EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Coden USA: EJBPBO
Volume: 13
Issue: 11
Year: 2026

A REVIEW ON METHOD DEVELOPMENT AND VALIDATION OF SELECTED ANTINEOPLASTIC DRUG TREOSULFAN BY USING DIFFERENT ANALYTICAL TECHNIQUES

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DOI: https://doi.org/10.5281/zenodo.23803918

How to cite this Article: Dr. Santhosh Illendula[1]*, Kurumeti Divya[2], Mothe Praneeth[2], Vusala Tejaswi[2], Varipally Sai Sindhu[2], Vootkuri Nihathi[2], Jetamoni Mahesh[2], Dr. Suthakaran Raj[3] (2026). A Review On Method Development And Validation Of Selected Antineoplastic Drug Treosulfan By Using Different Analytical Techniques. European Journal of Biomedical and Pharmaceutical Sciences, 13(11), 49–58.

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Article Received on 24/09/2026 **Article Revised on 14/10/2026** **Article Published on 01/11/2026**

ABSTRACT

Tresosulfan a synthetic alkylating agent and bifunctional prodrug that undergoes pH dependent conversion to active epoxybutane derivatives responsible for its cytotoxic activity. It is used mainly as a myeloablative agent in conditioning regimens prior to hematopoietic stem-cell transplantation and has also been investigated in the treatment of various malignancies. Owing to its therapeutic importance and the need for accurate drug quantification, development of a simple, rapid, sensitive, and reliable analytical method for the estimation of Treosulfan is essential. The present study is focused on the development and validation of an analytical method for the estimation of Treosulfan using Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC). Method development involves optimization of chromatographic parameters such as stationary phase, mobile phase composition, flow rate, injection volume, detection wavelength, column temperature, and run time to obtain satisfactory separation and symmetrical peak shape with good resolution. The developed method is intended to provide adequate sensitivity and reproducibility for the quantitative determination of Treosulfan in pharmaceutical formulations. The developed analytical method is validated according to ICH guidelines, with respect to parameters including specificity, linearity, accuracy, precision, range, limit of detection (LOD), limit of quantification (LOQ), robustness, and system suitability. The method is expected to demonstrate good linearity over the selected concentration range, satisfactory accuracy and precision, low LOD and LOQ, and robustness against small deliberate changes in chromatographic conditions.

KEYWORDS: Method development, method validation, RP-HPLC, Pharmaceutical analysis, chromatography method, ICH guidelines, Quality control.

INTRODUCTION

Analytical chemistry is a branch of chemistry which deal with identification of components and determination of number of components of elements or samples or mixture. Analytical method development and method validation is key role in drug discovery, method development and production of various pharmaceutical dosages form. These methods used to confirm the identity, transparency, effectiveness, and protection of drug products. The goal of the HPLC method is to separate, calculate the main active drug, any response

impurities, all available synthetic inter-mediates and any degradants.

SPECTROSCOPY

Spectroscopy is the branch of science that deals with the study of the interaction between matter and electromagnetic radiation (light or energy).

Types of spectroscopy

1. UVVisible spectroscopy
2. Infrared (IR) spectroscopy
3. Raman spectroscopy
4. Nuclear Magnetic Resonance (NMR) spectroscopy
5. Atomic Absorption Spectroscopy (AAS)
6. Atomic Emission Spectroscopy (AES)
7. Fluorescence spectroscopy
8. X-ray spectroscopy
9. Microwave spectroscopy
10. Electron spectroscopy

UV VISIBLE SPECTROSCOPY

Introduction

UV Visible Spectroscopy is an important instrumental analytical technique used in pharmaceutical analysis. It is based on the absorption of ultraviolet and visible radiation by molecules. It is widely used for the identification, determination of concentration, and analysis of drugs in pharmaceutical formulations.

Definition

UV Visible Spectroscopy is an analytical technique that measures the absorption of ultraviolet and visible light by a substance as a function of wavelength.

Ultraviolet region: 200-400 nm
Visible region: 400-800 nm

Principle

When UV or visible light passes through a sample, the molecules absorb radiation of specific wavelengths. This absorption causes electrons to move from a lower energy orbital to a higher energy orbital.

Components of a UV-Visible spectrophotometer

Light source: Deuterium lamp is used for the UV region, and tungsten-halogen lamp is commonly used for the visible region.
Monochromator: Selects the required wavelength of light.
Sample holder: Holds the sample in a cuvette.
Detector: Detects the transmitted light and converts it into an electrical signal.
Display system: Displays absorbance or transmittance.

Advantages

Simple and rapid technique.
Requires a small quantity of sample.
Non-destructive in many applications.
Good sensitivity for suitable compounds.
Useful for routine pharmaceutical analysis.

Conclusion

UV Visible Spectroscopy is a simple, reliable, and widely used instrumental method in pharmaceutical analysis. It plays an important role in drug identification, quantitative estimation, quality control, and stability studies.



Fig 01: Uv Visible Spectroscopy Instrumentation.

CHROMATOGRAPHY

The chromatography derived from the word chromo means colour and graphs means to write i.e., colour groups are shaped in the process which are measured or analysed. These colour groups are shaped due to the separation of separate complexes. Under these conditions, standards and analytical procedures for these drugs might not be available within the pharmacopoeias. Thus, it becomes necessary, to develop newer analytical methods for such drugs. The object of the characterization is to generate a reliable, accurate and interpretable set of information describing the sample. The analysis is vital in any product or service, and it is also important in drug development.

Chromatography is a procedure that is used for separating a complex mixture into its individual particular fractions or components. It is a separation technique and the separated compounds can be identified by using any analytical technique like UV-visible,

Infrared, Mass spectroscopy, NMR etc. "Chromatography derives its name from two words as chromo means colour and graphy means to write i.e. colour bands are formed in the procedure which are measured or analyzed. These colour bands are formed due to the separation of individual compounds. Analytical chemistry deals with methods for identification, separation, and quantification of the chemical components of natural and artificial materials.

Types of chromatography

Paper Chromatography
Thin Layer Chromatography (TLC)
Column Chromatography
Gas Chromatography (GC)
High-Performance Liquid chromatography
Ion-Exchange Chromatography
Size-Exclusion Chromatography
Affinity Chromatography

HPLC

HPLC is the method of choice for checking peak purity of new chemical moieties, reaction monitoring and evaluating new formulations. The official test methods that result from these processes are used by quality control laboratories to ensure the identity, purity, potency, and performance of drug products. In the modern pharmaceutical industry, high-performance liquid chromatography (HPLC) is the major and integral analytical tool applied in all stages of drug discovery, development, and production. The Goal of HPLC method is to try & separate, quantify the main drug, any reaction im-purities, synthetic intermediates and any degradation products. HPLC principle is the solution of sample is injected into a column of porous material (stationary phase) and liquid phase (mobile phase) is pumped at higher pressure through the column. The principle of separation followed is the adsorption of solute on stationary phase based on its affinity towards stationary phase.

Main features of HPLC

High resolution
Small diameter, Stainless steel, Glass column
Rapid analysis
Relatively higher mobile phase pressure
Controlled flow rate of mobile phase

Classification of HPLC can be done as

(Preparative HPLC and analytical HPLC (based on scale of operation)
(Affinity chromatography, adsorption chromatography, size exclusion chromatography, ion exchange chromatography, chiral phase chromatography (based on principle of separation)
(Gradient separation and isocratic separation, (based on elution technique)
(Normal phase chromatography and reverse phase chromatography (based on modes of operation).

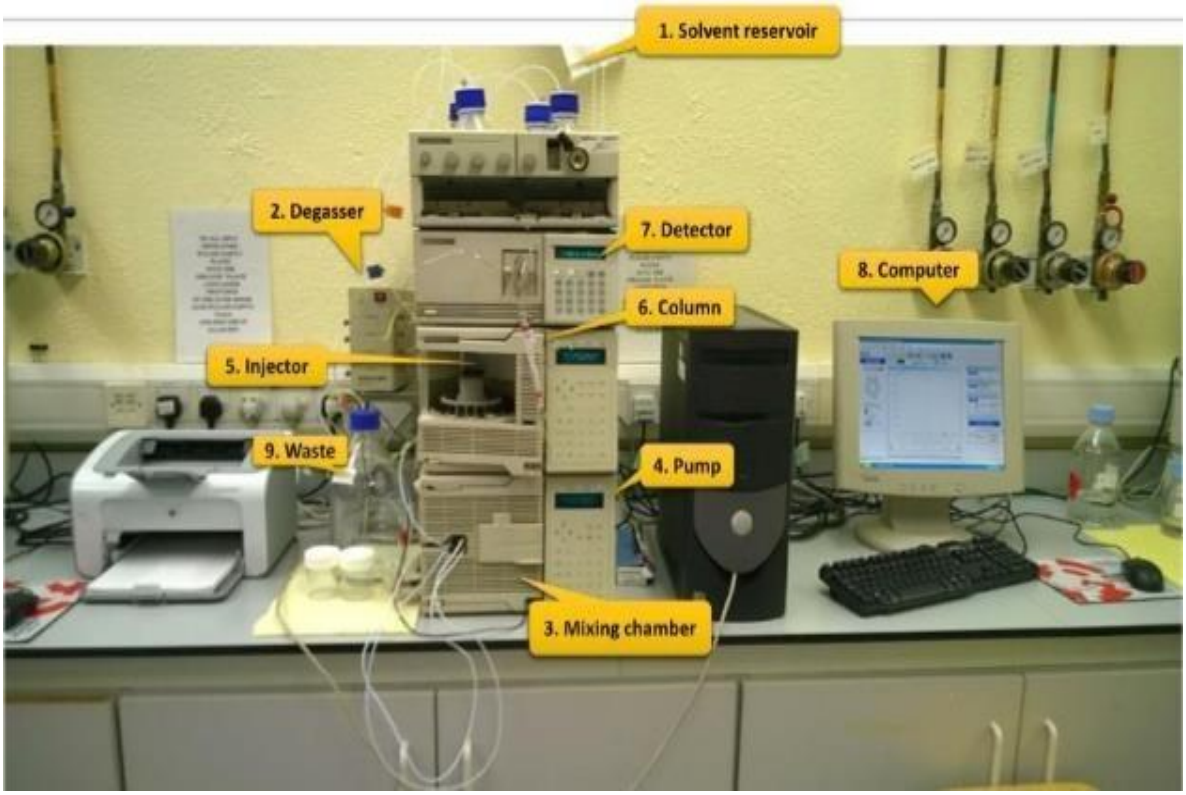


Fig 02: Hplc Instrumentation.

GAS CHROMATOGRAPHY

Gas chromatography is an analytical laboratory technique used to separate, identify, and quantify the different chemical components of a mixture.

Principle

In GC, the sample is vaporized and carried through a chromatographic column by an inert carrier gas. Different components interact differently with the stationary phase and therefore travel through the column at different rates, resulting in separation.

Main Components of GC Instrument

Carrier gas system: Supplies an inert gas such as helium, nitrogen, or hydrogen. Carries the vaporized sample through the column.
Injector: Introduces the sample into the instrument. The sample is rapidly vaporized.
Column: The main site of separation.
Common types include: capillary columns and packed columns.
Oven: Maintains and controls the temperature of the column. Temperature can be constant or programmed.
Detector: Detects compounds as they leave the column.

Common detectors include

FID Flame Ionization Detector
TCD Thermal Conductivity Detector
ECD Electron Capture Detector.

Data system: Records detector signals and produces a chromatogram. Provides information such as retention time and peak area.

Conclusion

Gas Chromatography is an important analytical technique for separating and analyzing volatile substances. It is widely used in pharmaceutical industries, particularly for residual solvent analysis, purity testing, and identification of volatile compounds.

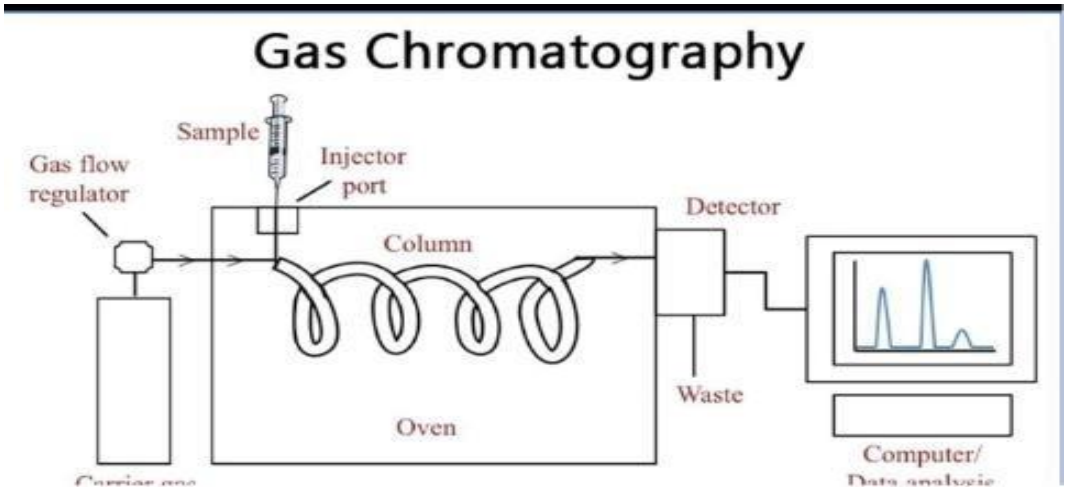


Fig 03: Gas Chromatography Instrumentation.

LIQUID CHROMATOGRAPHY MASS SPECTROSCOPY

Definition

Liquid Chromatography Mass Spectrometry (LCMS) is a powerful analytical technique that combines liquid chromatography (LC) for separation of components with mass spectrometry (MS) for identification and determination of their molecular masses.

Principle

LC: Separates compounds in a mixture based on their interaction with the stationary phase and mobile phase.
MS: Converts compounds into ions and separates the ions according to their mass-to-charge ratio (m/z).

Main Components

Solvent reservoir Contains the mobile phase.
Pump Delivers mobile phase at high pressure.
Injector Introduces the sample.
LC column Separates the sample components.
Ionization source Converts molecules into ions; commonly ESI (Electrospray Ionization).
Mass analyzer Separates ions according to their m/z ratio.
Detector Detects the separated ions.
Data system Records and processes chromatograms and mass spectra.

Working

The sample is injected into the LC system.
The mobile phase carries the sample through the column.
Different compounds are separated based on their interactions with the column.
The separated compounds enter the MS through an ionization source.
Molecules are converted into charged ions.
The mass analyzer separates ions according to their m/z values.
The detector measures the ions.
The computer produces a chromatogram and mass spectrum for identification and quantification.

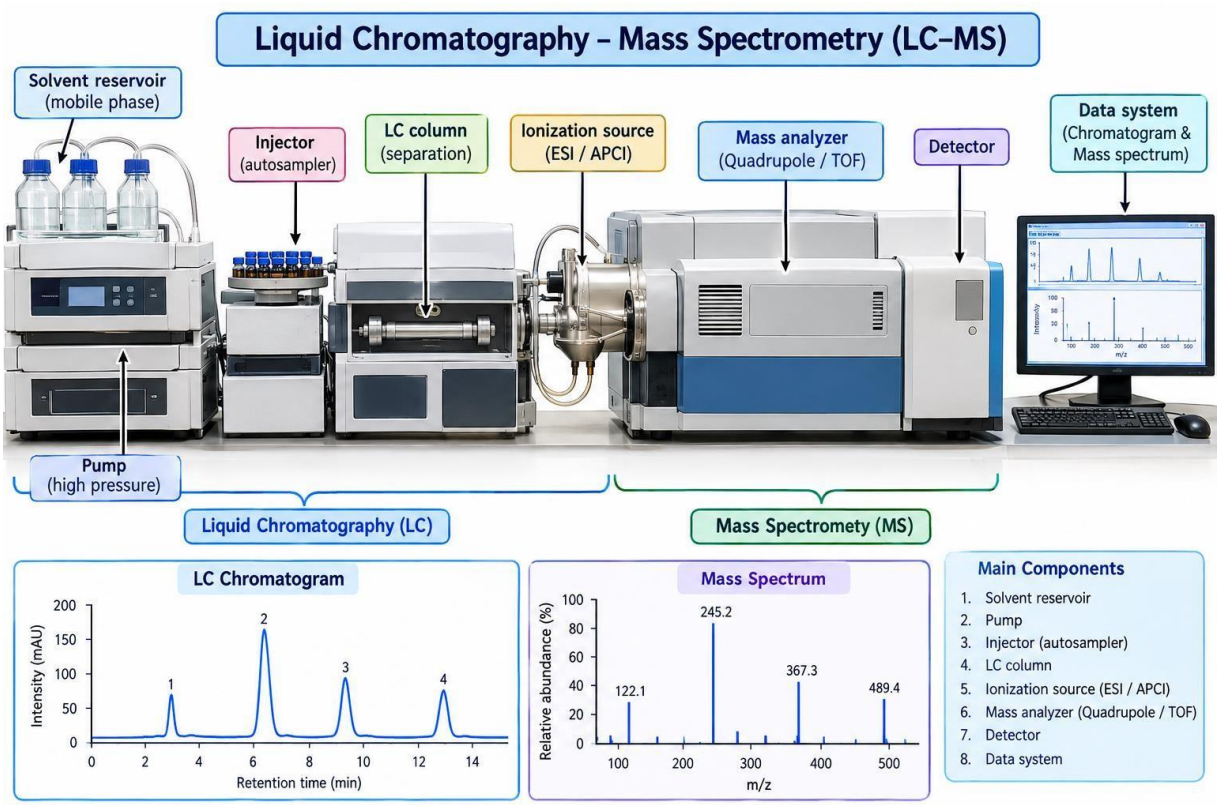


Fig 04: Liquid Chromatography Mass Spectroscopy Instrumentation.

Phases of chromatography

Normal phase chromatography

In Normal Phase mode the stationary phase is po-lar and the mobile phase is non-polar in nature. In this technique, non-polar compounds travel faster and are eluted first. This is because of the less affinity between the non-polar compounds and the stationary phase. Polar compounds are retained for longer times because of their higher affinity with the stationary phase. These compounds, due to affinity with stationary phase take more times to elute.

Reversed phase chromatography

It is the popular mode for analytical and preparative separations of compound of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this mode the stationary phase is non polar hydrophobic packing with octal or octa-decyl functional group bonded to silica gel and the mobile phase is polar solvent. The polar compound gets eluted first because of less affinity for stationary phase and non-polar compounds are retained for longer time. As most of the d rugs and pharmaceuticals are polar in nature, they are not retained for longer times and hence elute faster.

Analytical Method Development

Analytical method development and validation play important roles in the discovery, development and manufacture of pharmaceuticals. These methods used to ensure the identity, purity, potency and safety of drug products. The goal of the HPLC method is to separate, quantify the main active drug, any reaction impurities, all available synth-etic inter-mediates and any degradants.

Need for developing a method

Available method may be too expensive, time consuming or energy intensive, or that may not be easily automated.

Existing method may be too much error, contami-nation prone or they may be unreliable.

There may be need for an alternative method to confirm, for legal or scientific reasons, analytical data originally obtained by existing methods.

There may not be a suitable method for a particu-lar analyte in the specific sample matrix.

Existing method may not provide adequate sensi-tivity.

For regulatory requirements it is required.

Newer instrumentation and techniques may have evolved that provide opportunities for improved methods, including improved analyte identific- ation or limit of detection, greater accuracy or bet-ter return on investment.

Requirements for new Method Development

The drug or drug combination may not be official in any pharmacopoeias.

A proper analytical procedure for the drug may not be available in the literature due to patent regulations.

Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation ex-cipients.

Analytical methods for a drug in combination with other drugs may not be available.

The existing analytical procedures may require costly reagents and solvents. It may also in-volve cumbersome extraction and separation procedures and these may not be reliable.

Steps in Method Development

Physicochemical properties of drug molecule
Selection of chromatographic conditions
Developing the approach of analysis
Preparation sample
Method optimization
Method validation

Preparation of Sample Solutions For Method Development

The drug substance being analyzed should be stable in solution (diluent). During initial method development, preparations of the solutions in amber flasks should be performed until it is determined that the active component is stable at room temperature and does not degrade under normal laboratory conditions. The sample solution should be filtered; the use of a 0.22 or 0.45 µm pore-size filter is generally recommended for removal of particulates. Filtration is a preventive maintenance tool for HPLC analyses.[8-11] Sample preparation is a critical step of method development that the analyst must investigate. The effectiveness of the syringe filters is largely determined by their ability to remove contaminants/insoluble components without leaching undesirable artifacts (i.e., extractables) into the filtrate. If any additional peaks are observed in the filtered samples, then the diluent must be filtered to determine if a leachable component is coming from the syringe filter housing/filter.

Method optimization

The experimental conditions should be optimized to get desired separations and sensitivity after getting appropriate separations. Stability indicating assay experimental conditions will be achieved through planned/systemic examination on parameters including pH (if ionic), mobile phase components and ratio, gradient, flow rate, temperature, sample amounts, Injection volume and diluents solvent type.

Validation of method

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for its intended use. The methods validation process for analytical procedures begins with the planned and systematic collection by the applicant of the validation data to support analytical procedures.[12] All analytical methods that are intended to be used for analyzing any clinical samples will need to be validated. The validation of analytical methods is done as per ICH guidelines. Components of.

Method Validation

The following are typical analytical performance characteristics which may be tested during methods validation.

Accuracy is the nearness of a measured value to the true or accepted value. Accuracy indicates the deviation between the mean value found and the true value. lt is determined by applying the method to samples to which known amounts of analyte have been added. These should be analysed against standard and blank solutions to ensure that no interference exists. The accuracy is then calculated from the test results as a percentage of the analyte recovered by the assay. It may often be expressed as the recovery by the assay of known, added amounts of analyte.

The precision of an analytical method is the degree of agreement among individual test results obtained when the method is applied to multiple sampling of a homogenous sample.

Precision is a measure of the reproducibility of the whole analytical method It consists of two components- repeatability and intermediate precision.

Repeatability is the variation experienced by a single analyst on a single instrument. It does not distinguish between variation from the instrument or system alone and from the sample preparation process. During validation, repeatability is performed by analysing multiple replicates of an assay composite sample by using the analytical method. The recovery value is calculated. Intermediate precision is the variation within a laboratory such as different days, with different instruments, and by different analysts.[15-16] The precision is then expressed as the relative standard deviation. Accuracy and precision are not same, as the diagram below indicates this method can have a good precision but not get an accurate result.

Linearity is an analytical procedure to obtain a response that is directly proportional to the concentration (amount) of analyte in the sample. If the method is linear, the test results are directly or by well-defined mathematical transformation proportionalto concentration of analyte in samples within a given range. Linearity is usually expressed as the confidence limit around the slope of the regression line.

Detection Limit

The Detection limit (DL) or limit of detection (LOD) of an individual procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. In analytical procedures that exhibit baseline noise, the LOD can be based on a signal-to-noise (S/N)ratio (3:1), which is usually expressed as the concentration of analyte in the sample.(book) The signal-to-noise ratio is determined by: s = H/h WhereH= height of the peak corresponding to

the component. h = absolute value of the largest noise fluctuation from the baseline of the chromatogram of a blank solution.

Quantitation Limit

The limit of Quantitation (LOQ) or Quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. For analytical procedures such as HPLC that exhibit baseline noise, the LOQ is generally estimated from a determination of S/N ratio (10:1) and is usually confirmed by injecting standards which give this S/N ratio and have an acceptable percent relative standard deviation as well. Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present such as impurities, degradation products, and excipients. Specificity measures only the desired component without interference from other species that might be present; separation is not necessarily required. Range is defined as the interval between the upper and lower concentrations of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity. Robustness is defined as the measure of the ability of an analytical method to remain unaffected by small but deliberate variations in method parameters (e.g. pH, mobile phase composition, temperature and instrumental settings) and provides an indication of its reliability during normal usage.

Determination of robustness is a systematic process of varying a parameter and measuring the effect on the method by monitoring system suitability and/or the analysis of samples. System Suitability Determination is the evaluation of the components of an analytical system to show that the performance of a system meets the standards required by a method These parameters can be calculated experimentally to provide a quantitative system suitability test report: number of theoretical plates (efficiency), capacity factor, separation (relative retention), resolution, tailing factor, relative standard deviation (precision). These are measured on a peak or peaks of known retention time and peak width.

DRUG PROFILE

TREOSULFAN

IUPAC NAME: (2S,3S)-2,3-dihydroxybutane-1,4-diyl bis(methanesulfonate).

CHEMICAL FORMULA:$C_6H_{14}O_8S_2$

MOLECULAR FORMULA: 278.30 g/mol.

STRUCTURE

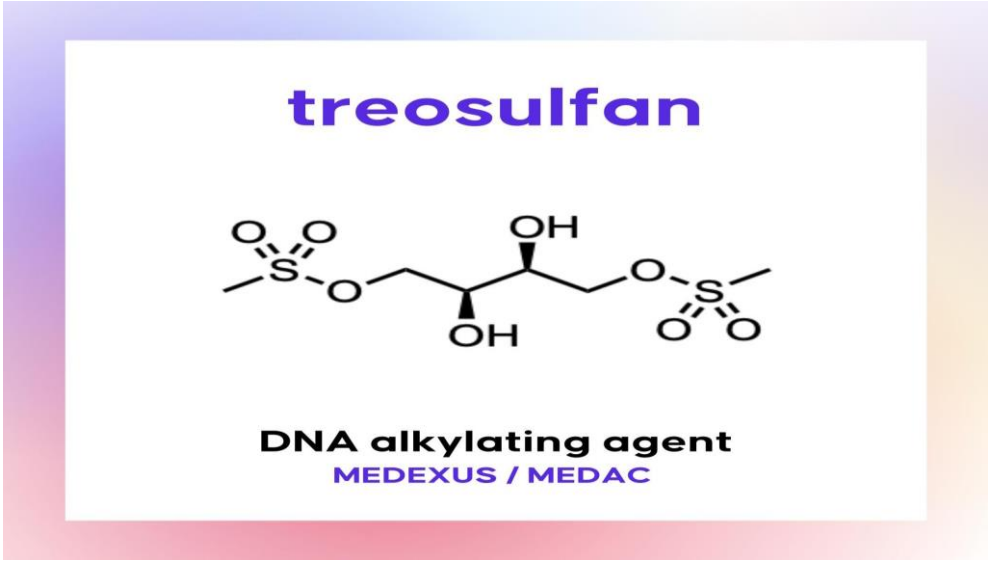


Fig.5: Structure of Treosulfan.

PHARMACODYNAMICS

Treosulfan is a bifunctional alkylating prodrug that spontaneously converts into active epoxide metabolites to exert cytotoxic, myeloablative, and immunosuppressive effects.

MECHANISM OF ACTION

Treosulfan - specifically its active epoxide metabolites - function as a DNA alkylating agent, which is likely responsible for its cytotoxic activity. DNA alkylation involves the substitution of alkyl groups for hydrogen atoms on DNA, allowing for cross-linking within the DNA molecule resulting in cytotoxic, mutagenic, and carcinogenic effects.

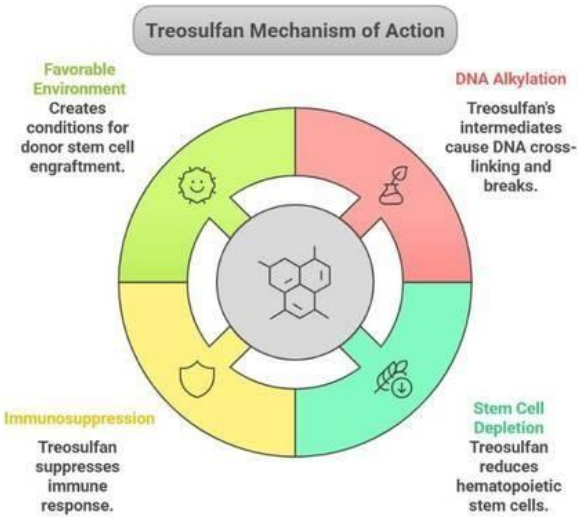


Fig 06: Mechanism of action of treosulan.

VOLUME OF DISTRIBUTION
The mean volume of distribution of treosulfan in adult patients is approximately 41 liters (or about 17 to 34–47 liters, corresponding to roughly 0.25–0.5 L/kg).

PROTEIN BINDING
Treosulfan exhibits negligible or very low plasma protein binding.

METABOLISM
Treosulfan is a prodrug that undergoes a spontaneous, non-enzymatic conversion into its active epoxide metabolites under physiological pH and temperature conditions.

ROUTE OF ELIMINATION
Treosulfan is primarily eliminated through the kidneys (renal excretion) and spontaneous, non-enzymatic chemical conversion. Unlike its structural analog busulfan, treosulfan does not undergo significant hepatic (liver) metabolism, giving it a distinct pharmacokinetic and safety profile. Core Routes of Elimination Renal Excretion: A significant portion–typically 15% to 40%–of the administered treosulfan dose is excreted unchanged in the urine. This occurs via dose-dependent glomerular filtration, followed by a tubular reabsorption rate of approximately 60%. The majority of this renal clearance happens rapidly, with about 89% of the unchanged fraction cleared within the first 8 hours Spontaneous Chemical Conversion: The remaining portion of the drug is eliminated from systemic circulation through a spontaneous, non-enzymatic conversion under normal physiological conditions (body temperature and pH). As a prodrug, treosulfan breaks down into its active, alkylating metabolites: a monoepoxide intermediate and ultimately active L-diepoxybutane. These active epoxides are then subsequently cleared from tissues, primarily by epoxide hydrolase enzymes in organs like the liver and lungs.

HALF LIFE
Treosulfan is cleared from the blood rapidly, with a short average elimination half-life of 1.5 to 2 hours.

Table 1: Different analytical techniques on TREOSULFAN.

S.NO	DRUG NAME	ANALYTICAL TECHNIQUE	DESCRIPTION OF TECHNIQUE	AUTHOR & YEAR
1.	Treosulfan	RP-UPLC	System: RP-UPLC with UV detection; LC–MS/MS Mobile phase: Acetonitrile : ammonium acetate (pH 2.5)/formic acid = 40:60 (v/v) Column: Waters Acquity UPLC BEH Shield RP-18, 50 × 1.0 mm, 1.7 µm Flow rate:0.2 mL/min Injection volume:5 µL Detection wavelength:239 nm pH:2.5 Linearity:$r^2 > 0.999$ LOD; Met acceptable criteria; LOQ: Met acceptable criteria; Accuracy:99–101% recovery Precision:%RSD < 1%	Sudha Divya Madhuri Kallam, Mithun Rudrapal 2026

2.	Treosulfan	LC-MS/MS	Sample: Treosulfan bulk drug/API and related Substances. System: LC–MS/MS; ACQUITY UPLC PLUS coupled with Xevo TQ-XS MS Run time:4 minutes Linearity:3.13–100 µg/mL LOQ:3.13 µg/mL Sample:25 µL human plasma Sample preparation: Protein precipitation using	Jeayeon Ryu et al., 2026

3.	Tresosulfan	HPLC-MS/MS	Acetonitrile: Internal standard:Treosulfan-D4 Detection: MRM: Treosulfan m/z 296.2 → 87.1; Treosulfan-D4 300.2 → 91.1 System: HPLC–MS/MS (High-Performance Liquid Chromatography–Tandem Mass Spectrometry) Column: Zorbax Eclipse Plus C18 Mobile phase: Formate buffer (pH 4.0) : Acetonitrile = 95:5 (v/v) Detection: Triple-quadrupole MS/MS, ESI positive mode, MRM Sample: Human plasma	Danielak D twardosz et.al 2018
4	Treosulfan	HPLCMS/MS	System: RP-HPLC-UV System: Shimadzu HPLC system with UV detector Mobile phase: Methanol : Water = 65:35 (v/v) Column: End-capped C18, 250 × 4.6 mm, 5 µm Flow rate:1.0 mL/min Detection wavelength:254 nm Linearity:2.5–50 µg/mL Correlation coefficient:0.9987 Accuracy: Intra- and inter day accuracy <8% Precision: Intra- and inter day precision <8% Sample: Human plasma Sample preparation: Derivatization with 10% sodium diethyldithiocarbamate Internal standard: Dinitro biphenyl Analytes: Treosulfan + its epoxy metabolites	Sundar rao koyyalamudi. Et. Al 2015

5	Tresosulfan	-HPLC	System: HPLC with refractive index Analytes: Treosulfan, S,S-EBDM and S,S-DEB Internal standard: Acetaminophen Sample preparation: Samples were directly injected into the HPLC system Column: Reversed-phase column Mobile phase: Acetate buffer pH 4.5 + acetonitrile Detection: Refractive index detection Calibration range – TREO:20–6000 µM Calibration range – S,S-EBDM:34–8600 µM Calibration range – S,S-DEB:50–6000 µM Precision: Intra- and inter day precision fulfilled analytical criteria Accuracy: Intra- and inter day accuracy fulfilled analytical criteria.	Franciszek k. Glowka et.al 2013

Table 2: BRAND NAMES.

S.NO	BRAND NAME	API	MANUFACTURING COMPANY
1	Trecondi	Treosulfan	Medac Gmph, Germany
2	Grafapex	Treosulfan	Medexus pharma ,Inc
3	Trecondyv	Treosulfan	Medexus pharmaceuticals, Inc./ medac
4	Treoall	Treosulfan	Hetero healthcare limited, India
5	Emtreo	Treosulfan	Emcure pharmaceutical limited.., india

CONCLUSION

A survey of literature reveals that The literature review indicates that treosulfan is an important alkylating agent used as part of conditioning regimens before hematopoietic stem cell transplantation (HSCT). It has demonstrated significant therapeutic potential in patients undergoing transplantation, with its pharmacokinetic profile being an important consideration because of interindividual variability. Various analytical techniques have been reported for the estimation and quantification of treosulfan, including HPLC and LC-MS/MS methods. These methods provide reliable determination of treosulfan in pharmaceutical and biological samples, with LC-MS/MS offering high sensitivity and selectivity for pharmacokinetic and therapeutic drug-monitoring applications.

Overall, the reviewed literature provides a useful foundation for the development and validation of a simple, accurate, precise, sensitive, and robust HPLC method for the estimation of treosulfan, following appropriate ICH analytical validation guidelines.

ACKNOWLEDGEMENT

The authors are grateful to the management of Vijaya college of pharmacy, munaganoor, Hyderabad for providing the facilities to carry out the present work.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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