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Novel Stability Indicating Reverse Phase HPLC Method for Estimation of Thiotepa In Its Formulation

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ABSTRACT

A Novel stability-indicating method was developed and validated by Reverse phase Hplc method for the estimation of Thiotepa in Lyophilized form. The simple, Accurate Assay method was developed by PDA Detector at 215nm and column(YMC,150mmx4.6mm, 3.5 µm,12nm).The mobile phase A consisted of (3.5g of potassium Dihydrogen phosphate/1liter: 4.2g of Disodium hydrogen Phosphate/2liters)Buffer : Acetonitrile (85:15v/v) and The mobile phase B consisted of (3.5g of potassium Dihydrogen phosphate/1liter: 4.2g of Disodium hydrogen Phosphate/2liters)Buffer : Acetonitrile (50:50v/v) gradient flow rate at 1.0 mL/min for 30minutes. Samples was stored at 5°c temperature in sample cooler and Column oven temperature was maintained in the method at 30°c Respectively. The retention time of Thiotepa was 11.313 minutes. The proposed method was developed and validated with respect to specificity, accuracy, precision, linearity, Analyte Stability. Specificity of the Assay method shows no interference from Diluent and degrading products formed by alkaline, acidic, oxidative, Water Hydrolysis conditions. At Each level, %Recovery of Thiotepa in formulations was obtained to be in a range of 98-102%. The linearity of the assay was established in the range of 159.915-479.744µg/mL with correlation coefficient (R^2) > 0.9999. This method was shows simple, precise, more accurate, stability indicating and reliable determination of Thiotepa for drug stability assay in pharmaceutical studies.

Keywords: Thiotepa, RP HPLC, Method Development, Method Validation, Stability indicating method.

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INTRODUCTION

Thiotepa is an anticancer agent. Chemically Thiotepa is N, N'N'triethylenethio phosphoramide [1]and a chemotherapeutic member of the alkylating agent group. The chemical name for Thiotepa is Tris(1-aziridinyl)phosphine sulphide and has a molecular weight of 189.22 . Thiotepa has the molecular formula C6H12N3PS. Thiotepa structural formula[2]:

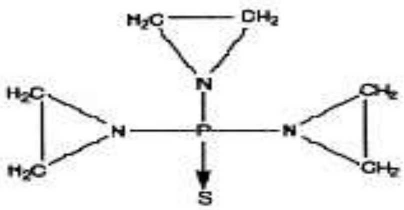

Figure 1: Thiotepa Structure

Thiotepa is a chemically and pharmacologically to nitrogen mustard[3]. The pharmacological action of Thiotepa is release of ethylenimine radicals which is attached to the guanine base of DNA, at the number 7 nitrogen atom of the imidazole ring. They stop tumor growth by cross linking guanine nucleobases in DNA double-helix strands, directly attacking DNA. This makes the strands unable to uncoil and separate. So this is necessary in DNA replications, the cells can no longer divide [4]. These drugs act non-specifically [1].

When Thiotepa reconstituted with sterile water for injection, the resulting solution has a pH of approximately 5.5 to 7.5. Thiotepa is unstable in acid medium and stable in alkaline medium [5].

Thiotepa for Injection, USP has been used in tumours [3]:

1. Adenocarcinoma of the breast.
2. Adenocarcinoma of the ovary.
3. It is used as conditioning for Bone marrow transplantation. Its main toxicity is myelosuppression[1].
4. Carcinoma of the urinary bladder.

MATERIALS AND METHOD

Chemicals and Reagents

Thiotepa active pharmaceutical ingredient (API) and lyophilized formulation of Thiotepa were procured from Mylan pharmaceuticals, Hyderabad, Andhra Pradesh, India. Analytical grade trifluoroacetic acid was purchased from Merck, Mumbai, HPLC grade acetonitrile and Methanol was procured form Rankem. 0.45µm membrane filter was purchased from Milli- pore. The entire experiment was performed using "class A" volumetric glassware and HPLC grade water.

Instrumentation

Determination of Thiotepa was performed by using Waters HPLC (Milford, MA, USA) PDA system[7] consisting of a quaternary solvent manager, a sample manager, column-Oven, and photodiode array detector. This system was controlled and output signal was monitored by Waters empower software3.0. Sartorius semi micro balance was used for all weighing and Thermoscientific pH meter was used for buffer pH adjustment and sonication was carried out by using fast clean ultrasonic bath. All samples were filtered through 0.45 µm membrane Millipore filters.

Chromatographic conditions

The analysis was carried out by using high performance liquid chromatography (HPLC). The Thiotepa was separated on YMC ODS-A column, 150mm length, 4.6mm internal diameter packed with porous silica of particle size 3µm, at 30°C column oven temperature. The flow rate was maintained at 1.0 mL minute−1. The separation was achieved by Gradient elution with a run time of 30 minutes. The mobile phase was filtered through a 0.45µm Millipore filter, before use. UV detection was performed at 215nm. The sample injection volume was 10 µL. Diluent was used as 2-8°c maintained Water.

System suitability solution Preparation:

Weighed and transferred 10.16mg of Thiotepa into 4ml Vial, added 2ml methanol, 50µl of 0.1% phosphoric acid and heated at 65°c for 50seconds cooled this solution, then added 1.0ml methanol and mixed well. Transferred 1ml of above solution into 10ml volumetric flask and added volume up to mark with diluents and mixed well

Standard solution preparation:

Weighed and transferred 30.56 mg of thiotepa Standard into 100ml volumetric flask, dissolved and diluted up to mark with diluents and mixed well.

Preparation of Test solution:

For 15mg/vial:

Reconstitute 5 Sample vials with diluents, pooled together into 25ml volumetric flask, diluted to volume with diluents and mixed well.(sample stock solution).

Further dilute 5ml of the above solution into 50ml volumetric flask and diluted to volume with diluents and mixed well.

For 100mg/vial:

Reconstitute 2 Sample vials with diluents, pooled together into 50ml volumetric flask, diluted to volume with diluents and mixed well. (Sample stock solution).

Further dilute 4ml of the above solution into 50ml volumetric flask and diluted to volume with diluents and mixed well.

Analytical Method Validation*:*

Validation studies were performed according to the ICH guidelines[6]. The method was validated for specificity (interference study and forced degradation study), linearity, System precision, method precision, accuracy, LOD&LOQ and standard &sample solution stability. The specificity was determined by analyzing the chromatograms of Lyophiled sample in comparison with those obtained for Thiotepa standard solution aiming at confirming that none of the ingredients interfere with the quantitation of the drug.

The linearity was determined by a least-square linear regression routine using the compound peak area and concentration of the working standard solutions prepared at seven concentration levels (159.915, 255.864, 287.846, 319.829, 351.812, 383.795and 479.744µg/mL). Determination of the correlation coefficient and intercept values by method according to the ICH guidelines [6].

Calibration curve method was established the lower concentrations LOD and LOQ. lower concentrations of Thiotepa were prepared in linearity range and injected in six replicates. Average peak area of six analyses was plotted against concentration. LOD and LOQ were expressed as 3.3 × Syx/*b* and 10.0 × Styx/*b*, respectively, where *b* is the mean slope of the linear regression curves and Styx is residual variance due to regression. The precision was assessed at intraday and interday precision[6]. The intraday precision was determined by Diluted standard solution, injected six times on the same day.

The Intermediate precision was obtained by injecting standard solution prepared at the same concentrations on different days by different operators. Results were reported in terms of relative standard deviation (RSD). The accuracy was established by using addition method at different levels: 50, 100, and 150%.The mean recovery of Thiotepa of the target concentration was calculated and accepted with 100 ± 2%.To determine the stability of sample and standard. Sample and standard solutions were injected and result was reported in terms of relative standard deviation (RSD).

Forced Degradation Study.

Forced degradation studies were carried out to provide some information about the drug. Stability and to validate the specificity of the Thiotepa, quantification of the assay. Demonstrated this by carrying out forced degradation of sample with HCl, NaOH, H_2O_2, and water. Prepared samples as per Test preparation and injected into HPLC with photodiode array detector.

i) As such sample

Reconstituted 1 sample vial (100mg/ml) with 20ml water, transferred in 25ml volumetric flask, Diluted up to mark with diluents and mixed well. Further transferred 4ml of above solution in to 50ml volumetric flask, diluted up to mark with diluents and mixed well.

ii) Acid degradation

Taken one sample vial (100mg/vial), reconstituted with 20ml of water and added 1ml of 0.02N HCl solution, kept the sample solution at room temperature for 15minutes then added 1ml of 0.02N NaOH solution to neutralize the solution, transferred whole solution in to 25ml volumetric flask make up to the volume with diluents and mixed well.

Further transferred 4ml of above solution in to 50ml volumetric flask, diluted up to mark with diluents and mixed well.

iii) Alkali degradation

Taken one sample vial (100mg/vial), reconstituted with 20ml of water and added 1ml of 0.25N NaOH solution, kept the sample solution in water bath at 60°c for 20minutes then added 0.8ml of 0.25N HCl solution to neutralize the solution, transferred whole solution in to 25ml volumetric flask make up to the volume with diluents and mixed well.

Further transferred 4ml of above solution in to 50ml volumetric flask, diluted up to mark with diluents and mixed well.

iv) Peroxide degradation

Taken one sample vial (100mg/vial), reconstituted with 20ml of water and added 0.5ml of 3%H_2O_2 solution, kept the sample solution in water bath at 60°c for 30minutes ,transferred whole solution in to 25ml volumetric flask make up to the volume with diluents and mixed well.

Further transferred 4ml of above solution in to 50ml volumetric flask, diluted up to mark with diluents and mixed well.

v) Water degradation

Taken one sample vial (100mg/vial), reconstituted with 20ml of water and kept the sample solution on hot plate at 80°c for 10minutes, transferred whole solution in to 25ml volumetric flask make up to the volume with diluents and mixed well. Further transferred 4ml of above solution in to 50ml volumetric flask, diluted up to mark with diluents and mixed well.

RESULTS AND DISCUSSION

Method Development and optimization

The analytical method is directly taken from vendor DMF (USP)(18), in this Nucleosil

150*4.6mm, 5µm and high concentration buffer were used but we observed the life time of column is less and chloroethyl analog impurity was not eluted within the run time (i.e. 25min) hence we optimized the method. Nucleosil 150*4.6mm, 5µm was used to provide an separation but efficient chromatographic separation was obtained on YMC ODS-A (150*4.6) mm, 3µm. Various buffer, mobile phase systems were prepared and used to provide an appropriate chromatographic separation, but the proposed mobile phase- A containing 3.5g of potassium Dihydrogen phosphate/1liter: 4.2g of Disodium hydrogen Phosphate/2liters: Acetonitrile in the ratio of 85:15 (v/v) and mobile phase- B containing 3.5g of potassium Dihydrogen phosphate/1liter: 4.2g of Disodium hydrogen Phosphate/2liters: Acetonitrile in the ratio of 50:50 (v/v) gave a better resolution. PDA detector was used at 215 nm for detection. The flow rate of 1 ml/min was suited for the drug and impurity with respect to Retention time and resolution of peaks after the several flow rates tested. The retention time of Thiotepa was found to 11.313min. Column efficiency for separation was indicated by plate count. System suitability parameters like Number of theoretical plates, tailing factor and Resolution are meeting ICH requirements [6].

Method Validation.

The proposed method was used for validated by determining its performance characteristics regarding System suitability/ system precision, specificity[6](Interference study and Forced degradation study), linearity, Method precision, accuracy, Standard & solution stability, filter variability and Robustness.

System precision/System Suitability

System precision/system suitability is assessed from the five replicate injections of the standard preparation from the same vial. The results of system precision/system suitability are summarized in Table 1. The analytical procedure is precise with respect to chromatographic system.

Table 1: Results of System Suitability/System precision

Injection#	**Thiotepa Peak**	
	Retention time	Area
1	11.318	2617947
2	11.313	2617322
3	11.320	2616993
4	11.306	2613283
5	11.306	2616939
Mean	11.313	2616497
%RSD	0.1	0.1
Tailing factor	1.1	
Theoretical plate	17956	

Method Precision

To evaluate the method precision, six individual samples were prepared for assay and analyzed the samples as per the analytical procedure. Intermediate precision expresses with in-laboratories variations such as Different days, Different analysts, Different columns, Different equipment's etc. Intermediate precision is established by doing same exercise as system and method precision by different analyst on different day using different column and different equipment. The same lot/Batch of Standard and sample were used within the laboratory. The results of intraday/ interday precision for assay are tabulated in Table 2 and 3.the results found comparable indicates the method is precise and rugged with respect to variation analyst to analyst, day to day, column to column and equipment to equipment for its intended use.

Table 2: Results of Intraday precision

Injection#	**Thiotepa Peak**	
	Retention time	**%ASSAY**
1	11.319	102.6
2	11.302	103.8
3	11.330	101.8
4	11.303	103.5
5	11.307	104.1
6	11.330	103.9
Mean	11.315	103.3
%RSD	0.1	0.9

Table 3: Results of Interday precision

Injection#	**Thiotepa Peak**	
	Retention time	%ASSAY
1	11.317	102.1
2	11.308	103.6
3	11.321	102.8
4	11.304	103.2
5	11.307	103.8
6	11.326	102.9
Mean	11.314	103.1
%RSD	0.1	0.6

Specificity

Specificity by interference study

Specificity has been evaluated by assuring no interference observed at the retention time of main analyte in the chromatograms obtained from the diluents, placebo and Known impurities by injecting diluents, placebo solution, Standard solution, sample solution and spiked solution as per the Analytical procedure[6]. All peaks are well separated from Thiotepa peak.

Specificity by Forced degradation

As shown in Table 4, Acidic degradation led to the faster effect on Thiotepa degradation with about 93.5% Thiotepa remaining only after 15 minutes. After exposure to alkaline Degradation, about 96.0 % of Thiotepa was degraded showing pH effects on Thiotepa stability (Table 4). In oxidative condition & water hydrolysis conditions, Thiotepa was found more stable with degradation. At ambient temperature, the drug was stable for at least 2 days. Each Degradation sample Purity angle is less than the purity threshold for Thiotepa peak.

Table 4: Results of Specificity by Forced degradation

Stress condition	%Assay	%Total degradation (with respect to as such)	Purity angle	Purity threshold
As such(unstressed)	102.0	NA	0.300	1.261
alkali	93.5	8.5	0.232	1.199
acid	96.0	6.0	0.228	1.202
peroxide	99.2	2.8	0.343	1.290
Water Hydrolysis	99.8	2.2	0.297	1.292

Linearity

The linearity of Thiotepa is established by analyzing Linearity solutions of Different concentrations from 50% to 150% of working concentration of method for assay. The Linearity curve is plotted for area versus concentration. The results summarized in Table 5. Hence the analytical procedure is linear within the concentration range from 50% to 150% (159.915µg/mL to 479.744µg/mL) for Thiotepa.

Table 5: Linearity Results of Thiotepa

Linearity Level	Thiotepa (µg/ml)	Area
50%	159.915	1396319
80%	255.864	2210573
90%	287.846	2491556
100%	319.829	2778205
110%	351.812	3034322
120%	383.795	3321815
150%	479.744	4130643
Correlation coefficient(R): 0.9999		
Y-intercept: 26722.5472		
Slope : 8565		
Y-intercept bias at 100% level: 0.9		

Accuracy

The accuracy[6] of an analytical procedure expresses the closeness of agreement between the value that is accepted either as a conventional true value or an accepted reference value and the value found.

To demonstrate the accuracy of assay test method, drug substance is spiked quantitatively into placebo from 50% to 150% of working concentration of test concentration at each level with triplicate preparation and analyzed using the test method. The results for bendamustine HCl is tabulated in Table 6. The results are well within the acceptance criteria; hence the method is accurate for its intended use.

Table 6: Results of Accuracy for thiotepa

Accuracy Level	Sample #	Amount added(mg)	Amount(founded(mg)	%Recovery	Average % Recovery	%RSD
50%	1	15.106	15.230	100.8	101.2	0.6
	2	15.108	15.251	101.0		
	3	15.103	15.389	101.9		
100%	1	30.209	30.410	101.2	100.8	0.3
	2	30.204	30.381	100.7		
	3	30.212	30.373	100.6		
150%	1	45.136	45.012	99.7	99.8	0.1
	2	45.148	45.085	99.9		
	3	45.152	45.089	99.9		
Overall %Recovery						100.6
Overall %RSD						0.7

Range

The range of the analytical method is considered from Linearity, Precision and Accuracy of the method [6]. Based on the Linearity, Method Precision and Accuracy data, Range of the method is 50% to 150% of test concentration.

Stability of analyte in solution

Stability of Analyte in solution is evaluated for the Standard and Sample solutions. The Standard and Sample Solution are prepared and analysed as per analytical procedure. A portion of these solutions were preserved at room temperature and Refrigerator (2-8°C), analyzed at Different time intervals from the time of preparations. The results are calculated from initial versus over a period of time. The results are summarized in Table 7 and 8. The Data indicates that the Standard and Sample Solutions are stable up to 48 hours at room temperature and refrigerator (2-8°c) conditions.

Table 7: Stability of Standard and sample solution at Room temperature

Time Interval	Room Temperature

	Standard		**Sample**	
	Area	**%Difference**	**Area**	**%Difference**
Initial	2912072	NA	3230076	NA
20Hrs	2872282	1.4	3196359	1.0
24 Hrs	2889223	0.8	3213181	0.5
44Hrs	2871189	1.4	3183609	1.4
51Hrs	2844199	2.3	3163600	2.1

Table 8: Stability of Standard and sample solution at Refrigerator (2-8°C)

Time Interval	**Refrigerator(2-8°c)**			
	Standard		**Sample**	
	Area	**%Difference**	**Area**	**%Difference**
Initial	2912072	NA	3230076	NA
24Hours	2908874	0.1	3219817	0.3
36 hours	2920158	-0.3	3231547	0.0
52Hrs	2912108	0.0	3197120	1.0

Filter Variability

Filter variability is evaluated for sample solution. The sample solution was prepared and analyzed as per the analytical procedure. A portion of these solutions were filtered through 0.22µm PVDF Filter and 0.22µm nylon Filter and analyzed as per methodology. The results are summarized in Table 9.

Table 9: Filter Variability Results

Type of filter	**%Assay**	**%Difference**
Unfiltered	101.2	NA
0.22µm PVDF filter	100.3	0.9
0.22µm nylon filter	100.6	0.6

CONCLUSION

A simple, precise cost effective and rapid stability-indicating high-performance liquid chromatographic method was developed and validated for the determination of Thiotepa in pharmaceutical dosage forms. The analytical method was found to specific, linear, precise, accurate, and robust, stability indicating and suitable for intended use. Chromatographic separation was achieved on a reverse phase C18 column (YMC,150mmx4.6mm, 3.5 µm,12nm) using mobile phase A consisted of (3.5g of potassium Dehydrogen phosphate/1liter: 4.2g of Disodium hydrogen Phosphate/2liters)Buffer : Acetonitrile (85:15v/v) and The mobile phase B consisted of (3.5g of potassium Dihydrogen phosphate/1liter: 4.2g of Disodium hydrogen Phosphate/2liters)Buffer : Acetonitrile (50:50v/v) gradient flow rate at 1.0 mL/min for 30minutes. The retention time of Thiotepa was 11.313 minutes. The proposed method was validated as per ICH.

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