou, China) were used for this study. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Wenzhou Medical College and were in accordance with the Guide for the Care and Use of Laboratory Animals. Rabbits were intravenously given with sulpiride via marginal ear vein at a dose of 10 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, 0.0833, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 16, 24 h after dosing. The samples were immediately centrifuged at 1,660 g for 5 min. The plasma obtained (100 µL) was stored at -20 °C until analysis.

RESULTS AND DISCUSSION

Method Development

Electrospray ionization (ESI+) source was applied in this work, as it exhibited more sensitivity and better reproducibility for sulpiride compared with an atmospheric pressure chemical ionization (APCI+) interface. It was amazing that the sensitivity of SIM mode was much higher than the multiple reaction monitoring (MRM) mode with S/N of 12/1 for SIM mode and 2/1 for MRM mode at the concentration of LLOQ (10 ng/mL). This phenomenon maybe caused by the unstable daughter ions of sulpiride in MRM mode. Therefore, we chose the SIM as the detection mode in this study.

The liquid chromatographic conditions were developed to separate as many interference compounds as possible from the analytes. Different columns, such as Zorbax SB-C18 (50 mm × 2.1 mm, 3.5 µm) and Zorbax Extend-C18 (50 mm × 2.1 mm, 3.5 µm) were compared for the analysis. A Zorbax Extend-C18, 3.5 µm particle column from Agilent, demonstrating better selectivity and proper retention for sulpiride and IS, was chosen for the separation. The mobile phase was optimized by evaluating methanol-water and acetonitrile-water. Acetonitrile-water showed the lower background noise than methanol-water, while the addition of formic acid to mobile phase improved ionization efficiency. As a result, acetonitrile-0.2% formic acid in water 40:60 (v/v) was used as mobile phase, resulting in an acceptable run time of 3.0 min.

In our initial studies, protein precipitation with acetonitrile and methanol was tried, however, this approach caused ion suppression for sulpiride. Liquid–liquid extraction with ethyl acetate was used for plasma samples, offering a relatively clean sample.

Selectivity and Matrix Effect

Figure 2 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with sulpiride and IS, and a plasma sample obtained 2 h post intravenous administration. No interfering endogenous substances were observed at the retention times of the analyte and IS. The ME at concentrations of 20, 200, and 2000 ng/mL were 90.6, 89.4 and 92.5% for sulpiride (n = 6), respectively. The ME for IS (100 ng/mL) was 106.7% (n = 6).

Calibration Curve and Sensitivity

Typical equation of the calibration curves for sulpiride was: \( y = (0.0065±0.00057) C -0.0905±0.17511, r = 0.9980±0.000636 (n = 3) \). Where \( y \) represents the ratios of analyte peak area to that of IS and \( C \) represents the plasma concentration.

The LLOQ for the determination of sulpiride in plasma was 10 ng/mL. The precision and accuracy at LLOQ were 15.3% and 92.2% for sulpiride, respectively. The limit of detection (LOD), defined as a signal-noise ratio of 3, was 3 ng/mL for sulpiride in plasma.

Precision, Accuracy and Recovery

The precision of the method was determined by calculating RSD for QCs at three concentration levels over three validation days. Intra-day and inter-day precision was 12% or less for sulpiride. The accuracy of the method was
ranged from 94.1 to 108.7 % for sulpiride. The mean extraction efficiency of sulpiride was better than 76.7 %. The recovery of the IS (100 ng/mL) was 88.2 ± 3.4 %.

Assay performance data are presented in Table 1. The above results demonstrate that the method validation values are within the acceptable range and the method is accurate and precise.

### Stability
The auto-sampler, freeze-thaw and long-term (30 days) stability results indicated that sulpiride and IS were stable under the storage conditions described above since the accuracy were within 85-115% of their nominal values (Table 2).

### Application of the Method
The method was applied to a pharmacokinetic study in rabbits. Pharmacokinetic analyses and plasma concentration versus time data were analyzed by DAS software (Version 2.0, Wenzhou Medical College, China). The data of sulpiride plasma concentrations-time were fitted using DAS ver. 2.0 and the values of AIC (Akaike’s Information Criterion) and $R^2$ were calculated. As the results showing that the value of AIC is minimum of all, while $R^2$ is most close to 1 under the two-compartment model (weight coefficient = 1), we take the two-compartment model to analyze the data. Pharmacokinetic parameters were calculated by using two compartmental model and results are summarized in Table 3. The mean plasma concentration-time curves after intravenous administration of sulpiride (10 mg/kg) in 6 rabbits are shown in Figure 3.

#### Table 1. Precision, accuracy and extraction efficiency for sulpiride in rabbit plasma ($n = 6$).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Intra-day</th>
<th>Inter-day</th>
<th>Intra-day</th>
<th>Inter-day</th>
<th>Extraction efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulpiride</td>
<td>7.5</td>
<td>11.8</td>
<td>103.8</td>
<td>108.7</td>
<td>76.7 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>4.1</td>
<td>7.5</td>
<td>101.5</td>
<td>97.5</td>
<td>83.7 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>5.2</td>
<td>5.0</td>
<td>94.1</td>
<td>96.7</td>
<td>79.8 ± 2.4</td>
</tr>
</tbody>
</table>

#### Table 2. Summary of stability of sulpiride and IS under various storage conditions ($n = 3$).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Added</th>
<th>Measured Concentration</th>
<th>RSD (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-20 °C, 30 days</td>
<td>20</td>
<td>21.6 ± 1.6</td>
<td>7.6</td>
<td>108.0</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>1978.5 ± 150.4</td>
<td>6.5</td>
<td>98.9</td>
</tr>
<tr>
<td>(IS) 100</td>
<td></td>
<td>104.6 ± 7.9</td>
<td>4.8</td>
<td>104.6</td>
</tr>
<tr>
<td>3 freeze thaw</td>
<td>20</td>
<td>22.1 ± 1.7</td>
<td>8.6</td>
<td>110.5</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>1895.5 ± 144.1</td>
<td>6.7</td>
<td>94.8</td>
</tr>
<tr>
<td>(IS) 100</td>
<td></td>
<td>96.3 ± 7.3</td>
<td>5.3</td>
<td>96.3</td>
</tr>
<tr>
<td>Autosampler ambient</td>
<td>20</td>
<td>19.6 ± 1.5</td>
<td>4.3</td>
<td>98.0</td>
</tr>
<tr>
<td>12 h</td>
<td>2000</td>
<td>2056.4 ± 156.3</td>
<td>3.2</td>
<td>102.8</td>
</tr>
<tr>
<td>(IS) 100</td>
<td></td>
<td>97.1 ± 7.4</td>
<td>2.8</td>
<td>97.1</td>
</tr>
</tbody>
</table>

#### Table 3. The main pharmacokinetic parameters after intravenous administration of sulpiride (10 mg/kg) in 6 rabbits. $t_{1/2\alpha}$ half-life of distribution; $t_{1/2\beta}$ half-life of elimination; $CL$, plasma clearance; $C_{max}$ maximum plasma concentration; $t_{max}$, time to reach maximum plasma concentration; AUC$_{0\rightarrow t}$, the area under the plasma concentration-time curve from 0 to $t$; AUC$_{0\rightarrow \infty}$, the area under the plasma concentration-time curve from 0 to infinite.

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>Sulpiride (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{1/2\alpha}$ (h)</td>
<td>0.132 ± 0.048</td>
</tr>
<tr>
<td>$t_{1/2\beta}$ (h)</td>
<td>1.552 ± 0.402</td>
</tr>
<tr>
<td>$CL$ (L/h)</td>
<td>2.299 ± 0.160</td>
</tr>
<tr>
<td>$C_{max}$ (ng/mL)</td>
<td>6321.0 ± 1455.2</td>
</tr>
<tr>
<td>AUC$_{0\rightarrow t}$ (h ng/mL)</td>
<td>3727.37 ± 154.87</td>
</tr>
<tr>
<td>AUC$_{0\rightarrow \infty}$ (h ng/mL)</td>
<td>4363.06 ± 291.04</td>
</tr>
</tbody>
</table>

Figure 3.
REFERENCES


CONCLUSION

A sensitive, simple and specific LC-MS method with liquid–liquid extraction for the determination of sulpiride in rabbit plasma was developed and validated. The method was validated to meet the requirements for pharmacokinetics evaluation of the sulpiride in rabbit plasma after intravenous administration.

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Figure 2. Representative LC-MS chromatograms of sulpiride (1) and IS (2): (a) blank plasma; (b) blank plasma spiked with sulpiride (100 ng/mL) and IS (100 ng/mL); (c) a rabbit plasma sample 2 h after intravenous administration of sulpiride (10 mg/kg).

Figure 3. Mean (± SD) plasma concentration time profile with two compartmental model after intravenous administration of sulpiride (10 mg/kg) in 6 rabbits.