High-performance liquid chromatography-electronspray ionization mass spectrometry for determination of tiopronin in human plasma

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Abstract
A simple and sensitive HPLC/ESI-MS method for the determination of tiopronin in human plasma was described. Vitamin C and 2-mercaptoethanol (2-Me) were used as the reducer and the stabilizer to release and stabilize tiopronin from a dimmer and mix forms with endogenous thiols in the treatment of plasma samples. The analytes were separated on a Johnson Spherigel analytical column packed with 5 μm C8 silica, using the formic acid aqueous solution (pH 4.5) including tris(hydroxymethyl) aminomethane (Tris) and 2-Me (0.5 and 1 mM, respectively) as a mobile phase. Cyclamate was used as the internal standard (I.S.) for the quantification of tiopronin. The correlation coefficient of the calibration curve were better than 0.998 in the range of 0.107–5.35 μg/ml in human plasma. The limit of quantification (LOQ) was 0.107 μg/ml (S/N 10:1, RSD 7.1%). The inter-day and intra-day accuracy was below 7.1 and 6.8%, respectively. As a preliminary application, this method has been successfully applied to the determination of tiopronin in the human plasma.

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1. Introduction

Tiopronin, N-(mercaptopropiony)-glycine (MPG, Thiola) (Fig. 1), is a synthetic thiol compound which has been used as a hepatoprotective agent [1], antidote to heavy metal poisoning [2] and a radioprotective agent [3]. It has also been successfully applied to prevent kidney stones, which may develop due to too much cystine in the urine and the extra cystine can be removed from the body by this medication [4], and treat rheumatoid arthritis [5]. However, tiopronin has also side effects that can be very serious. Loss of taste or stomach upset may occur. Laboratory tests will be done routinely while taking this medication to monitor for effectiveness and to prevent side effects.

For the determination of tiopronin in the biological matrices, many different methods have been reported, including gas chromatography–mass spectrometry [6], electrochemical detection [7] and high-performance liquid chromatography (HPLC). From the viewpoint of application, the HPLC coupling with different detectors was the most widely method for the analyses of tiopronin [8–12]. Springolo et al. [8] reported a HPLC-UV method for the determination of tiopronin in urine by reaction with the 2-furoyl chloride. Kagedal et al. [10], selecting N-(7-dimethylamino-4-methyl-3-coumarinyl) maleimide (DACM) as a fluorescent reagent for thiols, developed an HPLC fluorescent method for determining tiopronin in urine and plasma. Matsuura et al. [12] proposed a method for determination of tiopronin by LC-ESI-MS–MS using methyl acrylate for stabilization of thiol group.

However, there is still a challenge to analysts for the determination of tiopronin in the biological matrices because of its active thiol group. The thiol compounds can be easily oxidized to disulfides either as a dimmer or mix forms with endogenous thiols in the biological matrices [12]. A number of analytical methods have been reported with complicated sample preparation procedure.

In this paper, a relative simple, fast and sensitive HPLC/ESI-MS method for direct determination of tiopronin in human plasma was proposed. In the sample preparation process, Vita-
obtained from volunteers, and stored below 4 °C until used.

2. Experimental

2.1. Chemicals and reagents

Tiopronin and sodium cyclamate were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade acetonitrile, purchased from Tedia company Inc. (Fairfield, Ohio, USA), and ultrapure water, prepared by a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA), were used to prepare analytical-grade. Drug-free and drug-containing plasma were the mobile phase. Other reagents including 2-Me and Tris were selected as the suitable organic modifier in mobile phase. A good response should be taken into consideration. According to the weak retention behaviour of tiopronin on silica-based columns (C8, C18, –NH2 and –CN), the cyclamate was selected as the I.S.

2.2. LC/ESI-MS

The chromatographic separation was performed on a Waters Alliance 2695 (Milford, MA, USA) HPLC system equipped with a Johnson Spherigel analytical column (150 mm × 4.6 mm) (Dalian, China) packed with 5 μm C8 silica. Tiopronin and cyclamate were separated using 1 mM 2-mercaptoethanol and 0.5 mM Tris of formic acid aqueous (pH 4.5) as mobile phase. Liquid chromatography was performed at 30 °C and with a flow rate of 1 ml/min. The HPLC system coupled to a Micromass ZQ 2000 mass spectrometer with an electrospray ionization (ESI) interface. Mass spectra were operated in negative ion mode over a range of m/z: 70–350, and selective ion monitors (SIM) were at m/z: 162.3 and 178 for tiopronin and cyclamate ([M–H]−).

2.3. Standard solution and calibration curve

The stock standard solution of tiopronin was prepared by dissolving the analytical standard (10.7 mg) in 1 mM 2-Me aqueous solution (20 ml). The standard solution of cyclamate was prepared by dissolving the sodium cyclamate analytical standard (3.8 mg) in the solution of 99% acetonitrile aqueous (1000 ml).

All the stock solutions were stored in the refrigerator at −40 °C. Working standard solutions for the calibration curve of tiopronin were prepared in the concentration range of 0.107–5.35 μg/ml with drug-free human plasma. The concentration of I.S. in the calibration curve standards was 2.0 μg/ml. The standards were treated in the same process as the sample to be measured. A calibration curve was represented by plotting the peak-area ratios of the tiopronin to sodium cyclamate versus the concentrations of the calibration standard.

2.4. Sample preparation

0.5 ml of plasma samples obtained from volunteers after the administration of 600 mg of tiopronin were added into polypropylene tubes containing 0.2 ml of mercaptoethanol, 10 mg Vitamin C and 1 ml standard solution of I.S. After vortex mixed for 5–10 s, all the samples were deoxidized and deproteinized in ultrasonic for 30 min at 60 °C. Finally, all samples centrifuged at 27,000 × g for 10 min at 4 °C. A 10 μl aliquot of the supernatant of each sample was then directly injected on a Spherigel analytical column.

3. Results and discussion

3.1. Optimization of the chromatographic procedure

In order to develop a high sensitive method for separation and detection of tiopronin, the selection of I.S. and column style and the optimization of mobile phase and mass spectrometry response should be taken into consideration. According to the weak retention behaviour of tiopronin on silica-based columns (C8, C18, –NH2 and –CN), the cyclamate was selected as the I.S. with the similarly chromatographic character. Compared with the four styles of column, the retention time of the analytes on –NH2 and –CN columns was high sensitivity to the pH; so the result too difficult to replicate, and the peak shape of analytes separated on C8 was sharper and more symmetrical than that on C18.

Accordingly as the strong polarity and ionization of the tiopronin and cyclamate, a suitable counter ion agent should be chose. Dibutylamine, Tris and triethylamine were tested as the counter ion agent. The strongest effect of retention behaviour can easily obtained when dibutylamine was employed, followed by Tris and triethylamine. However, the serious suppression of ionization of analytes was observed by using dibutylamine, and the interference between tiopronin and endogenous substance was observed by using the triethylamine. Therefore, Tris agent was selected as the suitable organic modifier in mobile phase. A good effect on the resolution factor was observed when the amount of Tris of the mobile phase was increased, but the response of analytes become weakened when the concentration of Tris over 0.5 mM. By investigating the effect of 2-mercaptoethanol added in the mobile phase, we found that the 2-mercaptoethanol not only modify the peak symmetry but also improve the peak.
3.2. The status of tiopronin in plasma

During the experiment, we observed that the concentration of the free tiopronin in plasma decreased with the time elapsed. In order to find the relationship between the concentration of the free tiopronin in plasma and the stored time, we applied the method listed in Section 2.4 but without the addition of ascorbic acid. The concentration–time profile of free tiopronin in plasma is shown in Fig. 3. As shown in Fig. 3, tiopronin was labile in plasma, and was easily oxidized to disulphides either as a dimer or as mixed forms with endogenous thiols, which was in agreement with the paper of Leroy et al. [13]. It is necessary to release the tiopronin before determination. Therefore, we used the Vitamin C as the reducer in the sample preparation process. At the same time, the relationship between the deoxidizing time and yield of the free tiopronin in pretreatment step was also investigated. Three concentration levels of tiopronin spiked in drug-free human plasma were analyzed and presented in Fig. 4. From the results, 30 min of deoxidizing time is enough in pretreatment step and the Vitamin C is also a suitable reducer for freeing tiopronin from plasma.

3.3. Linearity and limit of quantification

A series of calibration standard sample solutions were injected into the analytic column under selected conditions to test the linearity of the calibration graphs for tiopronin. A linear relationship between drug concentration (X) and the peak-area ratios (Y) of the tiopronin to cyclamate was obtained in the ranges 0.107–5.35 μg/ml. The correlation coefficient (r²) was
Table 1
Calibration curve parameters ($n=5$)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Linear range ($\mu$g/ml)</th>
<th>Slope mean ± SD</th>
<th>Intercept mean ± SD</th>
<th>Correlation coefficient mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma sample</td>
<td>0.107–5.35</td>
<td>0.291 ± 0.010</td>
<td>0.0439 ± 0.0013</td>
<td>0.9994 ± 0.0045</td>
</tr>
</tbody>
</table>

Table 2
Inter- and intra-day precision and accuracy of the method for determination of tiopronin in human plasma ($n=6$)

<table>
<thead>
<tr>
<th>Added ($\mu$g/ml)</th>
<th>Measurement, mean ± SD ($\mu$g/ml)</th>
<th>RSD (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inter-day 0.107</td>
<td>0.109 ± 0.006</td>
<td>7.1</td>
<td>101.5</td>
</tr>
<tr>
<td>0.535</td>
<td>0.531 ± 0.034</td>
<td>6.4</td>
<td>99.3</td>
</tr>
<tr>
<td>5.35</td>
<td>5.371 ± 0.246</td>
<td>4.6</td>
<td>100.4</td>
</tr>
<tr>
<td>Intra-day 0.107</td>
<td>0.108 ± 0.007</td>
<td>6.8</td>
<td>100.9</td>
</tr>
<tr>
<td>0.535</td>
<td>0.537 ± 0.030</td>
<td>5.7</td>
<td>100.4</td>
</tr>
<tr>
<td>5.35</td>
<td>5.291 ± 0.209</td>
<td>3.9</td>
<td>98.9</td>
</tr>
</tbody>
</table>

Fig. 4. The influence of deoxidization time to freeing concentration of tiopronin in pretreatment process.

Fig. 5. Mean plasma concentration–time curves of tiopronin after oral administration of 600 mg tiopronin to 20 healthy volunteers.

Table 3
Stability of tiopronin in plasma QC samples

<table>
<thead>
<tr>
<th>Stability test</th>
<th>Nominal concentration</th>
<th>Mean ± SD</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short-term stability (24h)</td>
<td>0.107 $\mu$g/ml</td>
<td>0.106 ± 0.006</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>0.535 $\mu$g/ml</td>
<td>0.537 ± 0.013</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>5.35 $\mu$g/ml</td>
<td>5.342 ± 0.061</td>
<td>1.1</td>
</tr>
<tr>
<td>Freeze–thaw stability (three cycles)</td>
<td>0.107 $\mu$g/ml</td>
<td>0.105 ± 0.007</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>0.535 $\mu$g/ml</td>
<td>0.534 ± 0.015</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>5.35 $\mu$g/ml</td>
<td>5.353 ± 0.059</td>
<td>1.1</td>
</tr>
</tbody>
</table>

$\times=5$ for short-term and freeze–thaw stability.

Table 4
The pharmacokinetic parameters of tiopronin after oral administration of 600 mg of tiopronin

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Reference preparation</th>
<th>Experiment preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>RSD (%)</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (ng/ml)</td>
<td>3.407 ± 1.053</td>
<td>3.621 ± 1.049</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>5.4 ± 0.5</td>
<td>5.7 ± 0.6</td>
</tr>
<tr>
<td>$T_{1/2}$ (h)</td>
<td>2.7 ± 0.6</td>
<td>2.6 ± 0.5</td>
</tr>
<tr>
<td>AUC$_{0-24h}$ (h·ng/ml)</td>
<td>20.33 ± 4.13</td>
<td>20.315</td>
</tr>
</tbody>
</table>
more than 0.9994 \((Y = 0.291X - 0.0439)\), indicating a good linearity listed in Table 1. The LOQ of the method was established at 0.107 \(\mu g/ml\) (S/N 10:1, RSD 7.1%).

### 3.4. Method validation

The mean extraction recovery was measured by comparison of the peak-area ratios of the extracted samples with those of aqueous standard of the same concentration (0.107, 0.535 and 5.35 \(\mu g/ml\)). The result of the mean extraction recovery is 80.9%.

The precision and the accuracy of the method were confirmed by the six replicate determinations of plasma containing tiopronin at three level concentrations (0.107, 0.535 and 5.35 \(\mu g/ml\)). The respective data are presented in Table 2. As listed in Table 2, the method was found to be reproducible and accurate.

### 3.5. Stability

The three concentration levels (0.107, 0.535 and 5.35 \(\mu g/ml\)) of spiked samples were assessed after the storage at room temperature for 24 h, after three freeze and thaw cycles. The respective stability data of tiopronin are summarized in Table 3. The stability results presented in Table 3 indicate that the method for determination of tiopronin can be considered stable under the various conditions investigated.

### 3.6. Application

The proposed method was applied to the determination of tiopronin in human plasma samples for bioequivalence study. Mean plasma concentration–time profiles of tiopronin obtained from 20 volunteers after the oral administration of tiopronin (600 mg) were shown in Fig. 5 and the pharmacokinetic parameters were presented in Table 4, which approximately correspond to the literature reported by Carlsson M.S. (the concentration of TP peaked at 3–6 h and then decreased with a terminal half life \((T_{1/2})\) 1.8 h) [14].

### 4. Conclusion

Tiopronin was released from plasma by using the reducer of Vitamin C and mercaptoethanol in the sample preparation procedure, and which obtained stable recovery. The HPLC/MS method was developed and validated for determination of tiopronin in human plasma in the 0.107–5.35 \(\mu g/ml\) range. The method provided sufficient sensitivity with precision according to USA FDA criteria [15] for tiopronin and was successfully applied to a bioequivalence study of tiopronin in human plasma.

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