## STABILITY INDICATING RP-HPLC METHOD FOR THE DETERMINATION OF PIPERACILLIN AND TAZOBACTAM AND THEIR RELATED SUBSTANCES IN BULK AND PHARMACEUTICAL FORMULATION

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## ABSTRACT

A simple, sensitive and precise high performance liquid chromatographic method was developed for the determination of related substances of Piperacillin and Tazobactam in bulk and pharmaceutical formulation. The Impurities were well separated on a Hypersil ODS column (250mm X 4.6mm, 5 µm) by the gradient program using Phosphate buffer (pH 6.0) and Acetonitrile at a flow rate of 1.0 mL /min with detection wavelength at 220 nm. The developed method separates Piperacillin and Tazobactam from its related substances by 45 minutes run time. The calibration curve shows linearity in the concentration range of 0.3-75.5 µg/ml for Piperacillin, 0.3-3.3 µg/ml for Tazobactam, 0.4-3.1 µg/ml for Aminosulfinic acid, 0.4-17.2 µg/ml for Ampicillin, 0.4-7.9 µg/ml for D-phenylglycyl EDP,0.4-15.8 µg/ml for Piperacillin sulfoxide with a correlation coefficient 0.9997, 0.9993, 0.9998, 0.9999, 0.9992, 0.9996 respectively. The method was validated for specificity, linearity, accuracy, precision, robustness and solution stability. Recovery was found to be in the range of 75-110% and RSD is <2%. The proposed method was successfully applied for the determination of Piperacillin and Tazobactam and their related substances in bulk and pharmaceutical formulations.

**KEYWORDS:** Piperacillin(PIPER), Tazobactam(TAZO), HPLC, Related substances, Method Development.

## INTRODUCTION

Piperacillin is a broad spectrum antibiotic, chemically it is [2S-[2a,5a,6b(S*)]]-6-[[[[(4-ethyl-2,3-dioxo-1- piperazinyl) carbonyl] amino] phenyl- acetyl] amino-3,3 dimethyl-7-oxo-4-thia - 1-azabicyclo-[3,2,0]heptanes-2-carboxylic acid. It exerts bactericidal activity by inhibition of both septum and cell wall. Whereas Tazobactam is a β -lactamase inhibitor and its chemical formula is (2S,3S,5R)-3-methyl-7-oxo-3-(1H-1,2,3-triazol-1-ylmethyl)-4-thia-1-azabicyclo-[3.2.0]heptanes-2-carboxylic acid-4,4-dioxide. In vitro, piperacillin and tazobactam combination is active against a variety of gram-positive and gram-negative aerobic and anaerobic bacteria. Ampicillin(<0.2% w/W), D-phenylglycyl EDP (<0.1% w/W), Piperacillin sulfoxide(<0.2% w/W) are the major impurities of piperacillin and Aminosulfinic acid(< 1.0% w/W) is an impurity of tazobactam.

Several analytical methods were reported for the simultaneous estimation of Piperaciilin and Tazobatam include second-order derivative spectrophotometry, UV-spectrophotometric absorption correction method, HPLC. Literature survey revealed that very few methods were reported for the simultaneous estimation of PIPER and TAZO and their related substances. So, an attempt has been made to develop an accurate, precise and economic RP-HPLC method for the determination of related substances(1-6).
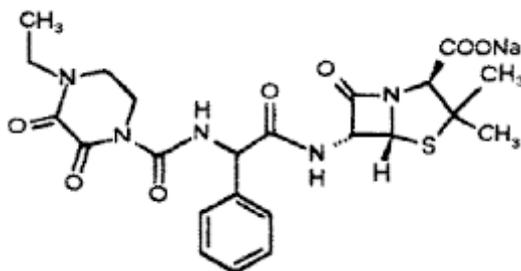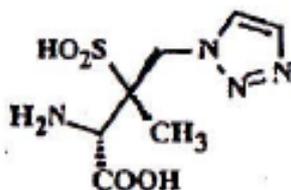


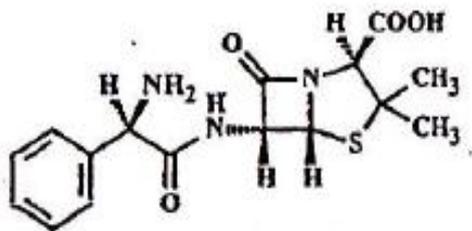**Fig.1 Structure of Piperacillin**



**Fig.2 Structure of Tazobactam**
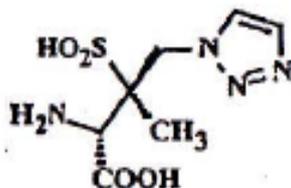
**Fig.3 Structure of Ampicillin**



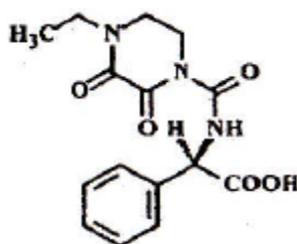**Fig.4 Structure of Aminosulfinic acid**



**Fig.5 Structure of D-phenylglycyl EDP**
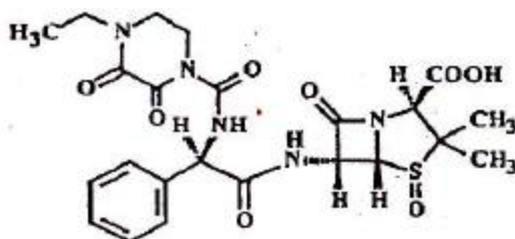


**Fig.6 Structure of Piperacillin sulfoxide**

**EXPERIMENTAL**

**MATERIALS AND METHOD**

**Chemicals**

Working reference standards of Piperacillin, Tazobactam acid and reference standards of ampicillin, aminosulfinic acid, D-phenyl glycel EDP, Piperacillin sulfoxide were gift samples from Aurabindo Pharma Pvt., Ltd., Hyderabad.  Orthophosphoric acid was purchased from Lancaster, 40% Tetrabutylammonium hydroxide aqueous solution, Acetonitrile, Water, sodium hydroxide, hydrochloric acid, hydrogen peroxide were purchased from Merck

specialities private limited, Mumbai. Piperacillin and tazobactam for injection- 4.5 gm was commercially purchased.

### Equipment

Alliance waters 2695 HPLC separation module provided with UV/Visible detector was used. Data acquisition was carried out by using Empower software. Separation of compounds was performed on Hypersil ODS $C_{18}$ (250mm×4.6mm, 5 µ).

### Chromatographic parameters

Mobile phase consists of solution A- a mixture of phosphate buffer: acetonitrile (98:2%v/V) was prepared, sonicated for 5 min and filtered through membrane filter (0.45 µm) then $P^H$ adjusted to (6±0.05) using NaOH solution. Solution B- Acetonitrile was alone used as solution B. Flow rate was 1.0 ml/min and injection volume was 20 µl. Run time was 45 min with a detection wavelength of 220 nm. Column temperature was maintained at $30^0$C.

### Preparation of Diluent

A mixture of phosphate buffer solution and Acetonitrile in the ratio of 98:2%v/V with $P^H$ adjusted to (6±0.05) was used.

### Preparation of stock and standard solution

Stock solutions of Piperacillin and Tazobactam were prepared by dissolving 10 mg of PIPER in 10 ml of diluent and 6 mg of TAZO in 10 ml of diluents respectively. From the above stock solutions 5 ml of PIPER and 3ml of TAZO were solutions were spiked in 100 ml volumetric flask and volume was made upto the mark with diluent to prepare a standard solution with concentration range of 18µg/ml for TAZO and 50µg/ml for PIPER.

### Preparation of Test solution

285 mg of dry powder (for injection) was weighed and transferred to 50 ml volumetric flask, to this 5 ml of acetonitrile was added and sonicated. Volume was made upto 100 ml with diluents and filtered through 0.45 µm or finer porosity membrane filter.

### VALIDATION OF THE HPLC METHOD

### System suitability

System suitability is commonly used to verify resolution, column efficiency, and repeatability of the chromatographic system to ensure its adequacy for a particular analysis. Standard solution of Piperacillin and Tazobactam(system suitability solution) was prepared and

injected into HPLC system in five replicates and chromatograms were recorded. The sysem suitability parameters, %RSD and calculated data was presented in Table-1.

**Precision**

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of homogeneous sample. Six preparations individually using single batch of PIPER and TAZO for injection spiked with related substances at 100% of their specification limit were prepared and injected into HPLC. Percentage RSD for peak areas of all components were calculated. Less than 10% RSD indicates the method was precise and data represented in Table-2.

**Accuracy**

Accuracy of an analytical method is the closeness of test results obtained by that method to the true value. To ascertain accuracy solutions were prepared in triplicate using PIPER and TAZO injection spiked with related substances at levels 50%,100% and 150% of specification as per proposed method and injected each solution into HPLC. The percentage recovery and RSD were calculated and the data was tabulated in Table-3.

**Limit of Quantification and Limit of Detection**

Limit of Quantification and Limit of Detection values of each of related substances were predicted from a separate linearity data performed at a lower concentration (below 50% specification level). Each predicted concentration was verified by preparing the solutions and injecting each solution six times into HPLC. Percentage RSD for PIPER, TAZO and related substances were calculated and data was given in Table-4.

**Linearity**

To determine the linearity a series of solutions were prepared using PIPER and TAZO working standards and their related substances at concentration levels from LOQ to 150% of specification and each solution was injected to HPLC. Correlation coefficient was calculated by plotting calibration curve using peak area ratio Vs concentration of the standard solution. Data was given in Table-5.

**Specificity**

All the solutions of related substances were prepared and injected individually to ensure that there is no interference of related substances at the retention times of PIPER and TAZO. Test

solution(control sample) and test sample spiked with known related substances were prepared and injected into HPLC. Data was recorded and given in Table-6.

**Robustness**

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness was done by changing the column temperature (± 5°C), flow rate (± 10%), Changing the wavelength (± 5 nm). Standard solution and test solution spiked with known related substances at specification level were prepared and injected into HPLC at different variable conditions. The data was recorded and given in Table-7.

**RESULTS AND DISCUSSION**

Various HPLC, UV, Ion pair HPLC were developed and published for estimation of Piperacillin, Tazobactam and their related substances in bulk and pharmaceutical formulations. Present work was aimed to develop a simple, accurate and precise RP-HPLC method for the determination of related substances in Piperacillin and Tazobactam injection in a single run with less run time so that the method can be utilised in routine quality control laboratories.

**Method development**

Based on the results obtained from various trials detection wavelength was selected at 220 nm since PIPER, TAZO and their related substances showing maximum absorbance at around 200-240 nm. Hypersil ODS 250 mm×4.6 mm×5µ column was selected as it shows better aqueous mobile phase compatibility. By using this column no merging of peaks was observed. The choice of buffer was made based on the $P^{ka}$ values of Piperacillin and Tazobactam. Phosphate buffer of $P^{H}$ (6±0.05) was selected as it improves peak shape. A mixture of Phosphate buffer and acetonitrile (solution A) and pure acetonitrile(solution B) was selected as mobile phase because this separate all the peaks. on the basis of solubility of all the impurities as well as PIPER and TAZO, the diluents was selected to be 98:2%v/V:buffer:acetonitrile. Column oven temperature was set at $30^{0}$C to activate the column and to achieve the early separation. Hence the optimized HPLC parameters were as follows: flow rate 10ml/min, injection volume 20 µl and a gradient program with mobile phase which consists of solution A and solution B. The developed method separates

piperacillin, Tazobactam and their related substances by 45 min run time. No interference due to diluents and excipients was observed.

## Method Validation

The method was validated as per ICH and USP guidelines.

## System suitability

The percentage RSD for peak areas of six replicate injections of the standard solution should be less than 5.0%. USP plate count for piperacillin peak should not less than 5000and USP tailing for PIPER should not more than 2.0 indicate the system was suitable for the proposed method.

## Precision

System precision was calculated by using six replicates of standard solution. The percentage RSD should not be more than 5.0%. Method precision was carried out by using single batch of PIPER and TAZO for injection spiked with related substances at specification level. The percentage RSD should be less than 10.0%. The data was given in Table-2.

## Accuracy

Recovery should be within 90.0% to 110.0% for related substances of 0.3 to 1.0% specification and 85.0% to 115.0% for related substances of 0.2 % specification indicates that the method is accurate.

## Limit of Quantification(LOQ) and Limit of Detection(LOD)

LOQ and LOD values of each related substances were predicted from a separate linearity data. The percentage RSD should be within the acceptance limit of <10.0 for LOQ and 33.0% for LOD. The determined LOQ and LOD values for PIPER, TAZO and their related substances are presented in Table-4.

## Linearity

Linearity curves of Piperacillin, Tazobactam and their related substances were  calibrated by using a series of solutions at concentration levels of LOQ to % of specification. Correlation coefficient which represents linearity should meet acceptance limit of >0.990. The regression values are shown in Table-5, with linearity curves of Piperacillin, Tazobactam and their related substances are represented in Figure 7-12.

## Specificity

Specificity of the method was demonstrated by injectioing diluents, test sample spiked with known related substances so that peaks of all the components should meet the acceptance criteria of peak purity< purity threshold. Forced degradation studies were carried out to conform the proposed method has stability indicating capability and selectivity. Piperacillin and Tazobactam for injection was stressed with acid(2M HCl), base(0.05 M NaOH), peroxide(10% $H_2O_2$), thermal($105^0C$), photolytic (10 K Lux) and humidity (92% RH)conditions respectively. Peak purity for all the mentioned conditions was established and peak purity should meet acceptance criteria of peak purity< purity threshold. From all the peak purity data of PIPER and TAZO peaks at every degradation sample shows that the peaks are homogenous and there are no co-eluting peaks conforms that the method is stability indicating and specific.

## Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate changes. The proposed method is robust as it meets the acceptance criteria of USP plate count for PIPER <5000 and USP tailing for PIPER peak is <2.0.

**Table -1 Results of System Suitability**

| System suitability parameters | Obtained value | Acceptance criteria |
|---|---|---|
| USP theorical plates for Piperacillin peak | 320802 | USP plate count NLT 5000 |
| Percentage RSD for PIPER from six replicate injections of standard solution | 1.0% | Percentage RSD NMT5.0% |
| Percentage RSD for PIPER from six replicate injections of standard solution | 0.3% | Percentage RSD NMT 5.0% |
| USP tailing for Piperacillin peak | 1.1 | Tailing NMT 2.0 |

**Table -2 Results for Precision and Intermediate Precision**

| Substance | Method precision | | Intermediate precision | |
|---|---|---|---|---|
| | % Impurity | % RSD | % Impurity | % RSD |
| Ampicillin | 0.236 | 0.8 | 0.221 | 0.5 |
| D-phenylglycyl EDP | 0.109 | 0.9 | 0.111 | 0.9 |
| Piperacillin sulfoxide | 0.258 | 0.4 | 0.231 | 0.0 |
| Aminosulfinic acid | 0.481 | 1.9 | 0.419 | 1.0 |

**Table -3 Results for Accuracy**

| Substance | % Recovery (Mean of Three Replicates) | | |
|---|---|---|---|
| | At 50% | At 100% | At 150% |
| Ampicillin | 102.9 | 103.2 | 103.1 |
| D-phenylglycyl EDP | 103.1 | 103.4 | 102.1 |
| Piperacillin sulfoxide | 101.9 | 103.0 | 103.3 |
| Aminosulfinic acid | 98.5 | 96.1 | 96.0 |

**Table -4 Results for LOD and LOQ**

| Substance | LOD(µg/ml) | %RSD | LOQ(µg/ml) | %RSD |
|---|---|---|---|---|
| Ampicillin | 0.208 | 13.2 | 0.416 | 5.8 |
| D-phenylglycyl EDP | 0.230 | 12.9 | 0.460 | 9.0 |
| Piperacillin sulfoxide | 0.215 | 13.3 | 0.430 | 5.0 |
| Aminosulfinic acid | 0.230 | 13.8 | 0.460 | 7.9 |

**Table -5 Results for Linearity**

| Substance | Linearity range (µg/ml) | Correlation coefficient $R^2$ |
|---|---|---|
| Piperacillin | 0.49 to 77.76 | 0.9997 |
| Tazobactam | 0.39 to 3.37 | 0.9993 |
| Ampicillin | 0.41 to 17.24 | 0.9999 |
| D-phenylglycyl EDP | 0.45 to 7.93 | 0.9992 |
| Piperacillin sulfoxide | 0.42 to 15.89 | 0.9996 |
| Aminosulfinic acid | 0.40 to 3.13 | 0.9998 |

**Table -6 Results for Forced Degradation Studies**

| Mode of Degradation | Conditions | Peak purity of Piperacillin | | Peak purity of Tazobactam | |
|---|---|---|---|---|---|
| | | Purity angle | Purity threshold | Purity angle | Purity threshold |
| Acid degradation | 2M HCl/initial | 0.050 | 0.276 | 0.097 | 0.097 |
| Base degradation | 0.05M NaOH/initial | 0.068 | 0.274 | 0.080 | 0.080 |
| Peroxide degradation | 10% $H_2O_2$/$85^0$C/5 min | 0.070 | 0.266 | 0.101 | 0.101 |
| Thermal degradation | $105^0$C /120 Hours | 0.107 | 0.296 | 0.524 | 0.524 |
| Photolytic degradation | 10K Lux/240 Hours | 0.046 | 0.258 | 0.059 | 0.059 |
| Humidity degradation | 92% RH/ $25^0$C/48 Hours | 0.029 | 0.258 | 0.027 | 0.027 |
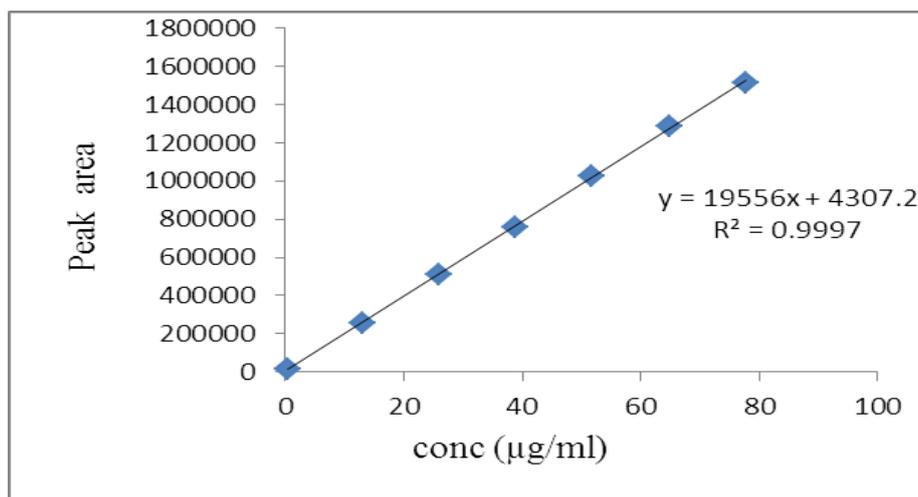
**Fig-7.Linearity Plot of Piperacillin**
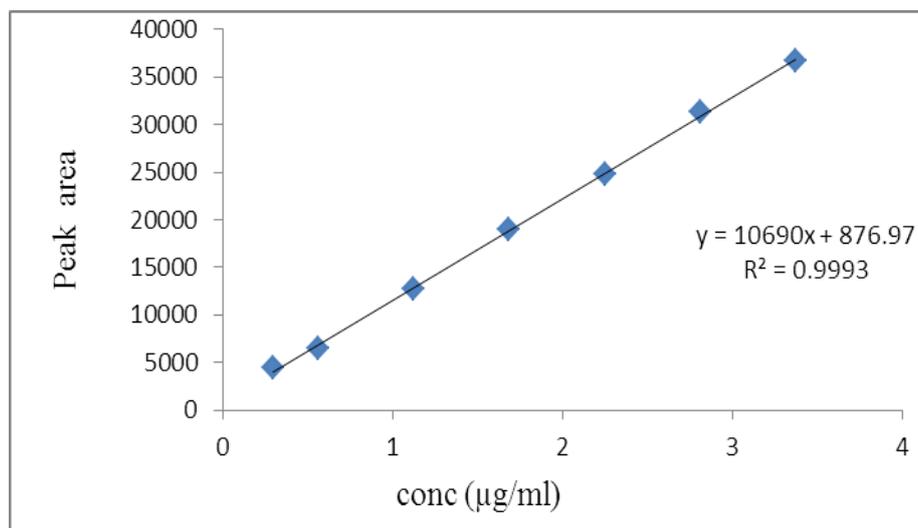


**Fig-8.Linearity Plot of Tazobactam**
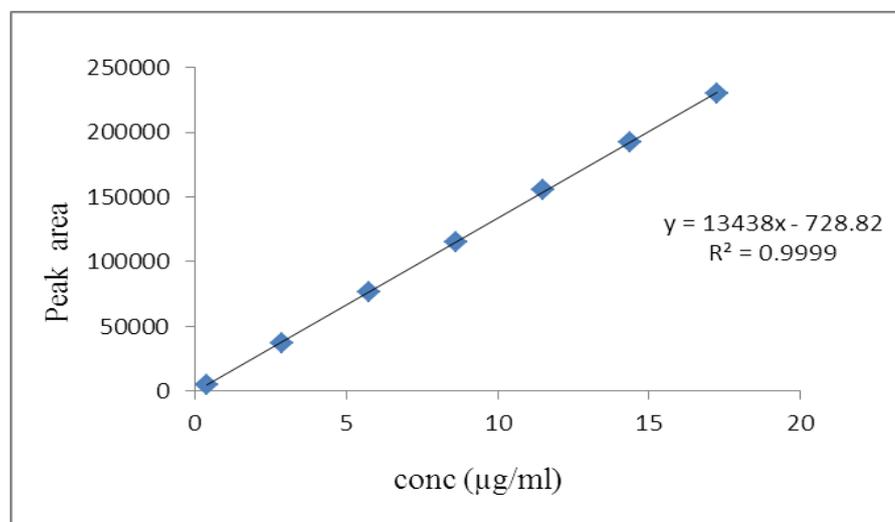


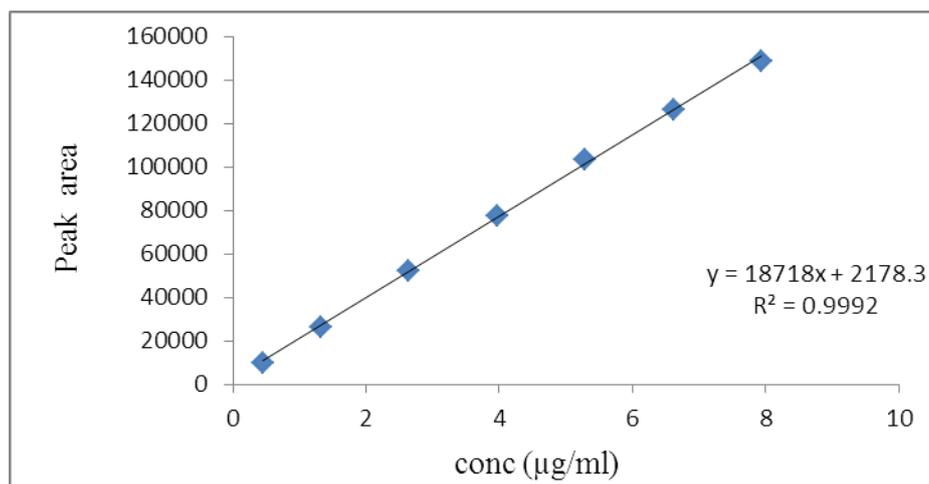**Fig-9.Linearity Plot of Ampicillin**
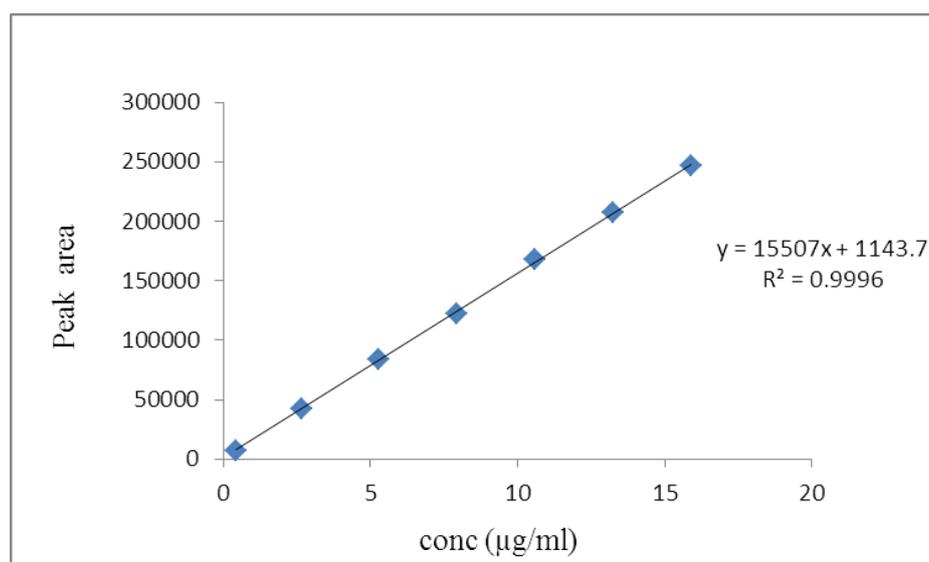
**Fig-10.Linearity Plot of D-Phenylglycyl EDP**

y = 18718x + 2178.3
R² = 0.9992



**Fig-11.Linearity Plot of Piperacillin Sulfoxide**

y = 15507x + 1143.7
R² = 0.9996
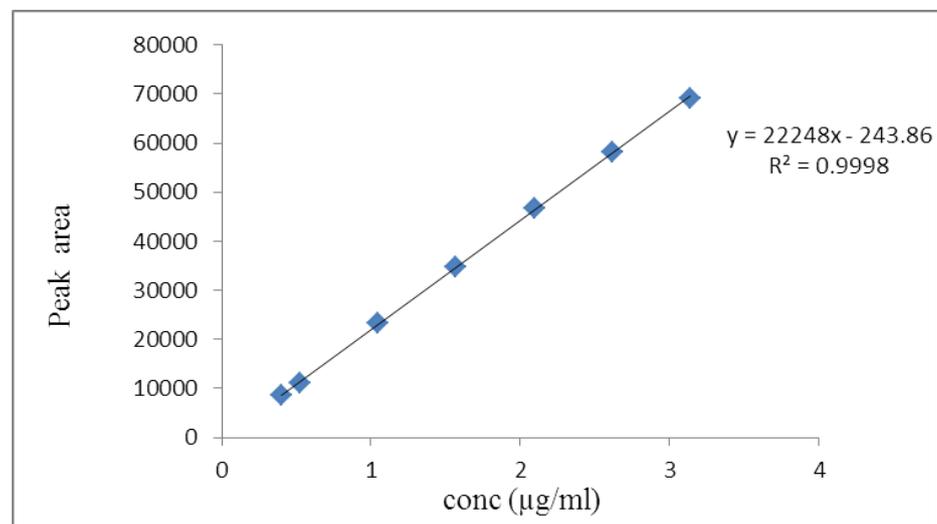


**Fig-12.Linearity plot of Aminosulfinic acid**

y = 22248x - 243.86
R² = 0.9998
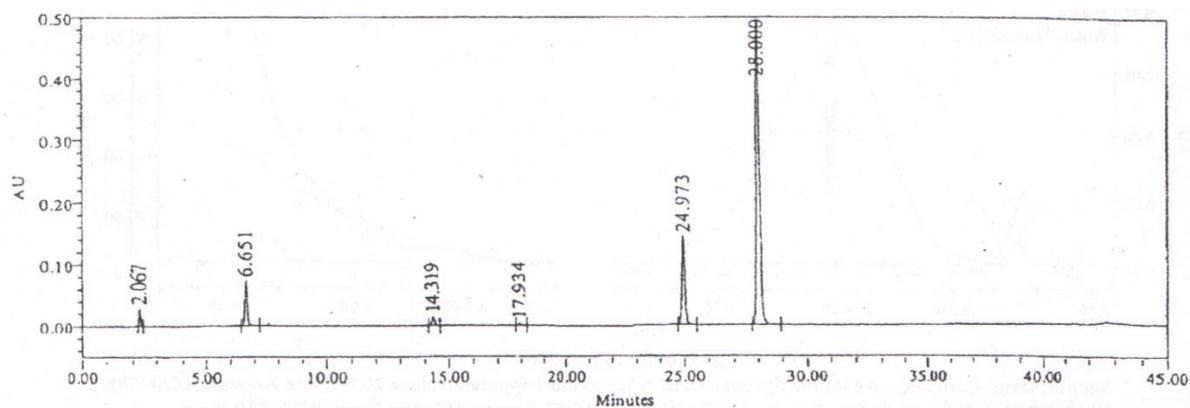
**Fig No-13 Chromatogram of Reference Solution (spiked with known related substances)**

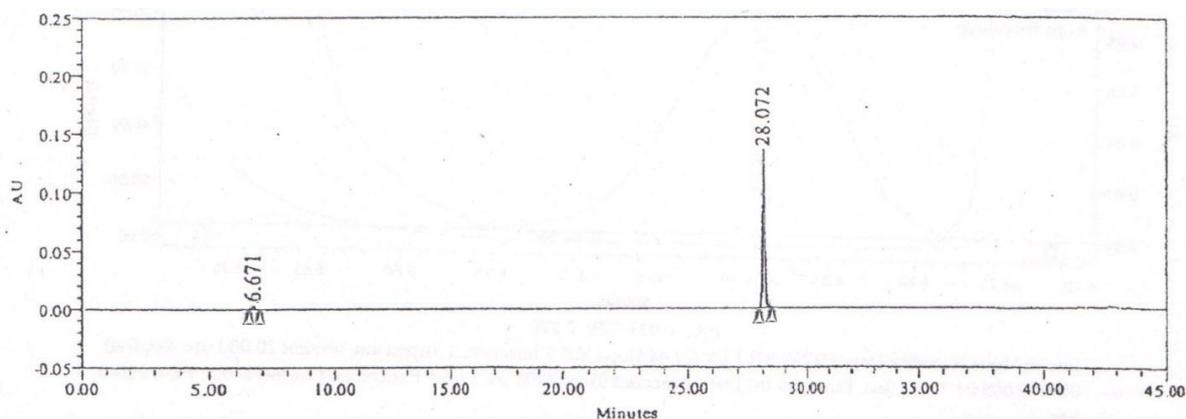| S. No | Name | RT(min) |
|-------|------|---------|
| 1 | Aminosulfinic acid | 2.067 |
| 2 | Tazobactam | 6.651 |
| 3 | Ampicillin | 14.319 |
| 4 | D-phenylglycyl EDP | 17.934 |
| 5 | Piperacillin sulfoxide | 24.973 |
| 6 | Piperacillin | 28.00 |



**Fig No-14 Chromatogram of System suitability**

**CONCLUSION**

The present study describes development and validation of a RP-HPLC method for determination of Piperacillin and Tazobactam and their related substances in bulk and pharmaceutical formulation. The developed method is simple, accurate, sensitive, precise and can be effectively utilized for routine analysis in quality control laboratories, research institutions and clinical pharmacokinetic studies as this method separates PIPER and TAZO from their related substances by 45 min run time. From overall observations it was concluded that the developed method was more simple, accurate, sensitive, precise and found to be within prescribed limits.

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