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Refinement and validation of a reverse phase-HPLC methodology for piperacillin quantification in therapeutic drug monitoring

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ABSTRACT

Piperacillin is a semisynthetic ureidopenicillin with enhanced activity against resistant Gram-negative bacteria and is commonly administered in combination with the β-lactamase inhibitor tazobactam. This combination exhibits broad-spectrum antibacterial activity and is widely used in the treatment of severe polymicrobial infections. Accurate quantification of piperacillin in biological matrices is essential for pharmacokinetic evaluation and therapeutic drug monitoring (TDM). Although previous studies have established optimized HPLC methods for piperacillin analysis, further refinement of chromatographic parameters is necessary to enhance method performance and suitability for application in complex biological matrices such as ultrafiltrate and plasma. This study aimed to refine and validate a reversed-phase high-performance liquid chromatography (RP-HPLC) method for the quantification of piperacillin in these matrices. Calibration curves were constructed over a concentration range of 5–100 µg/mL. Chromatographic separation was achieved using a C18 column with an isocratic phosphate buffer–acetonitrile mobile phase and diode array detection at 230 nm. The method demonstrated excellent linearity in standard solution and ultrafiltrate ($R^2 = 0.995$), while lower linearity was observed in plasma ($R^2 = 0.893$), likely due to matrix effects. Precision values (%RSD < 2%) confirmed acceptable repeatability and reproducibility. The refined method is suitable for quantifying piperacillin in ultrafiltrate and demonstrates acceptable performance in plasma, with further optimisation required to improve analytical sensitivity for tazobactam.

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1. INTRODUCTION

A fundamental component of modern medicine, antibiotics are essential for both treating and preventing bacterial illnesses. Antibiotics are widely used as a treatment for bacterial infections and are generally classified into two main types based on their mechanism of action: bactericidal, which kill bacteria, and bacteriostatic, which prevent bacteria from growing (Patel et al., 2024). Beta-lactams are an important group among antibiotics that include penicillin, ampicillin, rifamycin, and carbapenem. Antibiotics work through several mechanisms of action, such as the suppression of DNA replication, protein production, cell wall formation, and folic acid metabolism (Halawa et al., 2024). The spectrum of activity of antibiotics varies and targets infections that match the specific pathogens. Broad-spectrum antibiotics are used in empiric treatment when there are multiple or unidentified suspected pathogens, whereas narrow-spectrum antibiotics are preferred when the specific pathogen is known (Rhee et al., 2024). There are several classes of antibiotics categorized by either their chemical or molecular structure, including beta-lactams, aminoglycosides, tetracyclines, macrolides, oxazolidinones, sulfonamides, glycopeptides, and nitroimidazoles (Patel et al., 2024). A prominent class of antimicrobial drugs known as beta-lactam antibiotics is distinguished by the presence of a beta-lactam ring in its molecular structure. This category comprises several subclasses, including carbapenems, monobactams, cephalosporins, and penicillins (Pandey & Cascella, 2024).

Effective and precise analysis of these medications is required to preserve their effectiveness and guarantee safety in clinical settings, considering the rise of antibiotic resistance. The ability of bacteria, viruses, fungi, and parasites to withstand the effects of drugs that once effectively treated them is known as antimicrobial resistance (AMR), and it is a serious global health concern (Kırmusaoğlu, 2019). AMR has emerged since the discovery of the first antibiotic, penicillin, in the 1940s, as a result of the natural evolutionary processes of bacteria (Bo et al., 2024). Currently, AMR is a growing global health concern due to the irrational use, overuse, and misuse of antibiotics, which can result in increased healthcare costs, extended hospital stays, and potentially fatal outcomes. Immediate action is required to stop it from spreading further because this resistance raises morbidity, mortality, and healthcare expenses (Kırmusaoğlu, 2019). The catalytic activity of bacterial transpeptidases is efficiently inhibited by β-lactam antibiotics, which are also called penicillin-binding proteins (PBPs) (Bertonha et al., 2023). A class of β-lactam antibiotics called penicillins contains 6-aminopenicillanic acid nuclei (lactam and thiazolidine rings) with additional ringside chains (Abdelkawy et al., 2023).

Piperacillin, a piperazine derivative of ampicillin, as shown in Fig. 1, is categorized as an extended-spectrum penicillin. It was created in the 1970s along with other ureidopenicillins, including mezlocillin (Lima et al., 2020). This category consists of β-lactamase-resistant substances, natural penicillins, aminopenicillins, carboxypenicillins, and ureidopenicillins (El-Kimary et al., 2024). Each category has distinct characteristics. For example, β-lactamase-resistant compounds work well against bacteria that produce penicillinase, whereas aminopenicillins, carboxypenicillins, and ureidopenicillins have broader activity spectra that cover Gram-negative pathogens. The stereochemistry around the β-lactam ring is the same for all antibiotics of the penicillin class (Pahlavan et al., 2023). Piperacillin is a member of the semisynthetic ureidopenicillin group of penicillins, which was created to have broad-spectrum antipseudomonal action. These antibiotics are used to treat severe nosocomial infections, especially when paired with a β-lactamase inhibitor such as tazobactam (El-Kimary et al., 2024).

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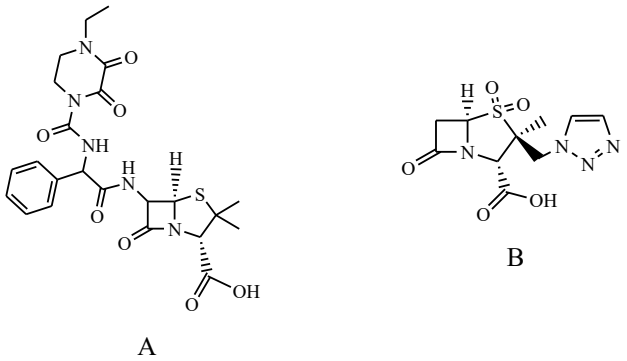

Fig 1. Chemical structure of (A) Piperacillin and (B) Tazobactam

Piperacillin's spectrum of activity is significantly broadened when combined with tazobactam because it makes this antibiotic more effective against β-lactamase-producing bacteria, including some multidrug-resistant strains. Furthermore, Gram-negative infections are often treated empirically with piperacillin–tazobactam (Sohani et al., 2024). Therefore, since piperacillin is an antipseudomonal penicillin, many methods and techniques have been developed to determine it in different samples such as medicines, food, the environment, and biological fluids to assess its pharmacokinetics or to ensure its use in therapy (El-Kimary et al., 2024).

There are several analytical methods that have been used to measure piperacillin in pharmaceutical formulations, biological samples, and environmental matrices. Spectrophotometry, for instance, has been discussed by Toral et al. (2012), who highlighted the ease of use and affordability of a derivative spectrophotometric approach for the simultaneous measurement of piperacillin and tazobactam. However, they also pointed out the drawbacks of using it on complex biological materials in terms of sensitivity and specificity. Additionally, liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Ferrari et al., 2019) is a reliable and quick technique for the simultaneous measurement of several antibiotics, including piperacillin in human plasma. The authors also mentioned that these techniques only require a minimal sample size, offer good repeatability, a short chromatographic time, and simple sample preparation. However, this method is limited by its high cost and complexity, which restricts its use to specialized laboratories. Currently, high-performance liquid chromatography (HPLC) is widely chosen for piperacillin analysis. Previous research stated that the HPLC method yields precise, accurate, and sufficiently stable results with an appropriate linearity range (Milla et al., 2020). A previous study by Aleya et al. (2024) optimized the pH conditions for the HPLC method to analyze piperacillin and tazobactam. This study identified pH 4 as the optimal condition for improving peak separation and resolution, using a phosphate buffer as the mobile phase. However, this method tested the water without any further validation or quantification steps in real samples. Thus, this method remains unverified for application to more complex biological matrices.

In another study, piperacillin and tazobactam in pharmaceutical formulations were successfully quantified, showing good linearity (R^2 = 0.996 and 0.997) and high precision, with extremely low %RSD values for retention times (less than 0.0026% for piperacillin and less than 0.00225% for tazobactam; Mangam et al., 2023). While this study confirms the method's reliability and consistency, the authors did

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not analyze biological samples such as plasma or ultrafiltrate. A study by Milla et al. (2020) quantified piperacillin and tazobactam in plasma samples using a Kinetex C18 column and a UV/Vis detector at 220 and 298 nm. Their method used a phosphate buffer at pH 3.15 and methanol in gradient mode, achieving high linearity with a correlation coefficient ≥ 0.999. However, this method uses different chromatographic conditions that differ from the method developed by Aleya et al., making direct comparison difficult (European Directorate for the Quality of Medicines & Healthcare, 2005).

Collectively, although piperacillin has been successfully quantified under various conditions, the applicability of previously optimized methods to more complex biological matrices such as ultrafiltrate and plasma remains insufficiently explored. Endogenous components in these matrices may alter chromatographic behaviour and affect analytical performance. Therefore, refinement of chromatographic parameters and further validation in relevant biological samples are necessary to ensure reliability for therapeutic drug monitoring applications, particularly at very low concentrations. Accordingly, this study aims to refine and partially validate a previously optimized reverse phase (RP)-HPLC method for the quantification of piperacillin in ultrafiltrate and plasma. By adapting chromatographic conditions and evaluating method performance in these matrices, this work seeks to bridge the gap between prior optimisation studies and practical clinical application, thereby improving analytical reliability for therapeutic drug monitoring of β-lactam antibiotics.

2. MATERIAL & METHODOLOGY

2.1 Sample preparation

The stock solutions for this study were prepared by dissolving 10 mg of piperacillin and tazobactam in 10 mL of deionized water to obtain a concentration of 1 mg/mL. A preliminary HPLC run of a 1 mg/mL solution was performed to confirm the presence and retention time of both piperacillin and tazobactam. The standard solutions of piperacillin and tazobactam were prepared from a 1 mg/mL solution by serial dilution into five concentration levels: 5, 10, 20, 50 and 100 µg/mL using the formula $C_1V_1 = C_2V_2$. Each standard solution was filtered through a 0.45 µm membrane filter into the HPLC vial for analysis and the calibration curve was constructed. The peak height obtained from each concentration was plotted to generate the standard curve, which was later used to quantify the antibiotics concentrations in ultrafiltrate and plasma samples. The ultrafiltrate samples were prepared by spiking blank ultrafiltrate with internal standard solutions of piperacillin and tazobactam at a 1:1 ratio directly in HPLC vials. The calibration curve of piperacillin and tazobactam in the ultrafiltrate matrix was constructed to observe whether it fell within the range of the standard calibration curve. The plasma samples were prepared by spiking 100 µL of plasma with 100 µL of internal standard solution, followed by the addition of 100 µL of acetonitrile into a microcentrifuge tube. The mixture was vortexed for 3 seconds and centrifuged at 1200 rpm for 5 minutes. Then, 500 µL of the supernatant was transferred to a clean microfuge tube and mixed with 500 µL of dichloromethane. The samples were vortexed and centrifuged again. Finally, the aqueous supernatant was transferred into an autosampler vial for HPLC analysis (Chavan & Desai, 2022). Additional concentrations of piperacillin and tazobactam were prepared to verify the reliability of the calibration curve. Two concentrations, 30 µg/mL and 70 µg/mL were chosen within the range of five concentrations used to construct the calibration curve. Each of these concentrations was spiked into ultrafiltrate and plasma, and the resulting peaks were observed to determine whether they fell under the linearity of the established

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standard calibration curve. The mobile phase used for the standard solution and ultrafiltrate consisted of phosphate buffer (KH_2PO_4) and acetonitrile (MeCN). The phosphate buffer was prepared by dissolving 6.8 g potassium dihydrogen phosphate in 800 mL of deionized water and the pH was adjusted to 4.0 using 0.1 M of hydrochloric acid (Veni et al., 2013). For the plasma samples, the phosphate buffer was prepared by dissolving 13.8 g of potassium dihydrogen phosphate in 800 mL of deionized water and the pH was adjusted to 6.5 using 1 M sodium hydroxide (Veni et al., 2013) Deionized water was then added to make up the final volume to 1 L.

2.2 Chromatographic conditions and data analysis

A RP-HPLC system (Agilent 1200) was used in this study, with sample injections performed via the autosampler. The elution for the standard solution and ultrafiltrate was performed in isocratic mode using a mobile phase consisting of phosphate buffer and acetonitrile at a ratio of 85:15 (buffer: MeCN) (Azman et al., 2024) and 96:4 (buffer: MeCN) (Chavan & Desai, 2022) for plasma. The buffer was adjusted to pH 4 for the standard and ultrafiltrate samples, and to pH 6.5 for plasma samples. The injection volume was set to 10 µL and the run time was programmed for 10 minutes per sample. Chromatographic separation was carried out using a C18 column (Zorbax, 150 mm x 4.6 mm, 5 µm) and the column temperature was maintained at 36 ºC. Detection was performed using a diode array detector (DAD) at a wavelength of 230 nm. All samples were injected in triplicate and the peak separation on the chromatogram was analyzed using ChemStation for HPLC data processing.

3. RESULTS

In this study, partial method validation was conducted to confirm that the previously optimised HPLC method is reliable for use in biological samples. Method validation is important to ensure that an analytical method produces results that are accurate, precise, and reproducible under defined conditions. In this study, the validation parameters evaluated were linearity, precision (intra-day and inter-day) and specificity.

3.1 HPLC analysis

In the standard solution chromatogram (Fig. 2A), both the piperacillin and tazobactam peaks were clearly observed with good resolution and peak shapes at 1.896 and 1.349 min, respectively. A higher concentration of the standard solution (1 mg/mL) was used to clearly visualize the separation of piperacillin and tazobactam using this method. In another study (Veni et al., 2013), piperacillin and tazobactam were analysed using a C18 reversed-phase HPLC column. The reported retention times were 2.11 minutes for piperacillin and 5.19 min for tazobactam. Although the pH was the same, the difference in elution order can be attributed to the higher organic solvent content in their study (80% organic solvent) compared to this study (15% organic solvent), which has a more aqueous mobile phase. A higher organic ratio decreases the retention of the hydrophobic piperacillin, causing it to elute before tazobactam. In addition, the retention time of piperacillin was quite similar to that observed in this study.

In the ultrafiltrate (Fig. 2B) and plasma (Fig. 2C) samples spiked with 10 µg/mL, only the piperacillin peak was observed. The piperacillin peak in both the ultrafiltrate and plasma remained sharp and consistent with

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almost the same retention time as the standard solution, which was at 1.866 min for the ultrafiltrate and 1.983 min for the plasma. The absence of the tazobactam peak in these samples may be associated with relatively low concentrations and potential matrix suppression effects in biological components, such as endogenous plasma proteins and phospholipids. In addition, the ultrafiltrate and plasma components possibly interfere with detectability, especially at lower concentrations (An et al., 2020). The 10 µg/mL concentration was selected because it yielded good results at lower concentrations. These findings suggest that this method is suitable for detecting piperacillin in biological matrices, even at lower concentrations.

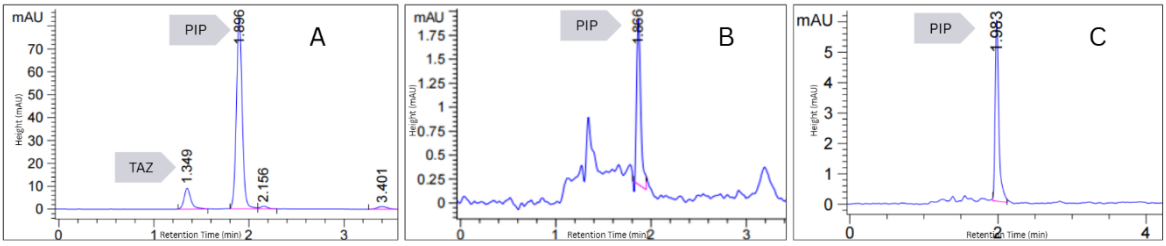

Fig 2. The chromatographic performance using a standard solution (1 mg/mL) for chromatogram (A), 10 µg/mL of ultrafiltrate for chromatogram (B) and 10 µg/mL of plasma for chromatogram (C). Abbreviations: TAZ: Tazobactam; PIP: Piperacillin.

3.2 Precision

Precision in HPLC analysis is important to ensure the method produces consistent and reproducible results under the same conditions across multiple measurements. Precision was evaluated through repeatability (intra-day) and inter-day analysis. Intra-day precision was analyzed by injecting samples in triplicate at three different concentration levels (10, 50 and 100 µg/mL) during three different times on the same day, as shown in Table 1A for ultrafiltrate and Table 1B for plasma to look for repeatability. The %RSD value for all concentrations that were below 2% indicate good repeatability. Specifically, the average %RSD value for each concentration spiked ultrafiltrate was found to be 0.109%, 0.075% and 0.047%, while for spiked plasma was 0.111%, 0.123% and 0.030%. According to International Council for Harmonisation (ICH) guidelines, %RSD below 2% are acceptable for precision and the low %RSD shown in this study demonstrates that the HPLC method provides consistent retention times at different concentration levels (International Conference on Harmonisation [ICH], 2005).

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Table 1. Intra-day of piperacillin and tazobactam in ultrafiltrate (A) and plasma (B).

A: ULTRAFILTRATE

Concentration level	Experiment	Injection 1	Injection 2	Injection 3	Mean ± SD (min)	% RSD
10 ug/ml	1	1.861	1.866	1.863	1.8633 ± 0.0025	0.134
	2	1.861	1.862	1.864	1.8623 ± 0.0015	0.081
	3	1.860	1.861	1.864	1.8617 ± 0.0021	0.113
	Average					0.109
50 ug/ml	1	1.857	1.859	1.860	1.8587 ± 0.0015	0.081
	2	1.862	1.859	1.861	1.8607 ± 0.0015	0.081
	3	1.862	1.864	1.864	1.8633 ± 0.0012	0.064
	Average					0.075
100 ug/ml	1	1.858	1.860	1.859	1.8590 ± 0.0010	0.054
	2	1.860	1.860	1.861	1.8603 ± 0.0006	0.032
	3	1.866	1.865	1.864	1.8650 ± 0.0010	0.054
	Average					0.047

B: PLASMA

Concentration level	Experiment	Injection 1	Injection 2	Injection 3	Mean ± SD (min)	% RSD
10 ug/ml	1	1.980	1.983	1.981	1.9813 ± 0.0015	0.076
	2	1.981	1.986	1.983	1.9833 ± 0.0025	0.126
	3	1.984	1.989	1.985	1.9860 ± 0.0026	0.131
	Average					0.111
50 ug/ml	1	1.981	1.987	1.983	1.9837 ± 0.0031	0.156
	2	1.982	1.986	1.983	1.9837 ± 0.0021	0.106
	3	1.983	1.987	1.984	1.9847 ± 0.0021	0.106
	Average					0.123
100 ug/ml	1	1.998	1.998	1.999	1.9983 ± 0.0006	0.030
	2	1.998	1.999	1.999	1.9987 ± 0.0006	0.030
	3	1.998	1.998	1.999	1.9983 ± 0.0006	0.030
	Average					0.030

The inter-day precision was assessed at three concentration levels which are 10, 50 and 100 µg/mL on three different days. Each day, triplicate injections were performed, and the mean ± SD and %RSD values are shown in Table 2A for ultrafiltrate and Table 2B for plasma. The average %RSD value for each concentration spiked ss was found to be 0.057%, 0.056% and 0.039%, while for spiked plasma was 0.119%, 0.158% and 0.039%. All %RSD values are below 2% and indicate that this HPLC method is reproducible and also stable over different days. The mean retention time for both ultrafiltrate and plasma showed minimal variation indicating the chromatographic conditions are stable. The minor difference can be due to slight day-to-day instrument variations or any environmental factors. Overall, the low %RSD values in both intra and inter-day represent the repeatability and precision of this method of analysis and comply with ICH Q2(R1) criteria (International Conference on Harmonisation [ICH], 2005).

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Table 2. Inter-day of piperacillin and tazobactam in ultrafiltrate (A) and plasma (B).

A: ULTRAFILTRATE

Concentration level	Day	Injection 1	Injection 2	Injection 3	Mean ± SD (min)	% RSD
10 ug/ml	1	1.865	1.867	1.866	1.8660 ± 0.0010	0.054
	2	1.871	1.873	1.871	1.8717 ± 0.0012	0.064
	3	1.863	1.864	1.865	1.8640 ± 0.0010	0.054
	Average					0.057
50 ug/ml	1	1.868	1.867	1.868	1.8677 ± 0.0006	0.032
	2	1.863	1.865	1.864	1.8640 ± 0.0010	0.054
	3	1.859	1.862	1.861	1.8607 ± 0.0015	0.081
	Average					0.056
100 ug/ml	1	1.868	1.868	1.867	1.8677 ± 0.0006	0.032
	2	1.866	1.865	1.864	1.8650 ± 0.0010	0.054
	3	1.860	1.860	1.861	1.8603 ± 0.0006	0.032
	Average					0.039

B: PLASMA

Concentration level	Day	Injection 1	Injection 2	Injection 3	Mean ± SD (min)	% RSD
10 ug/ml	1	1.736	1.736	1.739	1.7370 ± 0.0017	0.098
	2	1.947	1.946	1.950	1.9477 ± 0.0021	0.108
	3	1.983	1.986	1.989	1.9860 ± 0.0030	0.151
	Average					0.119
50 ug/ml	1	1.735	1.736	1.735	1.7353 ± 0.0006	0.035
	2	1.944	1.946	1.942	1.9440 ± 0.0020	0.103
	3	1.999	1.998	1.987	1.9947 ± 0.0067	0.336
	Average					0.158
100 ug/ml	1	1.735	1.734	1.735	1.7347 ± 0.0006	0.035
	2	1.946	1.945	1.946	1.9457 ± 0.0006	0.031
	3	1.995	1.993	1.994	1.9940 ± 0.0010	0.050
	Average					0.039

3.3 Specificity

Specificity in HPLC analysis is crucial to ensure the method can accurately identify and quantify the analyte of interest without interference from other components in the sample matrix. Specificity was evaluated by analyzing blank ultrafiltrate and plasma samples, then comparing their chromatograms with those of samples spiked with known concentrations of piperacillin and tazobactam which in this experiment are 30 and 70 µg/mL. The blank ultrafiltrate chromatogram in Fig. 3A showed a few minor peaks near the retention time of the analytes. However, Fig. 3B was the result observed for spiked ultrafiltrate with 30 µg/mL and this chromatogram shows one significant peak at 1.880 min, which was identified as piperacillin with no significant peak from blank ultrafiltrate that interferes with the analytes of the sample. Fig. 3C demonstrates that the 70 µg/mL spiked sample shows the peaks of piperacillin at 1.896 min.

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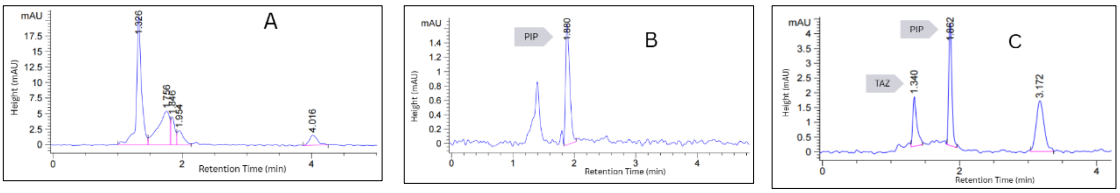

Fig 3. Specificity study for ultrafiltrate; (A) blank ultrafiltrate, (B) ultrafiltrate containing 30 µg/mL, (C) ultrafiltrate containing 70 µg/mL.

The significant peaks at 1.880 min and 1.862 min for the 30 µg/mL and 70 µg/mL spiked samples indicate that piperacillin can be distinguished from the background matrix peaks, even though there are minor peaks present in the blank ultrafiltrate. The presence of tazobactam can be detected starting from the ultrafiltrate spiked with 70 µg/mL of piperacillin and tazobactam at min 1.340. However, the blank chromatogram showed a peak at 1.326 min, which is very close to the expected peak of tazobactam. This may indicate slightly overlap or matrix interference. While this method shows good specificity of piperacillin under these method conditions, further optimisation may be needed in order to significantly detect tazobactam under matrix components.

Next, the specificity was also evaluated by comparing the chromatograms of blank plasma with plasma samples spiked at known concentrations of 30 and 70 µg/mL piperacillin and tazobactam solution. Chromatograms of blank plasma (Fig. 4A) show no significant interfering peaks at retention times corresponding to piperacillin and tazobactam but a small peak was observed at 2.206 min. However, this peak did not interfere with the detection of piperacillin and tazobactam. In the spiked plasma with 30 µg/mL (Fig. 4B) and 70 µg/mL (Fig. 4C), sharp peaks corresponding to piperacillin were clearly observed without overlapping with any matrix peaks at 1.990 and 1.986 min, respectively. These results indicate that the method is capable of detecting piperacillin and tazobactam in plasma accurately without any significant interference from plasma components. Even though the method shows good specificity for piperacillin under these conditions.

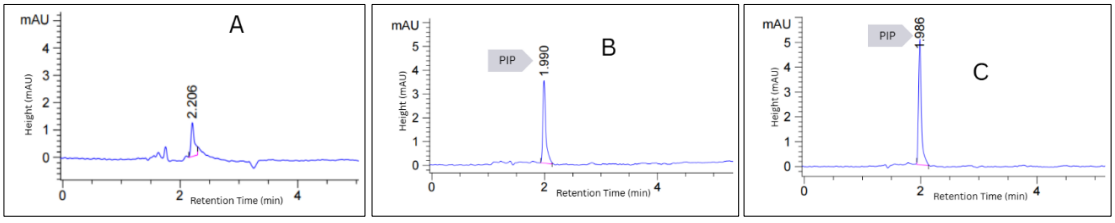

Fig 4. Specificity study for plasma; (A) blank plasma, (B) plasma containing 30 µg/mL, (C) plasma containing 70 µg/mL.

3.4 Linearity and accuracy

Linearity and accuracy in HPLC analysis are closely related, as good linearity ensures that the method can reliably quantify different concentrations of an analyte, while accuracy confirms that those quantifications reflect the true values. Linearity was evaluated by plotting calibration curves of peak area versus

concentration of piperacillin and tazobactam solution. Calibration curves were obtained at five different concentrations (5, 10, 20, 50 and 100 µg/mL) that were analysed in triplicate. The correlation coefficient (R^2) was calculated for each curve and $R^2 \geq 0.99$ is generally considered acceptable. Calibration was performed for the standard solution, spiked ultrafiltrate and spiked plasma samples. The calibration curve of the standard solution in Fig. 5A showed good linearity with R^2 of 0.995. Similarly, the ultrafiltrate calibration curve in Fig. 5B showed good linearity with R^2 of 0.995. However, the plasma calibration curve (Fig. 5C) showed slightly lower linearity of R^2 which is 0.893. For both the standard solution and ultrafiltrate calibration curves, a high correlation coefficient is observed. This indicates that the method provides good linearity over the concentration range and can reliably quantify piperacillin at different concentration levels in standard and ultrafiltrate samples.

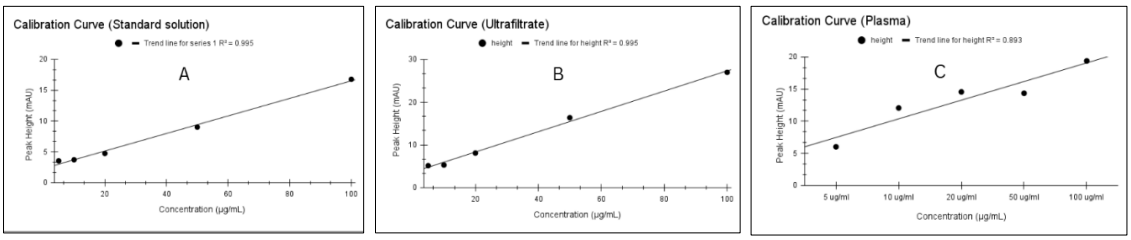

Fig 5. Calibration curve of standard solution (A), ultrafiltrate (B) and plasma (C).

However, the slightly lower R^2 value in plasma calibration curve indicates that the matrix component in plasma may have affected the response of the detector and peak area consistency. Biological matrices like plasma contain proteins and endogenous compounds that may interfere with analyte detection and may cause slight peak broadening, which leads to lower linearity. This study demonstrated that the previously optimized HPLC method is suitable for the quantification of piperacillin in ultrafiltrate with good linearity, precision, and specificity. For plasma quantification, an improved method based on the literature (20) was used to obtain better results for this partial validation. However, the method showed several limitations. First, the detection of tazobactam in biological matrices, especially at lower concentrations, remains challenging due to possible peak overlap near the expected analyte's retention time, indicating that further optimisation is needed. In addition, this study was only conducted on selected validation parameters, which are linearity, precision and specificity and did not include the other aspects that may strengthen the method's application in therapeutic monitoring. Lastly, further optimisation is recommended for the plasma method as the R^2 value for piperacillin in plasma samples was slightly lower than expected, suggesting that critical factors such as pH and mobile phase composition may need adjustment to achieve more consistent linearity and detection. In addition, further full validation is recommended according to the complete guideline of ICH Q2(R1) to confirm the method's reliability for applications in therapeutic drug monitoring.

4. DISCUSSION

This study aimed to improve and apply an RP-HPLC method for the quantification of piperacillin and tazobactam in the ultrafiltrate and plasma, which are important matrices for pharmacokinetic evaluation and therapeutic drug monitoring (TDM). The strong linearity observed in the standard solution and ultrafiltrate ($R^2 = 0.995$) indicates that the chromatographic conditions were appropriate for accurate

quantification. Similar findings were reported by Abdelkawy et al. (2023), demonstrated excellent linearity for piperacillin in plasma samples using HPLC-UV. The reduced linearity observed in the plasma ($R^2 = 0.893$) may be attributed to matrix effects. Biological matrices such as plasma contain proteins, phospholipids, and endogenous substances that can influence analyte recovery and detector response. Matrix-related variability has been widely reported in bioanalytical studies (An et al., 2020). Although protein precipitation was performed, residual matrix components may have contributed to peak variability. The excellent precision (%RSD < 2%) confirms that the method provides consistent retention times and peak responses, in agreement with the ICH Q2(R1) validation requirements (ICH, 2005). These findings suggest that the method is reproducible and suitable for routine quantification, particularly in the ultrafiltrate.

The ultrafiltrate represents the unbound fraction of piperacillin, which is pharmacologically active. Monitoring free β-lactam concentrations is clinically important because antibacterial efficacy is associated with the time that the free drug concentration remains above the minimum inhibitory concentration. Therefore, the demonstrated suitability of this method in the ultrafiltrate supports its potential application in therapeutic drug monitoring. However, the detection of tazobactam at lower concentrations remains challenging. Previous studies have shown that chromatographic optimisation, including adjustment of the mobile phase composition and pH, can significantly improve peak resolution (Abdelkawy et al., 2023). Further optimisation or advanced extraction techniques, such as solid-phase extraction, may enhance sensitivity and reduce matrix interference.

Overall, the findings indicate that the improved HPLC method is suitable for quantifying piperacillin in the ultrafiltrate, while analysis in the plasma still requires further optimisation. Comprehensive validation in accordance with International Counsil on Harmonisation guidelines, particularly ICH Q2(R1), is recommended prior to clinical application. It should also be noted that not all validation parameters outlined in ICH Q2(R1), specifically the limits of detection (LOD) and quantitation (LOQ), were evaluated in this study. Future work should include these parameters to ensure a more complete validation and to further confirm the method's reliability for therapeutic drug monitoring applications.

5. CONCLUSION

In conclusion, this study evaluated a previously reported HPLC method for the determination of piperacillin and tazobactam in standard solutions, ultrafiltrate, and plasma samples. The method demonstrated satisfactory repeatability in standard solutions and ultrafiltrate but failed to achieve acceptable performance in plasma, likely due to matrix effects and protein-binding interactions. Consequently, an independently validated method was adopted for plasma quantification to ensure accuracy and reliability. A key limitation of this study is the limited assessment of matrix complexity and the lack of sample preparation procedures specifically optimised for plasma analysis. The use of non-certified reference materials may have further constrained analytical traceability. Future studies should incorporate certified reference materials (CRM), standard reference materials (SRM), or pharmacopeial standards to enhance reliability. In addition, improved sample preparation, particularly through optimised protein precipitation or extraction techniques, may reduce matrix interference. Full validation, including forced degradation studies, stability assessments under varied conditions, and cross-platform evaluation across different chromatographic systems or

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detectors, would further strengthen method robustness and applicability. Furthermore, additional validation parameters recommended by the ICH Q2(R1), with LOD and LOQ, should be included in future work to achieve a more comprehensive evaluation (ICH, 2005). Overall, this study highlights the necessity of matrix-specific validation when extending HPLC methods to complex biological samples and provides a practical reference for future analytical method development.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

AUTHORS' CONTRIBUTIONS

Nur Miza Hanisah Hairil Nahar: Conceptualization, literature review, data collection, analysis, and preparation of the original draft. **Hannis Fadzilah Mohsin**: Supervision, conceptual guidance, methodological advice, review & editing of manuscript. **Ibtisam Abdul Wahab**: Methodological support, technical guidance, and manuscript review.

DECLARATION OF GENERATIVE AI IN THE WRITING PROCESS

During the preparation of this work, the authors used ChatGPT (OpenAI) and Grammarly to improve language, grammar, and readability. All content was reviewed and edited by the authors, who take full responsibility for the final manuscript.

ETHICS STATEMENT

Ethical approval was not required for this study as it did not involve human subjects, animal experimentation, or collection of personal/confidential data.

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