INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES
[ISSN: 0975-4725; CODEN(USA): IJPS00]
Journal Homepage: https://www.ijpsjournal.com

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Research Article

Development and validation of an RP-HPLC method for simultaneous estimation of Sulopenem Etzadroxil and Probenecid in bulk and Synthetic Mixture

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ARTICLE INFO

Published: 04 Jun. 2026
Keywords:
Sulopenem Etzadroxil, Probenecid, RP-HPLC, Accuracy, Linearity and Precision.
DOI:
10.5281/zenodo.20540747

ABSTRACT

Sulopenem Etzadroxil is an orally bioavailable prodrug of sulopenem, a broad-spectrum β-lactam antibiotic and is co-administered with Probenecid to enhance systemic exposure by inhibiting renal tubular secretion. Considering the clinical importance of this fixed-dose combination, the present work was undertaken to develop and validate a simple, rapid, accurate and precise reverse-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of Sulopenem Etzadroxil and Probenecid in bulk drug and synthetic mixture. Chromatographic separation was achieved using a Hypersil BDS C18 column (250 × 4.6 mm, 5 µm) with a mobile phase consisting of Methanol : Ammonium formate buffer (pH 3.2 adjusted with formic acid) in the ratio of 40:60 v/v. The mobile phase was delivered at a flow rate of 1.0 mL/min under isocratic mode, and detection was carried out at 272 nm, which corresponded to the isobestic point for both drugs. The total run time was 5 minutes. Under optimized conditions, Sulopenem Etzadroxil and Probenecid were eluted at retention times of approximately 2.4 minutes and 3.3 minutes, respectively, with good resolution and symmetrical peak shapes. The proposed method was validated as per ICH Q2 (R1) guidelines for parameters including specificity, linearity, accuracy, precision, robustness, limit of detection (LOD), limit of quantification (LOQ) and system suitability. The method showed linearity in the concentration range of 25–150 µg/mL for both drugs, with correlation coefficients (r^2) of 1.000 for Sulopenem Etzadroxil and 0.998 for Probenecid. Accuracy studies demonstrated percentage recovery in the range of 100.7–100.9% for Sulopenem Etzadroxil and 99.8–100.2% for Probenecid. Precision studies revealed %RSD values less than 2% for repeatability, intra-day, and inter-day

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Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

precision, indicating good reproducibility of the method. The LOD values were 1.89 µg/mL for Sulopenem Etzadroxil and 1.64 µg/mL for Probenecid, while LOQ values were 5.7 µg/mL and 4.99 µg/mL, respectively. Robustness evaluation confirmed that small deliberate variations in chromatographic conditions did not significantly affect method performance. In conclusion, the developed RP-HPLC method is specific, sensitive, economical, robust and reproducible, making it suitable for routine quality-control analysis of Sulopenem Etzadroxil and Probenecid in bulk drug and synthetic mixture and it can be effectively applied in pharmaceutical research and quality-control laboratories.

INTRODUCTION

1.1 Introduction to disease (Urinary Tract Infection)

- ❖ Urinary tract infections (UTIs) are among the most common bacterial infections in humans, representing a major public health concern worldwide.
- ❖ They account for approximately 40% of all hospital-acquired infections and are a leading cause of outpatient visits and antibiotic prescriptions.
- ❖ Globally, UTIs affect an estimated 150 million people annually, resulting in significant healthcare costs and morbidity.

1.2 Introduction of Drug

- ❖ Sulopenem Etzadroxil

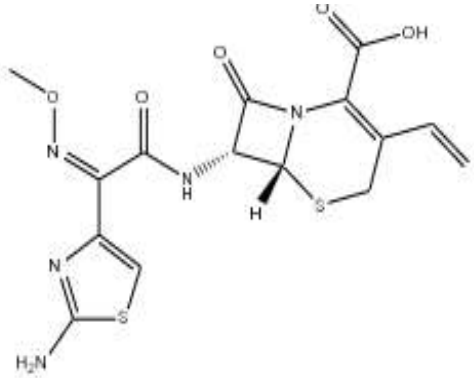

Fig : 1.1 Structure of Sulopenem Etzadroxil

- It is a prodrug of sulopenem (a penem β-lactam antibiotic).
- Molecular formula : C16H17N5O6S2
- Molecular weight : ~443.5 g/mol
- Mechanism:
  - ➢ Binds to penicillin-binding proteins (PBPs)
  - ➢ Inhibits bacterial cell wall synthesis
  - ➢ Leads to bacterial death (bactericidal action).

- ❖ **Probenecid**

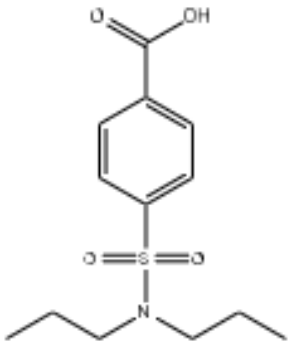

Fig : 1.2 Structure of Probenecid

- A renal tubular transport inhibitor
- Molecular formula : C13H19NO4S
- Molecular weight : 285.36 g/mol
- Mechanism:
  - ➢ Blocks organic anion transporters (OAT1/OAT3) in the kidney
  - ➢ Reduces excretion of sulopenem
  - ➢ Increases drug concentration in blood

2.Material and methods

Materials

- Drug Substance: Sulopenem Etzadroxil and Probenecid
- Brand name: Orlynvah

- Product name: Sulopenem etzadroxil and Probenecid Tablet Manufacturer: Iterum Therapeutics

Instrumentation:

Table 2.1 Apparatus and instrument for High Performance Liquid Chromatography

Sr No.	Instrument and Apparatus	Model
1.	HPLC	Waters Alliance HPLC system (e2695) with Empower 2.0 software
2	Digital balance (1 mg sensitivity)	Sartorius
3	pH meter	Eutech
4	Ultrasonicator	Unichrome UCA 701
5	0.2 micrometer membrane filter	Ultipor N66 Nylon
6	Glasswares	Borosilicate

Table: 2.2 Instrument specification for melting point apparatus

Make	Gallenkamp
Design no.	889339

Table: 2.3 Instrument specification for UV-visible spectrophotometry

Make	Shimadzu
Model	UV-1700
Type	Double beam spectrophotometer
Detector	Photodiode
Scanning Range	190-1100
Output	%T & Absorbance
Software	U.V. Probe

Table: 2.4 Reagents and materials for HPLC

Sr.No.	Name of APIs	Source
1	Sulopenem etzadroxil reference standard (purity ≥ 99.0%)	Medchem express
2	Probenecid reference standard (purity ≥ 99.0%)	DC chemicals
3	Acetonitrile (ACN) – HPLC grade	Merck, India
4	Methanol (MeOH) - HPLC grade	Merck, India

5	Formic acid – AR grade	SD Fine Chemicals
6	Ammonium formate	SD Fine Chemicals
7	Water - Milli-Q (18.2 MΩ·cm)	Millipore system

2.1Preparation of solutions Preparation of Mobile Phase

- The mobile phase, consisting of Methanol:ammonium formate buffer (pH 3.2 with formic acid) (40:60), was prepared, filtered, and degassed. This composition was chosen as the optimal mobile phase for both the drugs, as it exhibited excellent resolution and accurate peak characteristics.
- Ammonium formate buffer (10 mM, pH 3.2) was prepared by dissolving 0.77 g of ammonium formate in purified water, adjusting the pH to 3.0 with formic acid, and making up the volume to 1000 mL with water. The buffer was filtered through a 0.45 µm membrane filter prior to use.

Preparation of Standard Stock Solution

- Accurately weighed 10 mg of sulopenem etzadroxil and probenecid were separately transferred into volumetric flasks and dissolved in methanol 10 ml to obtain stock solutions. Pipette out 2 ml of this solution and dilute upto 10 ml to obtain 200 ppm for both the drugs.

Preparation of sample solution

- A synthetic mixture containing sulopenem etzadroxil and probenecid in the 1:1 ratio
- was prepared by accurately weighing the drugs (10 mg each), transferring them into a volumetric flask, and dissolving them in the methanol.
- The solution was sonicated to ensure complete dissolution and then filtered before analysis. Appropriate dilution with mobile phase was made to obtain final concentrations within the working range of the method.

Chromatographic condition

The chromatographic separation of sulopenem etzadroxil and probenecid was achieved on Hypersil BDS C18 130 A˚(250 × 4.6 mm, 5 µm) by using mobile phase composed of Methanol: ammonium formate buffer (pH 3.2 with formic acid) (40:60), at flow rate 1 ml/min with run time of 10 minutes. Detection of drug was carried out at 272 nm by using diluent as mobile phase.

2.2 Identification of drugs

Identification by Melting Point Determination

Melting point of drugs has been determined. The melting points of the compounds were taken by open capillary method

Table 2.1 Melting Point of Drugs

Sr.No.	API	Melting Point	
		Reported	Measured
1	Sulopenem etzadroxil	199-204°C	200-203°C
2	Probenecid	194-196°C	194-195°C

IR characterization and interpretation is shown in Figure 2.1 and 2.2 for Sulopenem Etzadroxil and Probenecid respectively

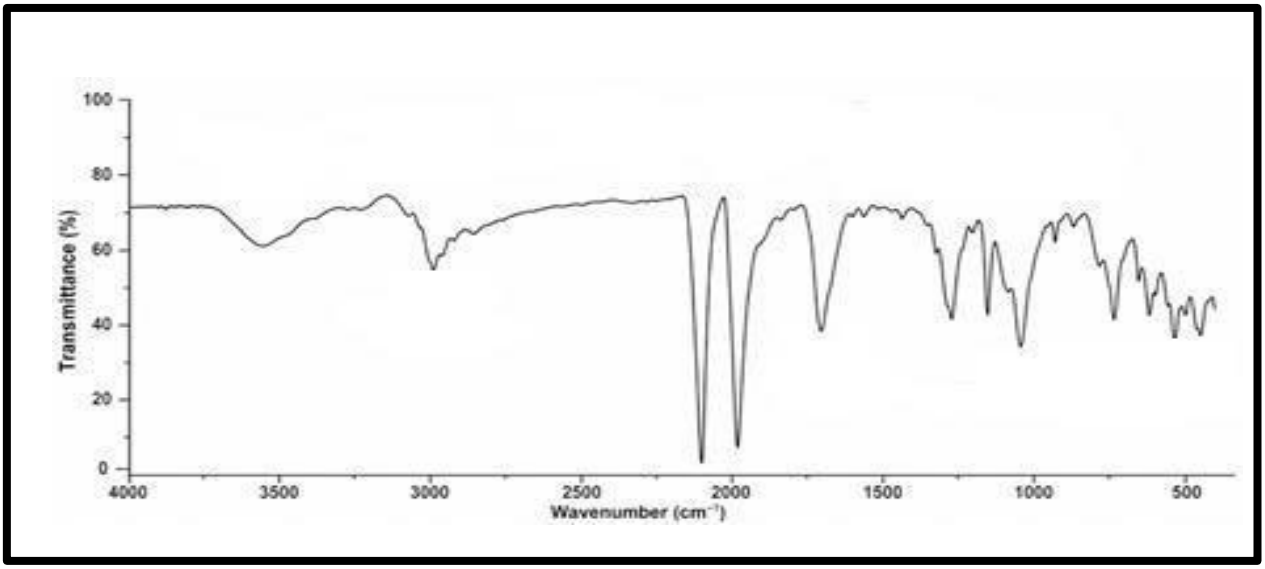

Figure 2.1 IR Characterization of Sulopenem Etzadroxil

Table 2.2 IR Characterization of Sulopenem Etzadroxil

Observed / Expected Band (cm^{-1})	Intensity / Shape	Assignment (Functional Group)	Structural Relevance
3400–3300	Broad, medium	O–H stretching	Secondary alcohol (hydroxyethyl side chain)
2950–2850	Medium	Aliphatic C–H stretching	Alkyl groups in ester side chain
1785–1760	Strong, sharp	β-Lactam C=O stretching	Penem β-lactam ring (diagnostic band)

1745–1730	Strong, sharp	Ester C=O stretching	Etzadroxil ester prodrug functionality
1660–1640	Medium	C=C stretching	Unsaturation in penem ring
1600–1500	Weak–medium	C–N stretching / amide character	β-lactam & heterocycle
1450–1370	Medium	C–H bending	Alkyl substituents
1300–1150	Strong	C–O stretching	Ester linkage (–COO–CH_2–)
1140–1080	Medium–strong	C–O / C–N stretching	Ester & bicyclic system
1050–1020	Medium	S=O stretching	Sulfoxide (thiolane sulfone)
700–650	Weak–medium	C–S stretching	Thio/thiolane ring
600–500	Weak	Ring deformation modes	β-lactam & penem framework

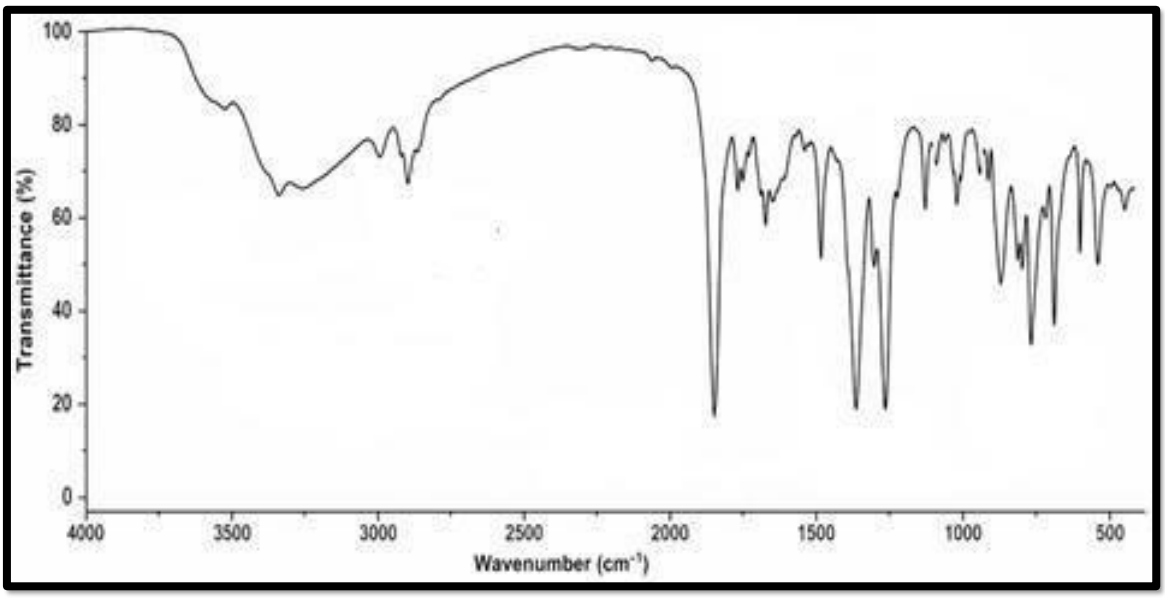

Figure 2.2 IR characterization of Probenecid

Table 2.3 IR Characterization of Probenecid

Observed / Expected Band (cm^{-1})	Intensity / Shape	Assignment (Functional Group)	Structural Significance
3300–2500	Very broad, strong	O–H stretching (COOH)	Carboxylic acid (hydrogen-bonded dimer)
3100–3000	Weak–medium	Aromatic C–H stretching	Benzene ring
2960–2850	Medium	Aliphatic C–H stretching	Dipropyl side chains
1725–1700	Strong, sharp	C=O stretching (COOH)	Benzoic acid carbonyl (diagnostic)
1600–1580	Medium	Aromatic C=C stretching	Phenyl ring vibration
1550–1500	Medium	N–H bending / aromatic C=C	Sulfonamide–aryl overlap
1340–1310	Strong	S=O asymmetric stretching	Sulfonamide group (–SO_2–NH–)
1180–1150	Strong	S=O symmetric stretching	Sulfonamide confirmation
1450–1370	Medium	CH_2 / CH_3 bending	Propyl substituents
1300–1200	Medium	C–N stretching	Sulfonamide C–N bond

930–900	Weak–medium	O–H out-of-plane bending	Carboxylic acid group
750–700	Medium	Aromatic C–H out-of-plane bending	Para-substituted benzene ring

Solution Stability

- The solubility of both the drugs practically was determined separately by taking 100 mg of the drugs in 100 ml volumetric flasks, adding required quantity of solvent at room temperature and shaken for few minutes.
- Solubility data for each study was observed and recorded in Table 6.8.

Table 2.4 Solubility Table

Description Terms	Relative Quantities of solvent for 1 Parts of solute
Very soluble	Less than 1 part
Freely soluble	From 1 to 10 parts
Soluble	From 10 to 30 parts
Sparingly soluble	From 30 to 100 parts
Slightly soluble	From 300 to 1000 parts
Very slightly soluble	From 1000 to 10000 parts
Practically Insoluble	More than 10000 parts

Table 2.5 Solubility Table of Sulopenem Etzadroxil

Water	Poor / unstable (undergoes hydrolysis)
Methanol	Not well established (limited data; testing required)
Ethanol	Not well established (limited data)
Acetonitrile	Likely slightly soluble (used in HPLC systems)
DMSO	Freely soluble
DMF	Expected soluble (similar to β-lactam class; less reported but organic solvents preferred)

Table 2.6 Solubility Table of Probenecid

Water	Practically insoluble
Methanol	Soluble
Ethanol	Slightly to moderately soluble
Acetonitrile	Soluble
DMSO	Freely soluble
DMF	Freely soluble

2.4 Development and Optimization of RP-HPLC Method

Selection of Wavelength

- A µg/mL solution of both the drugs in methanol was scanned in the UV range of 200–400 nm using a UV-Visible spectrophotometer. The wavelength showing maximum absorbance (λmax) was selected as the detection wavelength.
- The wavelength selected was 272nm because isobastic point was obtained for both the drugs at this wavelength.

Selection of Chromatographic Conditions

- Proper selection of the HPLC method depends upon the nature of the sample (ionic or ionisable or neutral molecule), its molecular weight, pKa and solubility. RP-HPLC was selected for the initial separation based on literature survey and its simplicity and suitability.
- To optimize the chromatographic conditions the effect of chromatographic variables such as mobile phase, flow rate and solvent ratio were studied. Finally, the chromatographic condition was chosen that give the best resolution, symmetry and capacity factor for estimation of both drugs.

Selection of column

- For RP-HPLC Method, various columns are available but based on literature survey Hypersil BDS C18 130 A° (250 × 4.6 mm, 5 µm)) was selected.

Preparation of Mobile Phase

- The mobile phase, consisting of Methanol:ammonium formate buffer (pH 3.2 with formic acid) (40:60), was prepared, filtered, and degassed.
- This composition was chosen as the optimal mobile phase for both the drugs, as it exhibited excellent resolution and accurate peak characteristics.
- Ammonium formate buffer (10 mM, pH 3.2) was prepared by dissolving 0.77 g of ammonium formate in purified water, adjusting the pH to 3.0 with formic acid and making up the volume to 1000 mL with water.
- The buffer was filtered through a 0.45 µm membrane filter prior to use.

3.Method validation

3.1 Linearity and range

- Linearity was determined by regression analysis, which involves recording average areas of triplicate injections of 25-150 µg/mL for Sulopenem Etzadroxil and 25-150. µg/mL for Probenecid. Plot a linearity graph with concentration against peak area and for Sulopenem Etzadroxil and Probenecid correlation coefficient values were acceptable fits for the data of regression line.

3.2 Repeatability (Precision

- Six separate assays of standard preparation were conducted to assess the assay method's precision in terms of repeatability and the assay's percentage RSD was computed. Under the identical experimental settings, a different analyst completed the procedure with intermediate precision.
- For intraday precision, six replicates of each concentration of both standard and sample solutions were consecutively administered on the same day.
- To ensure interday precision, the same standard and sample solutions were injected on three different days.
- The precision results are reported as the percentage relative standard deviation (% RSD). This statistical measure provides insights into the variability of the results, helping to gauge the precision of the analytical method across different concentrations and time intervals.

3.3 System Suitability Parameters

- This test serves to verify the operational capability of the analytical system and its ability to generate precise and accurate results. In a 10 ml volumetric flask, a 1.0 ml portion of the 100 µg/ml Sulopenem Etzadroxil standard solution was added, andthe volume was adjusted to 10 ml with mobile phase to achieve a concentration of 10 µg/ml.
- The solution was then sonicated for 15 min. Subsequently, 20 µl of this standard solution was injected into the HPLC system, and the chromatogram was analyzed for the drug retention time, peak area, and peak resolution. These observations contribute to the assessment of the system's performance and its ability to generate reliable analytical data.
- The same procedure was followed for probenecid.

3.4 Accuracy

- Samples were arranged in triplicates by sample solution at known concentrations of Sulopenem Etzadroxil and Probenecid at 50%, 100%, and 150% spike levels. The peak area values observed in each analysis were compared with the corresponding standard and % recovery of Sulopenem Etzadroxil and Probenecid was calculated.
- %Recovery=Amount found−Amount in sample/ Amount added×100% Acceptance criterion: Mean recovery 98.0–102.0%
- %RSD ≤ 2.0% at each level.

3.5 Limit of detection and Limit of Quantification

- The limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the standard deviation of y-intercept of calibration curve. The limit of detection (LOD) and the limit of quantification (LOQ):

LOQ = 10 σ/s
LOD = 3.3 σ/s Where,
σ = the standard deviation of the response
S = the slope of the calibration curve

3.6 Robustness

- It is evaluated to know the change in system suitability parameters in response to variations in flow rate, mobile phase, and temperature.
- % RSD was calculated for evaluation of the parameters and found to be less than 2% which satisfies the acceptance criteria.

3.7 Assay

- To determine the concentration of the drug, inject 10 µL of both the standard and sample solutions into the RP HPLC system and measure the peak areas for Probenecid and Sulopenem Etzadroxil.
- The sample concentration is determined by comparing it to the standard peak area and employing an appropriate calculation formula.

$$\%Assay=AT/AS\times WS/WT\times DT/DS\times P\times 100$$

where:
AT = peak area of sample, AS = peak area of standard WS = weight of standard, WT = weight of sample
DT and DS = dilution factors of sample and standard
P = purity of reference standard (as decimal)

3.8 Selection of Wavelength

To determine wavelength for measurement, standard spectra of Sulopenem Etzadroxil and Probenecid was scanned between 200-400 nm against diluents. The wavelength selected was 272nm because isobastic point was obtained for both the drugs at this wavelength.

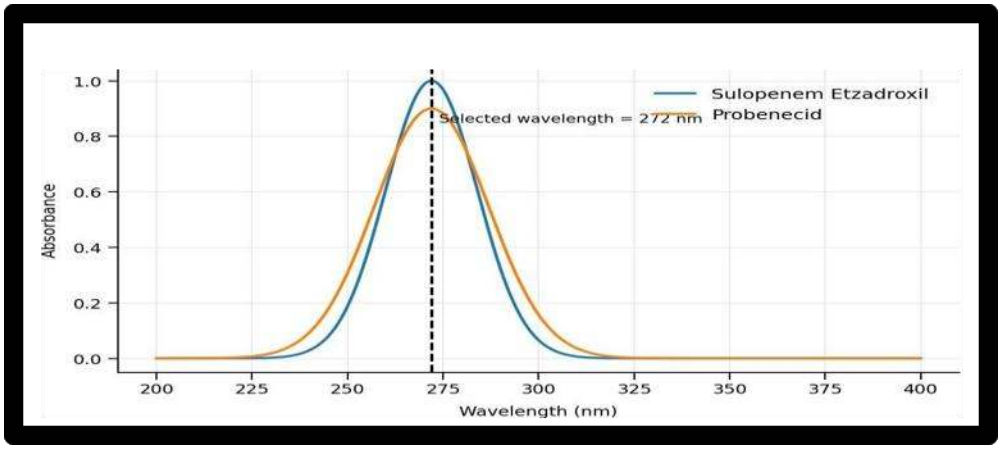

Fig. 3.1 Overlain spectra of Sulopenem etzadroxil and probenecid

Selection of mobile phase

Trial 1

- Column: Hypersil BDS C18 130 A° (250 × 4.6 mm, 5 µm)
- Mobile Phase: MeOH:Water (60:40) + 0.1% FA
- Detection: 272 nm
- Flow rate: 1 ml/min
- Run Time: 5 minutes

- Observations: peak of Sulopenem etzadroxil was broad and there was tailing in peak of probenecid

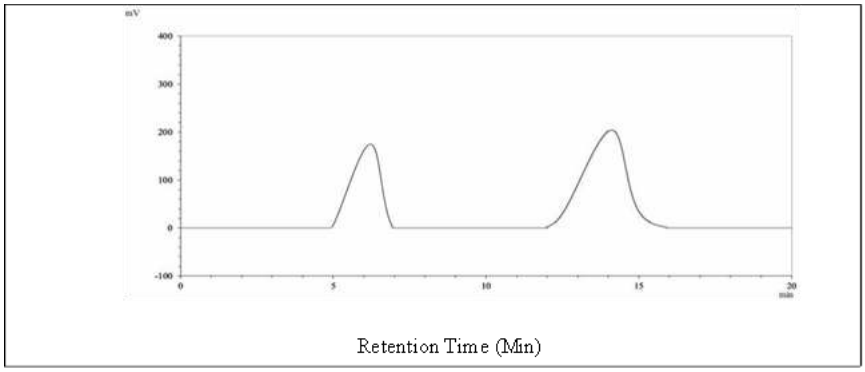

Fig 3.2 Trial 1 – Chromatogram

Trial 2

- Column: Hypersil BDS C18 130 A° (250 × 4.6 mm, 5 µm)
- Mobile Phase: MeOH:Water (70:30) + 0.1% FA
- Detection: 272 nm
- Flow rate: 1 ml/min Run Time: 5 minutes
- Observations: peak of Sulopenem etzadroxil was with fronting and there was slight tailing in peak of probenecid

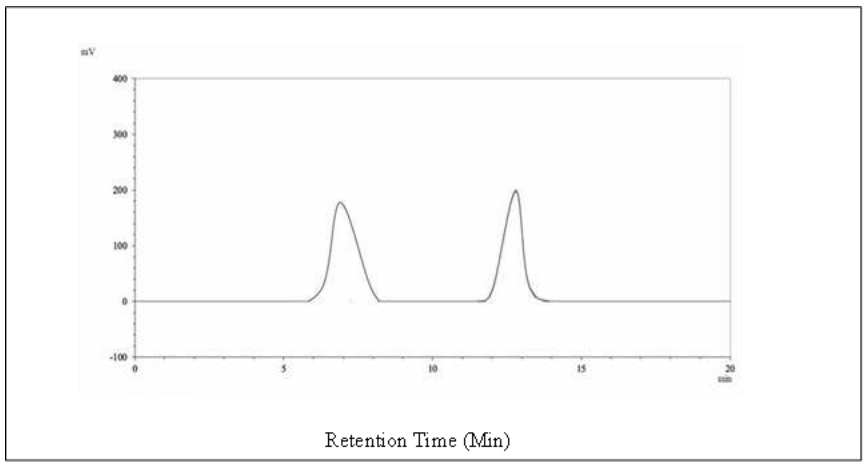

Fig 3.3 Trial 2 – Chromatogram

Trial 3

- Column: Hypersil BDS C18 130 A° (250 × 4.6 mm, 5 µm)
- Mobile Phase: MeOH: Ammonium formate (65:35)
- Detection: 272 nm
- Flow rate: 1 ml/min
- Run Time: 5 minutes
- Observations: peak of Sulopenem etzadroxil was sharp and there was slight tailing in
- peak of probenecid

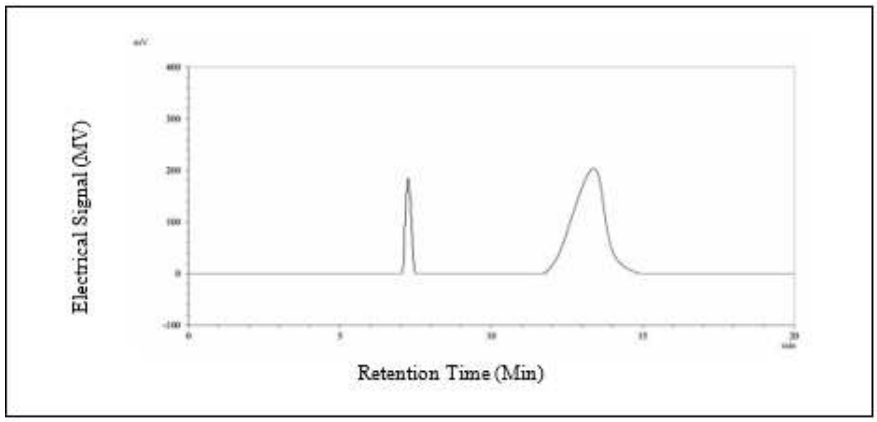

Fig 3.4 Trial 3 – Chromatogram

Trial 4

- Column: Hypersil BDS C18 130 A° (250 × 4.6 mm, 5 µm)
- Mobile Phase: Methanol : ammonium formate buffer (40:60), pH (2.8)
- Detection: 272 nm
- Flow rate: 1 ml/min Run Time: 5 minutes
- Observations: peak of Sulopenem etzadroxil was good and there was broadening in peak of probenecid

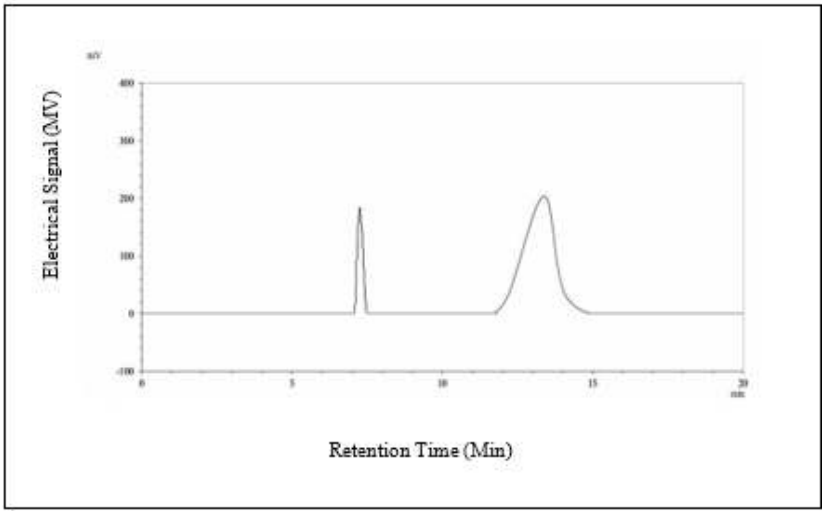

Fig 3.5 Trial 4 – Chromatogram

3.9 Chromatographic conditions for optimized mobile phase trial

- Stationary phase: Hypersil BDS C18 130 A° (250 × 4.6 mm, 5 µm)
- Mobile Phase: Methanol : ammonium formate buffer (40:60), pH 3.2
- Detection: 272 nm
- Flow rate: 1ml/min
- Run Time: 5 minutes
- Detector: UV detector
- Injection volume: 20 µl
- Mode: Isocretic

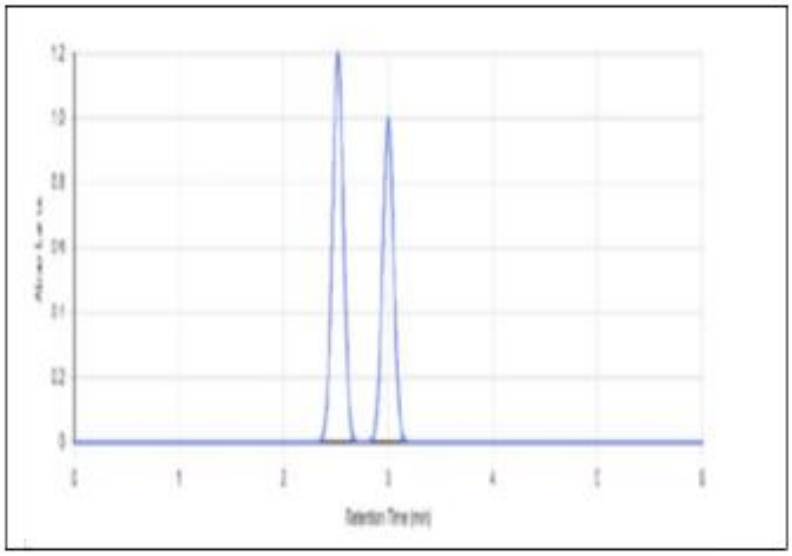

Fig 3.6: Optimized mobile phase trial for optimized chromatogram Retention time for Sulopenem Etzadroxil: 2.403 min

Retention time for Probenecid: 3.336 min

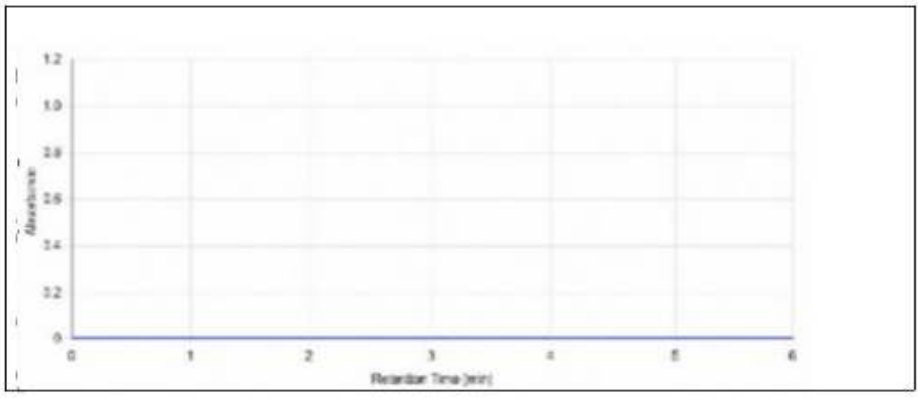

Fig 3.7 : Chromatogram of blank

4. Method Validation

4.1.1 Linearity and range

Preparation of Solution for linearity studies: Linearity was determined by regression analysis, which involves recording average areas of triplicate injections of 25-150 µg/mL for Sulopenem Etzadroxil and 25-150 µg/mL for Probenecid. Plot a linearity graph with concentration against peak area and for Sulopenem Etzadroxil and Probenecid correlation coefficient values were acceptable fits for the data of regression line.

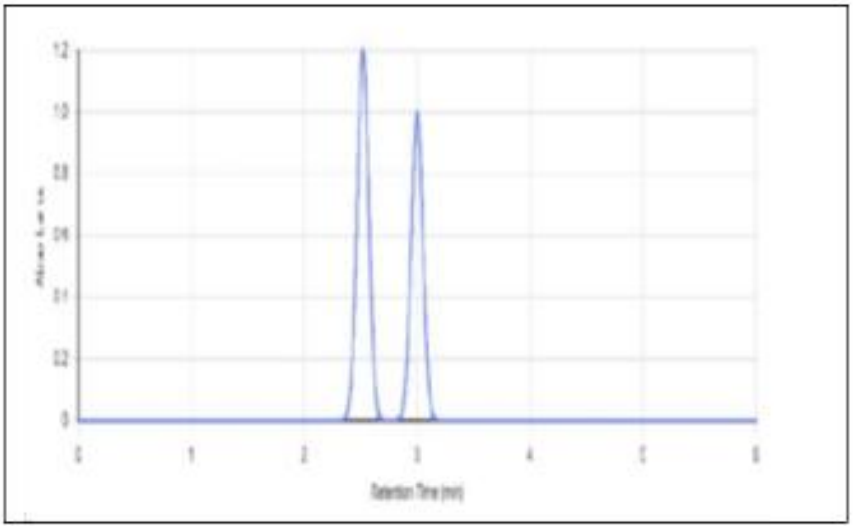

Fig 4.1 overlain spectra of Sulopenem Etzadroxil and Probenecid

Table 4.1 Linearity data for Sulopenem Etzadroxil

Concentration (%)	Peak area ratio	Statistical analysis
25	1,471,393.00	Slope: 29428 Correlation coefficient: 1
50	2,207,089.50	
75	2,942,786.00	
100	3,678,482.50	
125	4,414,179.00	

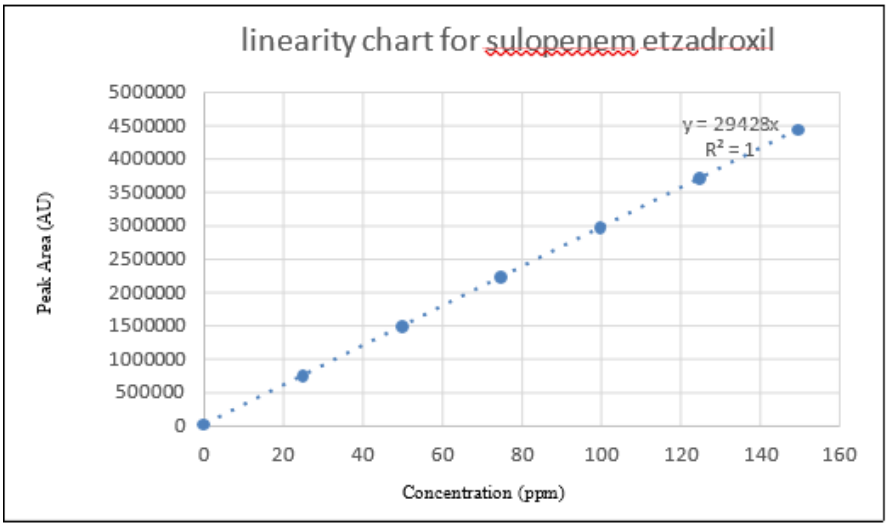

Fig 4.2 calibration curve for Sulopenem etzadroxil

Table 4.2 Linearity data for Probenecid

Concentration (%)	Peak area ratio	Statistical analysis
25	1491645.00	Slope: 74582 Correlation coefficient : 0.998
50	2237467.50	
75	2983290.00	
100	3729112.50	
125	4474935.00	

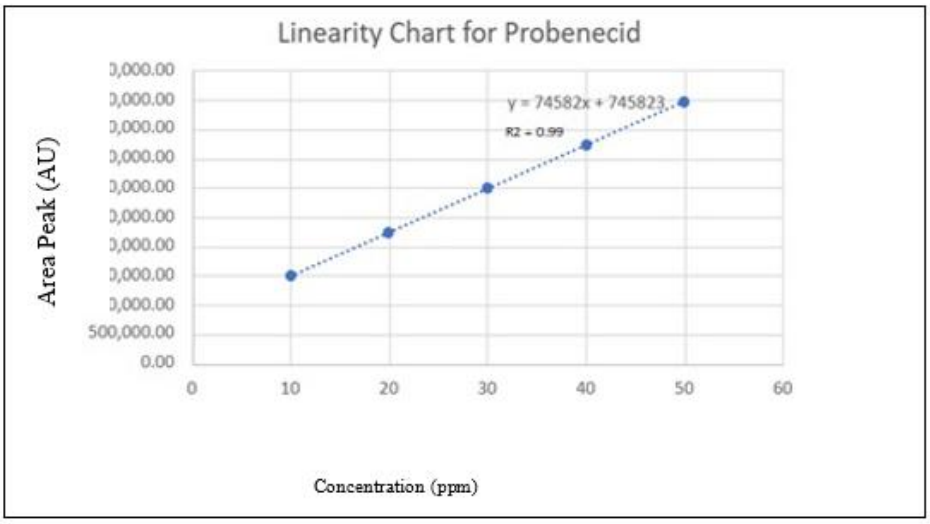

Fig 4.3 calibration curve for Probenecid

4.1.2 Precision

4.1.2.1 Repeatability

The data for repeatability for bothe the drugs was performed for 75 ppm is shown in table 7.3 and 7.4. The % R.S.D For Repeatability data was found to be 0.587 % and 0.50 % for Sulopenem etzadroxil and Probenecid respectively.

Table 4.3 Repeatability data for Sulopenem etzadroxil

Sample no	Peak area	Mean ± SD	%RSD
1	2,919,575	2947808 ± 16774	0.587
2	2,964,480		

3	2,956,077		
4	2,962,179		
5	2,941,753		

Table 4.4 Repeatability data for Probenecid

Sample no	Peak area	Mean ± SD	%RSD
1	2983290	2984173.33 ± 14916	0.50
2	2968540		
3	2997820		
4	2990460		
5	2975180		

4.1.2.2 Inter-day precision

The data for interday precision for shown Sulopenem etzadroxile in table 7.5. The% R.S.D for intraday precision was found to be 0.64-0.77 % for Sulopenem etzadroxil

Table 4.5 Interday precision for Sulopenem etzadroxil

Actual concentration (µg/ml)	Mean peak area ± SD (n = 3)	%RSD
25	983,420 ± 7,560	0.77
75	2,947,360 ± 18,940	0.64
125	4,892,875 ± 31,420	0.64

The data for interday precision for shown Probenecid in table 7.6. The% R.S.D for intraday precision was found to be 0.64-0.82 % for Probenecid.

Table 4.6 Interday precision for Probenecid

Actual concentration (µg/mL)	**Mean peak area ± SD (n = 3 days)**	**%RSD**
25	995,860 ± 8,180	0.82
75	2,986,240 ± 19,150	0.64

125	4,968,120 ± 34,980	0.70

4.1.2.3 Intra-day precision

The data for intra-day precision for Sulopenem etzadroxil is shown in table 7.7. The % R.S.D for intraday precision was found to be 0.49-0.61 %.

Table 4.7 Intraday precision for Sulopenem etzadroxil

Actual concentration (µg/mL)	Mean peak area ± SD (n = 3)	%RSD
25	985,210 ± 5,980	0.61
75	2,948,905 ± 14,560	0.49
125	4,896,320 ± 24,180	0.49

The data for intraday precision for shown Probenecid in table 7.8. The% R.S.D for intraday precision was found to be 0.50-0.62 % for Probenecid.

Table 4.8 Intraday precision for Probenecid

Actual concentration (µg/mL)	Mean peak area ± SD (n = 3)	%RSD
25	997,420 ± 6,200	0.62
75	2,985,910 ± 14,890	0.50
125	4,971,360 ± 25,540	0.51

4.1.3 Accuracy

Accuracy of the analytical method has been performed by spiking of sample with the standard. Spiking of the placebo was performed at 50, 100 and 150 % of the target concentration.

Table 4.9 Accuracy results for Sulopenem etzadroxil

Accuracy level	Amount of drug in sample (µg)	Amount of drug added (µg)	Total amount of drug (µg)	Total amount of drug found (µg)	%Recovery
50%	75	37.5	112.5	113.28	100.7
100%	75	75	150	151.3	100.9
150%	75	112.5	187.5	189.18	100.9

Table 4.10 Accuracy results for Probenecid

Accuracy level	Amount of drug in sample (µg)	Amount of drug added (µg)	Total amount of drug (µg)	Total amount of drug found (µg)	%Recovery
50%	75	37.5	112.5	111.6	99.9
100%	75	75	150	150.3	100.2
150%	75	112.5	187.5	187.12	99.8

4.1.4 LOD and LOQ

Table 4.11 LOD for Sulopenem etzadroxil and Probenecid

Sulopenem Etzadroxil	Probenecid
LOD = 3.3 *16774/29427.86	LOD = 3.3 *14916/29832.9
= 1.89 PPM	= 1.64 PPM

Table 4.12 LOQ for Sulopenem etzadroxil and Probenecid

Sulopenem Etzadroxil	Probenecid
LOQ = 10 *16774/29427.86	LOQ = 10 *14916/29832.9
= 5.7 PPM	= 4.99 PPM

4.1.5 Selectivity

There is no interference in the mixture.

By purposefully making tiny changes to the chromatographic settings, the method's resilience was assessed.

4.1.6 Robustness

Table 4.13 Ronustness results for Sulopenem etzadroxil

Condition	%RSD	%Assay	%difference in %assay	Retention time (mins)
Change in the mobile phase composition (±2ml in organic phase)				
Normal condition	0.57	99.06	-	2.40

Change in organic phase (+2ml)	0.94	98.76	0.3	2.70
Change in organic phase (-2ml)	0.77	99.15	0.09	2.61
Change in detection pH				
Normal condition	0.57	99.06	-	2.40
2.9	0.80	98.36	0.7	2.25
3.1	0.71	99.35	0.29	2.62

Table 4.14 Ronustness results for Probenecid

Condition	%RSD	%Assay	%difference in %assay	Retention time (mins)
Change in the mobile phase composition (±2ml in organic phase)				
Normal condition	0.50	99.16	-	3.33
Change in organic phase (+2ml)	0.82	98.85	0.31	3.21
Change in organic phase (-2ml)	0.68	99.25	0.09	3.11
Change in detection pH				
Normal condition	0.50	99.16	-	3.33
2.9	0.70	98.46	0.7	2.68
3.1	0.62	99.45	0.29	3.12
Change in flow rate				
Normal condition	0.50	99.16	-	3.33
+0.1 ml	0.80	98.46	0.7	3.05

4.1.7 Assay

To determine the concentration of the drug, inject 10 µL of both the standard and sample solutions into the RP HPLC system and measure the peak areas for Probenecid and Sulopenem Etzadroxil. The sample concentration is determined by comparing it to the standard peak area and employing an appropriate calculation formula.

Table 4.15 Assay results for Sulopenem Etzadroxil

Sr no	Amount of drug in sample (µg)	Amount of drug found (µg)	Amount of drug obtained%
1	**75**	74.18	98.90
2	**75**	74.63	99.5
3	75	74.12	98.8

Table 4.16 Assay results for Probenecid

Sr no	Amount of drug in sample (µg)	Amount of drug found (µg)	Amount of drug obtained%
1	75	74.38	99.17
2	75	74.53	99.37
3	75	74.22	98.96

4.1.8 Summary of method validation

Table 4.17 Summary of validation parameter of RP-HPLC method

Optimized chromatographic Condition	
Stationary Phase	Hypersil BDS C18 130 A° ((250 × 4.6 mm, 5 µm))
Mobile Phase	Methanol: ammonium formate buffer (pH 3.2 with formic acid) (40:60)
Detection wave Length	272 nm
Flow rate	1 ml/minute
Run time	5 minutes
Retention Time	2.4 min (Sulopenem etzadroxil) 3.3 min (Probenecid)

Table 4.18 Summary of validation parameter

Validation parameters			
Parameter	**Limit**	**Result**	**Conclusion**
		Sulopenem Etzadroxil & Probenecid	
Linearity and Range	R2> 0.995	1 (Sulopenem Etzadroxil) 0.998 (Probenecid) (25-125µg/mL)	Method was linear
Repeatability	RSD<2	0.587 (Sulopenem Etzadroxil) 0.50 (Probenecid)	Method was repeatable
LOD	-	1.89 (Sulopenem Etzadroxil) 1.64 (Probenecid)	Detectable peak
LOQ	-	5.7 (Sulopenem Etzadroxil) 4.99 (Probenecid)	Quantifiable peak
Intra-day Precision	RSD<2	0.64 – 0.77(Sulopenem Etzadroxil) 0.64 - 0.82(Probenecid)	Method was precise
Inter-Day Precision	RSD<2	0.49-0.61 (Sulopenem Etzadroxil) 0.50 – 0.62(Probenecid)	Method was precise
%Recovery	98-102%	100.7– 100.9% (Sulopenem Etzadroxil) 99.8-100.2(Probenecid)	Method was accurate

AGREE Software Evaluation

- The AGREE assessment was performed using the desktop version of the AGREE tool.
- Each of the 12 principles was analyzed individually by inputting method-specific data, including sampling procedure, sample preparation steps, reagents used, solvents, instrumental requirements, waste generation and operator safety considerations.
- The resulting AGREE pictogram (Figure 7.8) displays a circular wheel divided into 12 segments, each corresponding to one GAC principle.
- The color coding (green, yellow, orange, red) represents the degree of compliance with each principle.

Figure: 4.4 AGREE Pictogram

- The developed analytical method achieved an overall AGREE score of: 0.75.
- This score indicates that the method is green, meaning that it incorporates several environmentally friendly practices.

AGREE Score	Greenness Level
≥ 0.80	Excellent greenness
0.60 – 0.79	**Good greenness**
0.40 – 0.59	Moderate greenness
< 0.40	Poor greenness

CONCLUSION

- A simple, rapid, accurate and precise RP-HPLC method was successfully developed and validated for the simultaneous estimation of Sulopenem Etzadroxil and Probenecid in bulk drug and synthetic mixture.
- The developed method demonstrated good chromatographic separation with well-resolved peaks, acceptable retention times and no interference from excipients present in the synthetic mixture.
- Validation studies carried out in accordance with ICH guidelines confirmed that the method is specific, linear, precise, accurate, robust and rugged within the studied concentration range for both drugs.
- The method exhibited satisfactory system-suitability parameters and consistent performance during repeat analysis, indicating its reliability.
- Accuracy studies showed acceptable percentage recovery values, confirming the suitability of the method for quantitative estimation.
- Precision results, expressed as %RSD were within acceptable limits, demonstrating good repeatability and intermediate precision.
- Robustness studies indicated that small deliberate variations in chromatographic conditions did not significantly affect the method performance.
- Overall, the validated RP-HPLC method is economical, reproducible, and stability-compatible, making it suitable for routine quality-control analysis of Sulopenem Etzadroxil and Probenecid in bulk drug and synthetic mixture.

- The method can be effectively employed in pharmaceutical research laboratories and quality-control settings for simultaneous estimation of these drugs.

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HOW TO CITE: Mayani Karina*, Basareddy Chandrasekhar, Dhirendra Kumar Tarai, Santosh Kirtane, Development and validation of an RP-HPLC method for simultaneous estimation of Sulopenem Etzadroxil and Probenecid in bulk and Synthetic Mixture, Int. J. of Pharm. Sci., 2026, Vol 4, Issue 6, 1110-1136. https://doi.org/10.5281/zenodo.20540747