Bioanalytical determination of Treosulfan and its active metabolites

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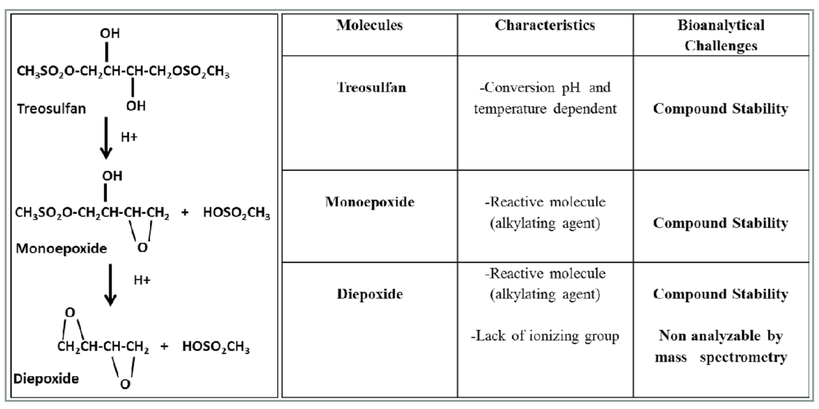
Introduction

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 alkylation agents to arrest the division of the fast 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 i) by the intrinsic instability of the compounds, ii) by the interconversion reactions that can occur during sample processing, iii) 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).



Molecules	Characteristics	Bioanalytical Challenges
Treosulfan	-Conversion pH and temperature dependent	Compound Stability
Monoepoxide	-Reactive molecule (alkylating agent)	Compound Stability
Diepoxide	-Reactive molecule (alkylating agent) -Lack of ionizing group	Compound Stability Non analyzable by mass spectrometry

Figure 1. Challenges for LC-MS/MS determination of the Treosulfan and Treosulfan expoxides.

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 Monoepoxide, the other was used for quantitation of the Diepoxide.

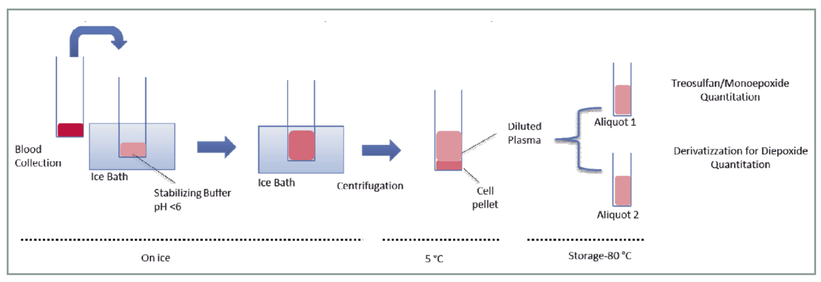


Figure 2. Strategy for sample collection and sample analysis.

Molecules	ULOQ ng/mL	LLOQ ng/mL
Treosulfan	500	50000
Monoepoxide	10	12000
Diepoxide	3	6000

Table 1. Upper and lower limits of quantitation.

Effects of the Temperature on Treosulfan/Monoepoxide Conversion During the Sample Work Up

For accurate quantitation of the Monoexpoxide metabolite it was necessary to control the degradation of the Treosulfan during the sample preparation. Initial experiments were performed in pure solutions. The impact of the conversion was evaluated by quantitation of Monoepoxide in solutions containing Treosulfan (ULOQ).

Figure 3 (A) shows pure solution of freshly dissolved Treosulfan contained only negligible traces of Monoepoxide. In solution, the Monoepoxide concentration increased by a factor of about 20 upon sample storage on ice for 8 hours (B). To determine the effects of temperature on the Treosulfan/Monoepxoxide conversion, evaporation of the sample (in 0.1% formic acid) at 40°C was performed. Elevated temperatures increased the level of Monoepoxide of 650 times (C).

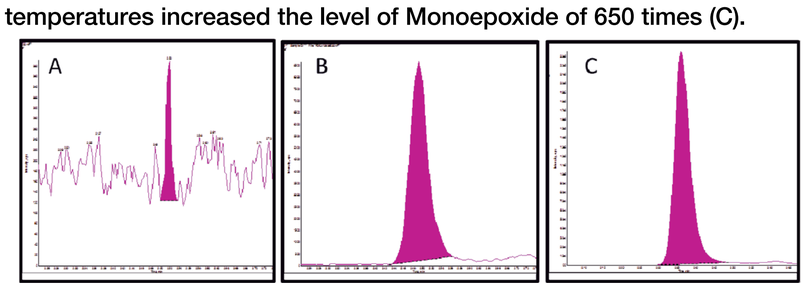


Figure 3. Effect of the temperature on the Treosulfan/ Monoepoxide conversion.

pH and temperature during sample workup need to be accurately controlled to avoid Treosulfan conversion into Monoepoxide.

Treosulfan/Monoepoxide Method Overview

An aliquot of stabilized diluted plasma was processed for Treosulfan and Monoepoxide determination. The entire procedure was performed on ice and under acidic conditions to minimize the Treosulfan/ Monoepoxide conversion.

Method details:

- 50 µL of diluted plasma (citrate)
- 20 µL of labeled Treosulfan and Monoepoxide (IS).
- 250 µL of Me-OH 0.1% Formic acid (ice cold) –Protein precipitation-.
- 10 µL of supernatant were diluted for Treosulfan determination.
- 230 µL of supernatant were concentrated for Monoepoxide determination.

ACCURACY AND PRECISION	RUN	QC LLOQ	QC LOW	QC MED	QC HIGH
INTRARUN	Run 1	103	103	95.5	96.6
	Run 2	102	97.0	94.9	94.8
	Run 3	95.1	95.3	90.3	90.0
INTRARUN	ACCURACY [%]	100	98.4	93.6	93.8
	CV [%]	9.2	5.9	6.8	4.0
STABILITY	**RUN**	**QC LOW**		**QC HIGH**	
FREEZE/THAW	Run 2	94.1		97.7	
24 HRS BENCHTOP	Run 2	72.8		61.4	

Table 2. Treosulfan Monoepoxide results.

ACCURACY AND PRECISION	RUN	QC LLOQ	QC LOW	QC MED	QC HIGH
INTRARUN	Run 1	109	103	96.8	102
	Run 2	101	91.4	90.5	93.1
	Run 3	96.3	100	92.3	98.7
INTRARUN	ACCURACY [%]	102	98.3	93.2	97.9
	CV [%]	7.6	7.7	10.7	6.4
STABILITY	**RUN**	**QC LOW**		**QC HIGH**	
FREEZE/THAW	Run 2	88.6		101	
24 HRS BENCHTOP	Run 2	85.8		90.4	

Table 3. Treosulfan results.

The LC-MS/MS methods developed for the determination of the 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 for Diepoxide determination.

Diepoxide was derivatizied with Diethyltiocarbamate 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 (citrate)
- 20 µL of labeled Diepoxide.
- Derivatization with 250 µL of DTC in phosphate buffer pH 6.2
- 250 µL of Me-OH 0.1% Formic acid (ice cold) –stop of the reaction and protein precipitation-
- Dilution of the supernatant for Derivatized Diepoxide detection

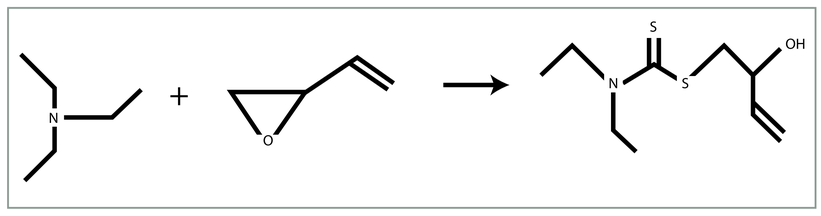


Figure 4. Derivatization of epoxide (general scheme).

ACCURACY AND PRECISION	RUN	QC LLOQ	QC LOW	QC MED	QC HIGH
INTRARUN	Run 1	115	104	107	102
	Run 2	120	104	105	109
	Run 3	104	99.7	98.1	100
	Run 4	117	106	104	96.1
INTRARUN	ACCURACY [%]	114	103	103	102
	CV [%]	12.6	5.0	5.1	7.3
STABILITY	**RUN**	**QC LOW**		**QC HIGH**	
FREEZE/THAW	Run 2	99.1		101	
24 HRS BENCHTOP	Run 2	38.2		36.5	

Table 4. Treosulfan Dieopoxide results.

The developed method for the determination of the detivatized 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 concentrations range.

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

Treosulfan was spiked at the ULOQ 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.

SAMPLE	PEAK AREA RATIO	% MONOEPOXIDE LLOQ PEAK AREA
MONOEPOXIDE IN TREOSULFAN ULOQ	0.0250	20.1
	0.0190	15.3
	0.0310	25.0
	0.0280	22.6
	0.0340	27.4
	0.0300	24.2
	AVERAGE	22.1
SD	0.0108	
CV [%]	8.67	
N	6	

Table 5.

Sample preparation for mass spectrometric determination of the Monoepoxide induces the conversion of Treosulfan spiked at ULOQ into Monoepoxide. 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 uloq or hiqc 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.

SAMPLE	PEAK AREA DIEPOXIDE	% of LLOQ DIEPOXIDE
TREOSULFAN ULOQ	32.1	1.70
	34.9	1.85
	56.1	2.98
MEAN		2.18
MONOEPOXIDE ULOQ	3952	210
	3916	208
	4921	261
MEAN		226
QC HIGH Treosulfan/ Monoepoxide	3673	195
	3450	183
	3838	204
MEAN		194
Reference Diepoxide LLOQ	1859	
	1625	
	1982	
	1936	
	2067	
	1844	
MEAN	1886	
SD	152	
CV [%]	8.04	
N	6	

Table 6.

Sample preparation for mass spectrometric determination of the Diepoxide does not induce the conversion of Treosulfan spiked at ULOQ into Diepoxide. The Monoepoxide spiked at the ULOQ in the samples converts to Diepoxide. In these samples the Diepoxide was estimated to be about 200 % of Diepoxide 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 reaction 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 analytes taking in account of their measured interconversion were determined.

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