Introduction

Treosulfan is a prodrug used for the treatment of several cancers. It is the precursor of L-diepoxybutane (Diepoxide) produced in vivo via the corresponding Monoepoxide. Treosulfan epoxides are reactive molecules and represent the active compounds that act as DNA alkylating agents to arrest the division of the last replicating tumour cells. The non-enzymatic conversion of Treosulfan to its epoxides is pH and temperature dependent and occurs under physiological conditions resulting in the simultaneous coexistence of the three compounds in the bloodstream. The accurate quantitation of these molecules, in plasma, is complicated by the intrinsically unstable nature of the compounds, especially the interconversion reactions that can occur during sample processing, i) by the absence of ionizable groups on the Diepoxide which therefore requires derivatization prior to its mass spectrometric determination. To accurately measure the concentration in plasma, it is essential to control the interconversion of the three compounds.

Here we describe a method for the analysis of Treosulfan, the Monoepoxide and the derivatized Diepoxide (Figure 1).

Strategy for Sample Collection and Stabilization

Figure 2 shows a schematic representation of the sample collection and sample analysis strategy. Blood was collected at the clinical sites and the compounds were immediately stabilized by the addition of buffer to lower the pH below 6. The diluted blood was always maintained on ice until the plasma fraction was stored at -80°C. Stability was demonstrated using this sample collection strategy. The true matrix for determination of Treosulfan and metabolites is best described as diluted plasma. The diluted plasma contained all three molecules: Treosulfan, Monoepoxide and Diepoxide. The plasma was divided in two aliquots of which one was used for the quantitation of the Treosulfan and its metabolites, the other was used for quantitation of the Diepoxide.

Table 1. Upper and lower limits of quantitation.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>ULOQ mg/ml</th>
<th>LLOQ mg/ml</th>
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<tbody>
<tr>
<td>Treosulfan</td>
<td>500</td>
<td>0.0005</td>
</tr>
<tr>
<td>Monoepoxide</td>
<td>10</td>
<td>0.0005</td>
</tr>
<tr>
<td>Diepoxide</td>
<td>5000</td>
<td>0.0005</td>
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</table>

Table 2. Treosulfan Monoepoxide results.

<table>
<thead>
<tr>
<th>Accuracy and Precision</th>
<th>RUN</th>
<th>QC LLOQ</th>
<th>QC LOW</th>
<th>QC MED</th>
<th>QC HIGH</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTERIM</td>
<td>Run 1</td>
<td>103</td>
<td>108</td>
<td>98.6</td>
<td>99.6</td>
</tr>
<tr>
<td></td>
<td>Run 2</td>
<td>126</td>
<td>107</td>
<td>94.9</td>
<td>94.6</td>
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<td></td>
<td>Run 3</td>
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<td>103</td>
<td>93.9</td>
<td>93.9</td>
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<tr>
<td>ACCURACY [%]</td>
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<td>100</td>
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<td>93.6</td>
<td>93.8</td>
</tr>
<tr>
<td>CV [%]</td>
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<td>9.2</td>
<td>5.9</td>
<td>6.8</td>
<td>6.7</td>
</tr>
</tbody>
</table>

Table 3. Treosulfan results.

| The LC-MS/MS methods developed for the determination of Treosulfan and Monoepoxide are precise and accurate over the quantification range. Experiments have showed poor stability of the Monoepoxide when maintained at room temperature. Treosulfan Diepoxide Method Overview

An aliquot of stabilized plasma was processed forDiepoxide determination.

Diepoxide was derivatized with Diethylthiocarbamate according to the reaction described in Figure 4. Sample preparation was performed on ice to limit the Treosulfan and Monoepoxide conversion into Diepoxide.

Method details:

- 50 µL of diluted plasma (lithrate)
- 20 µL of labeled Diepoxide.
- Derivatization with 250 µL of DTC in phosphate buffer pH 6.2
- 10 µL of supemantant were diluted for Diepoxide determination.
- 230 µL of supemantant were diluted for quantitation of the Diepoxide.

Table 4. Treosulfan Diepoxide results.

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Figure 4. Derivation of epoxide (general scheme).

The developed method for the determination of the derivatized Diepoxide is precise and accurate over the quantification range. Experiments show poor stability of the Diepoxide at room temperature.

Impact of Inter-Conversion on the Quantitation at the LLOQ Levels of Monoepoxide and Diepoxide

Instability of the analytes during sample preparation may affect the accuracy of the determined concentrations. To measure the effect of analyte instability, experiments were designed to determine the impact of possible molecule interconversion in the low concentration range.

Effect of High Concentration of Treosulfan on the Accurate Quantitation of Monoepoxide

Treosulfan was spiked at the LLOQ in diluted plasma. Samples were processed according to the method for the determination of the Monoepoxide. Six samples were processed independently and the area ratio was expressed as percentage of the Monoepoxide LLOQ measured in the same run. Results are presented in Table 5.

Table 5. Sample preparation for mass spectrometric determination of the Monoepoxide induces the conversion of Treosulfan spiked at LLOQ into Diepoxide. In those samples the Monoepoxide was estimated to be about 20 % of Monoepoxide LLOQ.

Effect of High Concentration of Treosulfan and Monoepoxide on the Accurate Quantitation of Diepoxide at the LLOQ

Treosulfan and monoepoxide were spiked either individually or together in diluted plasma at at or hsp level. Samples were processed according to the method for quantitation of the Diepoxide. Three samples were processed independently and the peak area of the Diepoxide detected was expressed as percentage of the Diepoxide LLOQ measured in the same run. Results are presented in Table 6.

Table 6. Sample preparation for mass spectrometric determination of the Diepoxide does not induce the conversion of Treosulfan spiked at ULOQ into Diepoxide. In these samples the Diepoxide was estimated to be about 200 % of Monoepoxide LLOQ.

Conclusion

It was demonstrated that a tight control of pH and temperature during sample preparation limits the interconversion between these analytes. The capacity of the method to accurately quantify the Monoepoxide and the Diepoxide at the lowest expected concentrations in presence of high concentration of Treosulfan and Monoepoxide respectively, in human plasma was evaluated. The data show that Treosulfan, during sample processing, converts to the Monoepoxide while it is stable during the derivatization for the Diepoxide. Interestingly, it was observed that only the Monoepoxide converts to the Diepoxide during the derivatization reaction. The maximum and the minimum quantifiable concentrations of the three were taken in account of their measured interconversion were determined.

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