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Developing and Validating a Stability-Indicating Method for the Analysis of Piperacillin and Tazobactam in Bulk and Dosage Forms in Human Plasma Using RP-HPLC.

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KEYWORDS
HPLC; Method Development; Method Validation; Forced Degradation; Piperacillin, Tazobactam.

ABSTRACT:
A meticulously developed and thoroughly validated High-Performance Liquid Chromatography (HPLC) method was designed for the simultaneous determination of Piperacillin and Tazobactam in a Fixed-Dose Combination. The chromatographic profiles were constructed using a mobile phase of Methanol: 0.1% orthophosphoric Acid (OPA) in water (85:15) with a flow rate of 0.7 ml/min. Employing a C18 Column (4.6 x 250 mm, 5µm particle size) as the stationary phase, detection at 231 nm facilitated reliable quantification. The method exhibited linear correlation within concentration ranges of 16-80 µg/ml for Piperacillin and 02-10 µg/ml for Tazobactam, with regression values of 0.9992 and 0.9999, respectively. Precision studies demonstrated % RSD values below 2% for both drugs across all selected concentrations. The limit of detection (LOD) and limit of quantification (LOQ) were determined as 0.258 µg/ml and 0.784 µg/ml for Piperacillin, and 0.143 µg/ml and 0.435 µg/ml for Tazobactam, respectively. Beyond method validation, stability studies subjected analyte solutions to acid, base, oxidative, and hydrolytic stress conditions. The validation process adhered to the guidelines outlined by the International Conference on Harmonization (ICH).

INTRODUCTION:

Piperacillin and Tazobactam, when used together, are a powerful treatment for various infections. The success and safety of this combination depend on maintaining the right levels of both substances in the blood. To study and optimize their use, it's crucial to have a precise and dependable method for measuring them simultaneously. In recent times, Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) has become a reliable technique for this purpose, especially when dealing with complex biological samples. This research focuses on developing and applying an RP-HPLC method to simultaneously measure Piperacillin and Tazobactam in plasma samples. In pharmaceutical research, having strong analytical methods is essential, and RP-HPLC stands out due to its flexibility, high sensitivity, and ability to separate different kinds of compounds. By using this method, our aim is to efficiently and selectively separate Piperacillin and Tazobactam, ensuring accurate and precise measurement in the complex environment of plasma. This research explores the details of creating and validating a Stability-Indicating Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) method. The main goal is to simultaneously identify Piperacillin and Tazobactam, crucial elements in pharmaceutical formulations, and guarantee their stability and reliability during the entire analysis [16-18].

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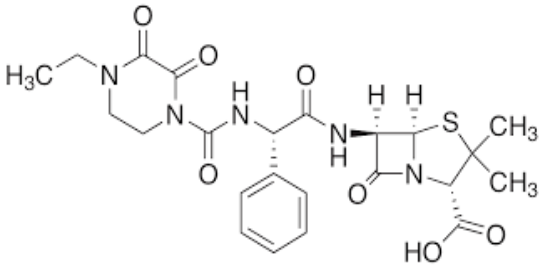

Figure 1: Structures of Piperacillin

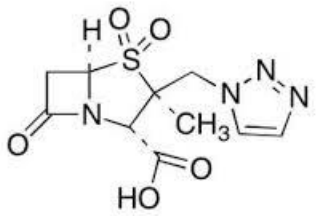

Figure 2: Structures of Tazobactam

MATERIALS AND METHODS

Chemicals and Reagents

Piperacillin and Tazobactam are sourced from Kopran Ltd. The Ortho-Phosphoric acid is procured from Avantor Performance Materials India Ltd. in Thane, Maharashtra, while Methanol is obtained from Merck Specialties Pvt. Ltd. in Shiv Sagar Estate 'A', Worli, Mumbai, Maharashtra. The marketed formulation of Piperacillin and Tazobactam, under the brand name Piperanem and containing Piperacillin 40 mg and Tazobactam 0.5 mg, is acquired from a local medical store.

Instrumentation:

For the analysis, an HPLC system from Agilent Technologies was employed, featuring a gradient system and a UV detector. The chromatographic separation was carried out using an Agilent C18 Column with dimensions of 4.6mm x 250 mm and a particle size of 5 µm. The analytical setup included a 940D pump, a 20 µl injection loop, a UV 740D Absorbance detector, and the Chemstation software for running and monitoring the analysis process.

Mobile Phase Preparation:

The mobile phase was created by blending methanol and water (with a pH of 2.8 adjusted using 1 % OPA) in a ratio of 85:15 v/v. The resultant mixture underwent filtration and degassing.

Preparation of standard Stock solution:

A standard solution was prepared by combining 80 mg of Piperacillin and 10 mg of Tazobactam. This standard mixture was then mixed with 100 ml of methanol and 2 ml of untreated human plasma. The resulting solution underwent vertical shaking for a duration of 30 minutes and was subsequently subjected to centrifugation at components.

5000 rpm for 1 hour. After centrifugation, the solution was filtered using membrane filters to obtain a clear organic solution. The filtered solution was then transferred into sample vials designed for HPLC analysis. These vials were subsequently loaded onto the HPLC instrument for the analytical run. [4-7]

Solution preparation for Assay:

To assess the content of Piperacillin and Tazobactam in a commercially available product (with a label claim of 4 mg Piperacillin and 0.5 mg Tazobactam), a 10 ml sample was mixed. To ensure thorough extraction, the mixture underwent sonication for 15 minutes. Subsequently, 0.1 ml of the supernatant was diluted to a total volume of 10 ml using the mobile phase. The resulting solution was then injected into the HPLC system, and the area corresponding to the drug peaks was recorded. [9-12].

RESULTS AND DISCUSSION:

Method Development:

The process of developing the method systematically employed a trial-and-error approach, experimenting with mobile phases of various compositions and proportions. Choosing an appropriate mobile phase is crucial in establishing an effective analytical method that achieves the best resolution of drug components. By manipulating the composition of the mobile phase and using a suitable column, we achieved the optimal separation of Piperacillin and Tazobactam. Numerous preliminary trials were conducted, incorporating different columns, buffers, and organic solvents in various proportions. These trials aimed to pinpoint the most effective conditions for achieving the best separation of the drug

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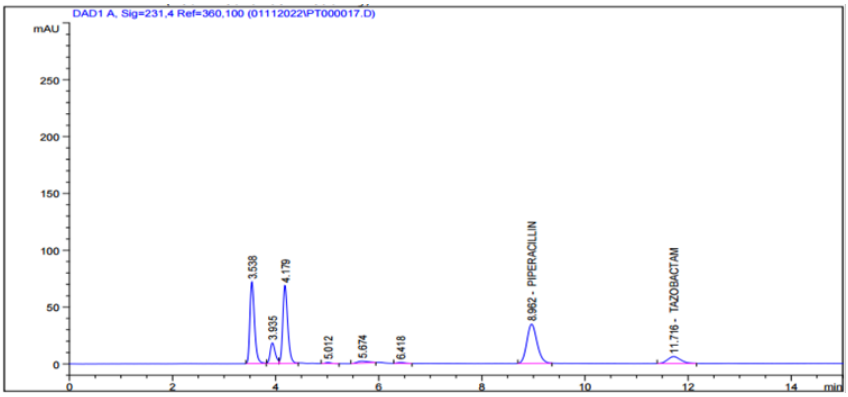

Figure 3: Standard Chromatogram of Piperacillin and Tazobactam

Table 1. Optimized Chromatographic conditions

Sr. No.	Instrument/Equipment	Optimized condition
1	HPLC	Agilent (S.K) Gradient System UV Detector
2	Software	Chemstation
3	Column	(Agilent C18 Column (4.6mm x 250mm)
4	Particle size packing	5 µm
5	Stationary phase	C-18 (Agilent)
6	Mobile Phase	Methanol: Water (0.1% with OPA) 85 : 15
7	Detection Wavelength	231 nm
8	Flow rate	0.7 ml/min

HPLC METHOD VALIDATION:

System suitability

The system suitability parameters for Piperacillin and Tazobactam were thoroughly examined to assess the resolution and reproducibility of the proposed chromatographic system. In the context of repeatability studies conducted on the RP-HPLC method for estimating Piperacillin and Tazobactam, the %RSD (Relative Standard Deviation) was determined to be less than 2%. This low %RSD value signifies a high degree of precision and consistency in the measurements, indicating the reliability of the analytical method. The results, as outlined in Table 2, underscore the robustness of the chromatographic system, affirming its suitability for the accurate and reproducible estimation of Piperacillin and Tazobactam in the given conditions.

Table 2. System suitability test parameter for Piperacillin and Tazobactam.

Method	RP-HPLC	
Drug	**Piperacillin**	**Tazobactam**
Conc. (mg/ml)	32	04
Peak area	940.33 945.36	209.77 214.26
Mean	942.85	212.02
Amount found (mg)	31.91	3.97
% Amount found	99.71	99.31
% RSD	0.38	1.50

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Linearity study:

The calibration experiment involved a comprehensive linear regression analysis of Piperacillin and Tazobactam, aiming to establish a clear relationship between peak areas and concentrations within defined ranges. Specifically, the concentration range was set at 16-80 µg/ml for Piperacillin and 02-10 µg/ml for Tazobactam. The calibration data for Piperacillin and Tazobactam are presented in Tables 3 and 4, respectively. The linear equations derived from the regression analysis were determined as follows: for Piperacillin, the equation is y = 29.456x - 3.2277, and for Tazobactam, the equation is y = 49.211x + 16.548. Here, 'x' represents the concentration, and 'y' signifies the area of the peak. The correlation coefficients associated with these linear equations were found to be 0.999 for both Piperacillin and Tazobactam. A correlation coefficient of 0.999 indicates an exceptionally strong and positive linear relationship between the variables, reinforcing the reliability of the calibration. Visual representation of the calibration curves for Piperacillin and Tazobactam can be observed in Figures 4 and 5, respectively.

Table 3: Linearity study of Piperacillin

Sr. No	Conc. µg/ml	Area I	Area II	Mean	SD	% RSD
1	16	474.9641	474.7326	474.85	0.16	0.03
2	32	939.0911	941.4206	940.26	1.65	0.18
3	48	1378.2039	1384.9537	1381.58	4.77	0.35
4	64	1911.5882	1908.2747	1909.93	2.34	0.12
5	80	2344.7697	2348.4821	2346.63	2.63	0.11

Table 4: Linearity study of Tazobactum

Sr. No	Conc. µg/ml	Area I	Area II	Mean	SD	% RSD
1	2	115.1708	112.284	113.73	2.04	1.79
2	4	214.8166	216.6244	215.72	1.28	0.59
3	6	310.4085	313.1601	311.78	1.95	0.62
4	8	410.6997	408.8635	409.78	1.30	0.32
5	10	505.6343	511.4912	508.56	4.14	0.81

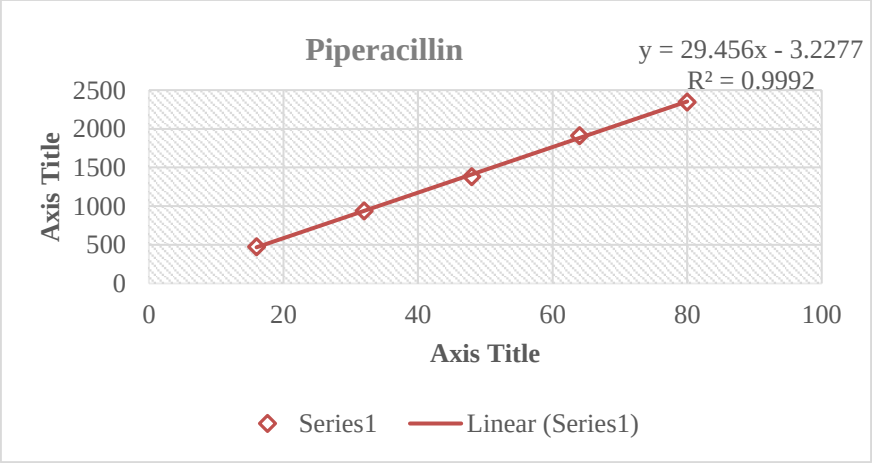

Figure 4: Calibration curve of Piperacillin

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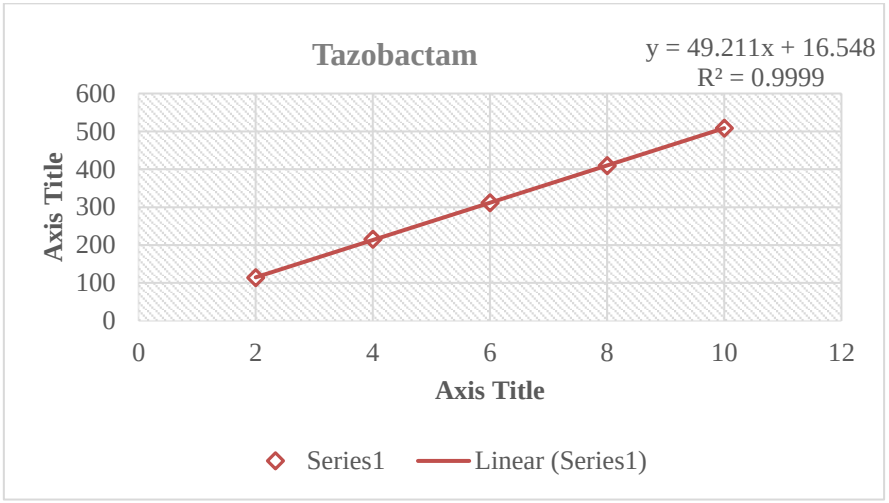

Figure 5: Calibration curve of Tazobactam

Accuracy:

To validate the accuracy of the developed method, % recovery studies for Piperacillin and Tazobactam were meticulously conducted. In this process, a predetermined concentration of standard drug (80%, 100%, and 120%) was intentionally added to pre-analyze vial formulations, followed by the analysis of the recovery. The results of these recovery studies are systematically presented in Table 5. To further establish the accuracy of the RP-HPLC method, a comparison with the Spectrophotometric method was undertaken through recovery studies at different concentration levels (80%, 100%, and 120%). The % recovery values obtained were found to fall within the range of 99-101%. This statistical validation provides strong evidence of the accuracy and reliability of the developed method in estimating Piperacillin and Tazobactam concentrations as depicted in Table 5.

Table 5. Accuracy Data for Piperacillin and Tazobactam

	Piperacillin			Tazobactam		
Level (%)	80 %	100 %	120 %	80 %	100 %	120 %
Amount added (ug/ml)	12.80	16.0	19.20	1.6	2.0	2.4
Absorbance Mean *±S.D.	849.63±0.02	942.98±0.03	1037.80±0.06	192.90±0.007	213.68±0.016	232.74±0.012
Amount recovered Mean *	12.74±0.02	15.91±0.03	19.12±0.06	1.58±0.12	02.00±0.094	02.39±0.64
% Recovery Mean *	99.53±0.3	99.44±0.16	99.63±0.70	98.99±1.6	100.31±0.94	99.73±0.5

*mean of each 3 reading for RP-HPLC method

Precision:

The methodology was established through the meticulous analysis of numerous replicate standards containing Piperacillin and Tazobactam. Each solution was subjected to triple analysis to meticulously capture any potential intra-day and inter-day variations in the results. The aim was to thoroughly examine the precision of the method under different conditions. To delve into the intricacies of precision, the results obtained from both intra-day and inter-day analyses were documented and are presented comprehensively in Table 6. This tabulated information offers a detailed insight into the consistency and reliability of the method across multiple analyses conducted within a single day (intra-day) and over different days (inter-day).

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Table 6: Intra-day and Inter-day Precisions of Piperacillin and Tazobactam

Method	Drug	Conc. (µg/ml)	Intra-day Precision		Inter-day Precision	
			Area Mean± SD	% Amount Found	Area Mean± SD	% Amount Found
RP-HPLC Method	Piperacillin	16	481.61	101.52	481.14	101.42
		48	1398.99	98.74	1404.79	99.15
		80	2366.98	100.33	2367.55	100.35
	Tazobactam	2	117.26	102.34	115.58	100.63
		6	308.39	98.85	307.18	98.44
		10	519.68	102.24	506.18	99.50

*Mean of each 3 readings for RP-HPLC method

Robustness:

The Robustness studies aim to assess the resilience of the analytical system. Robustness refers to the system's ability to remain unaffected by minor intentional variations in parameters. To gauge the robustness of the proposed method, slight yet intentional changes were introduced to the optimized method parameters. The impact of variations in the mobile phase composition, flow rate, and wavelength on the retention time and tailing factor of the drug peak was thoroughly examined. Specifically, the flow rate was adjusted by ±0.2 ml/min, and the mobile phase composition was altered to 84:16 and 86:14 proportions. Additionally, variations in wavelength, namely 232 nm and 230 nm (±2 nm) from the optimized chromatographic conditions, were implemented. The robustness parameters were found to be satisfactory, affirming the resilience of the analytical method. The robustness study involved modifying the flow rate (0.6 and 0.8 ml/min), adjusting the pH of the mobile phase composition (84:16 and 86:14), and changing the wavelength (230 nm and 232 nm). The %RSD for peak area, calculated to be less than 2%, is detailed in Table 7.

Table 7. Robustness Study of Piperacillin and Tazobactam.

Parameters	Conc. (µg/ml)	Piperacillin		Tazobactam	
		Area (mean ±SD)	% RSD	Area (mean ±SD)	% RSD
Flow rate 0.6 ml/min	64+8	2261.30±1.63	0.07	481.49±1.23	0.26
Flow rate 0.8 ml/min	64+8	1667.14±2.38	0.14	353.86±2.29	0.65
Mobile Phase 84 + 16 ml	64+8	1916.10±0.39	0.02	429.40±1.52	0.35
Mobile Phase 86 + 14 ml	64+8	1926.60±3.19	0.17	407.80±2.67	0.65
Wavelength 232 nm	64+8	1976.80±0.80	0.04	356.00±1.27	0.36
Wavelength 230 nm	64+8	1884.51±0.099	0.05	477.47±0.95	0.20

Limit of Detection:

Table 8 displays the minimum detection limits for Piperacillin and Tazobactam. The Limit of Detection (LOD) for Piperacillin was established at 0.258, while for Tazobactam, it was determined to be 0.143. These Limit of Detection (LOD) values confirm the method's appropriateness for detecting lower concentrations of Piperacillin and Tazobactam. The outcomes substantiate the sensitivity of the developed method, asserting its capability for precise determination even at lower concentrations.

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Table 8: Limit of Detection Piperacillin and Tazobactam

Piperacillin	Tazobactam
Formula LOD = 3.3×avg S.D/Slope	Formula LOD = 3.3×avg S.D/Slope
Avg.SD = 2.31	Avg.SD = 2.14
Slope = 29.45	Slope = 49.21
LOD = 3.3 X 2.31/ 29.45= 0.258	LOD = 3.3 X 2.14/49.21 = 0.143

Limit of Quantification:

Table 9 showcases the minimum quantification limits for Piperacillin and Tazobactam. The Limit of Quantification (LOQ) for Piperacillin was established at 0.784, while for Tazobactam, it was determined to be 0.435. These LOQ values confirm the method's appropriateness for accurately quantify lower concentrations of Piperacillin and Tazobactam. The outcomes validate the sensitivity of the developed method, asserting its capability for precise determination even at lower concentrations of these substances.

Table 9: Limit of Quantification Piperacillin and Tazobactam

Piperacillin	Tazobactam
Formula LOQ = 10×avg S.D/Slope	Formula LOQ = 10×avg S.D/Slope
Avg.SD = 2.31	Avg.SD = 2.14
Slope = 29.45	Slope = 49.21
LOD = 10 X 2.31/ 29.45 = 0.784	LOD = 10 X 2.14/49.21 = 0.435

Analysis of Marketed Formulation:

The analysis of the commercial formulation containing Piperacillin and Tazobactam was conducted, and the percentage purity was calculated. The mean % assay values were determined to be 100.75 for Piperacillin and 99.53 for Tazobactam, respectively. Detailed assay results are provided in Table 10, and the corresponding chromatograms are illustrated in Figure 6.

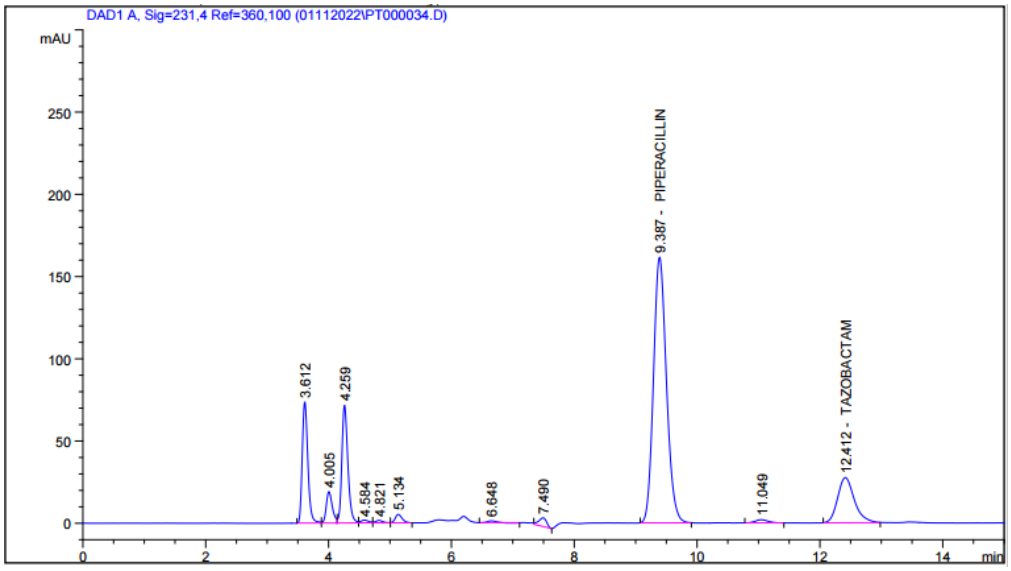

Fig 6: Chromatogram of Marketed formulation

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Table 10: Assay of Marketed Formulation

Conc. µg /ml	Area I	Area II	Mean	Amount Found	% Found	SD	%RSD
Piperacillin							
80	2372.871	2367.971	2370.42	80.60	100.75	3.465	0.146
Tazobactam							
10	505.340	507.321	506.33	9.95	99.53	1.401	0.277

FORCED DEGRADATION STUDY:

A standard sample of Piperacillin and Tazobactam underwent acidic, alkaline, oxidative, and hydrolytic degradation. The degradation remained within the acceptance criteria, demonstrating the stability-indicating properties of the method. The results of stress degradation for Piperacillin and Tazobactam are presented in Tables 11 and 12.

Table 11: Forced degradation study of Piperacillin

After 1 hour					
Sr. No	Degradation	Area of Standard	Area of degraded sample	Degraded up to %	% degradation
1	Acid	1909.93	1683.71	88.16	11.84
2	Basic	1909.93	1752	91.73	8.27
3	H_2O_2	1909.93	1741.15	91.16	8.84
After 2 hours					
1	Acid	1909.93	1536.3026	80.44	19.56
2	Basic	1909.93	1593.8081	83.45	16.55
3	H_2O_2	1909.93	1532.94	80.26	19.74
4	Hydrolytic	1909.93	1901.07418	99.54	0.46

Table 12: Forced degradation study of Tazobactam

After 1 hour					
Sr. No	Degradation	Area of Standard	Area of degraded sample	Degraded up to %	% degradation
1	Acid	409.78	367.52	89.69	10.31
2	Basic	409.78	386.18	94.24	5.76
3	H_2O_2	409.78	358.7392	87.54	12.46
After 2 hours					
1	Acid	409.78	328.342	80.13	19.87
2	Basic	409.78	345.3856	84.29	15.71
3	H_2O_2	409.78	307.3472	75.00	25.00
4	Hydrolytic	409.78	400.06	97.63	2.37

Conclusion:

In conclusion, the development and validation of a stability-indicating method for the analysis of Piperacillin and Tazobactam in both bulk and dosage forms within human plasma using RP-HPLC have been successfully undertaken. The meticulous design of the method involved careful consideration of parameters such as mobile phase composition, flow rate, and column characteristics to ensure optimal separation and accurate quantification. The stability-indicating nature of the method was established through systematic validation studies, adhering to stringent guidelines. The validation process encompassed various critical aspects, including linearity, precision, repeatability, robustness, and the determination of limits of detection and quantification. The analytical method exhibited a linear

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response within the specified concentration range, demonstrating its suitability for accurate determination. The precision and repeatability studies revealed consistent and reliable results, attesting to the robustness of the method. Furthermore, the stability studies conducted under various stress conditions added a layer of confidence in the method's ability to withstand challenging situations and provide accurate results. The RP-HPLC method has proven to be not only precise and reliable but also sensitive and selective, making it well-suited for routine analysis of Piperacillin and Tazobactam in diverse sample matrices.

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