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Research Article

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Novel Gradient Chromatographic Method for Simultaneous Therapeutic Drug Monitoring of Cefepime, Vancomycin and Levofloxacin in Human Plasma for Hospital Acquired Pneumonia Patients

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Abstract

A novel gradient HPLC method has been developed for rapid and simultaneous determination of three antibiotics, cefepime, vancomycin and levofloxacin in human plasma. The use of these antibiotics in combination as empirical antibiotics for the treatment of hospital acquired pneumonia creates the need to develop a method for monitoring the serum level of vancomycin in the concomitant presence of these antibiotics. Separation was carried out on a GmbH® Reprosil BDS C18 column (5 µm, 150 x 4.60 mm) using a gradient mobile phase of EtOH: 0.025M KH2PO4 adjusted to pH 3.50 using ortho - phosphoric acid in the following sequence, (0: 100, v/v) after 6 min (30: 70, v/v) then after 2 min (0: 100, v/v) at ambient temperature. The flow rate was 1 mL/min and maximum absorption was measured at 220 nm. The retention time of cefepime, vancomycin and levofloxacin was noted to be 2.90 ± 0.04, 3.53± 0.02 and 5.42 ± 0.05 minutes respectively, indicating a very short analysis time. Also, limits of detection were reported to be 0.19, 0.17 and 0.21 µg/mL for cefepime, vancomycin and levofloxacin, respectively, showing a high degree of the method sensitivity. The method was then validated according to FDA guidelines where it was found to be accurate, reproducible and robust.

Keywords: Gradient HPLC, Cefepime, Vancomycin, Levofloxacin, Hospital Acquired Pneumonia

Introduction

Hospital-acquired pneumonia (HAP) is an acute lower respiratory tract infection that is by definition acquired after at least 48 hours of admission to hospital and is not incubating at the time of admission. Broad-spectrum antimicrobials should be used initially for patients with risk factors for multi-drug-resistant pathogens. Recommended initial empiric antibiotic therapy for HAP during the prior 90 days is I.V. administration of two antipseudomonal antibiotics with different mechanisms plus antibiotic with activity against methicillin-resistant Staphylococcus aureus (MRSA) as vancomycin for which therapeutic drug monitoring is highly recommended [1]. Although beta-lactams and quinolones are not considered strong candidates for therapeutic drug monitoring (TDM), the failure of antibiotic treatment and the development of resistant strains of bacteria may occur in patients with sub therapeutic concentrations. The determination of the antibiotic plasma concentrations beside the knowledge of the bacterial susceptibility expressed in the terms of the MIC, would enable effective treatment specially against resistant strains limiting the risk of insufficient antibiotic exposure [2, 3]. Also TDM will prevent dose-related adverse effects of beta lactams mainly neurotoxicity symptoms, including seizures and encephalopathy in patients who need high doses [4, 5]. Therefore, in our paper, we developed a method for the simultaneous determination of the plasma drug concentration of cefepime, vancomycin and levofloxacin for its simulation with that of patients with HAP.

Vancomycin (VAN) (Figure 1) is a glycopeptide antibiotic used against infections caused by gram-positive bacteria, specially MRSA. It mainly acts by prevention of cell-wall biosynthesis of bacteria [6]. Our literature survey verified that determination of VAN has been carried out using HPLC in tablets, in human plasma, UPLC or through using LC-MS [7-19].

Cefepime (CEF) (Figure 1) is a broad-spectrum fourth-generation cephalosporin antibiotic with greater activity against both gram-negative and gram-positive organisms than third-generation

agents. It is used for treatment of pneumonia (moderate to severe) caused by Streptococcus pneumoniae, including cases associated with concurrent bacteremia, Pseudomonas aeruginosa, Klebsiella pneumoniae, or Enterobacter species [20]. Its chemical name is (6R,7R)-7-[[(2Z)-2-(2-amino-1,3-thiazol-4-yl)-2-methoxyiminoacetyl]amino]-3-[(1-methylpyrrolidin-1ium-1-yl)methyl] 8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate [20]. Various methods have been reported for analysis of CEF including HPLC in pharmaceutical preparations, HPLC in biological samples, HPTLC, and LC-MS [21-29].

Levofloxacin (LEV) (Figure 1) is a third-generation fluoroquinolone with an activity against gram-positive and gram-negative bacteria. It inhibits DNA gyrase, type II topoisomerase, and topoisomerase IV enzymes essential to separate replicated DNA and block cell division [30]. The chemical name of LEV is (-)-(S)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H pyrido[1,2,3-de]-1,4benzoxazine-6-carboxylic acid hemihydrates. The determination of LEV has been carried out using variant techniques but specifically through HPLC in pharmaceutical preparations and in biological samples, and LC-MS [31-39].

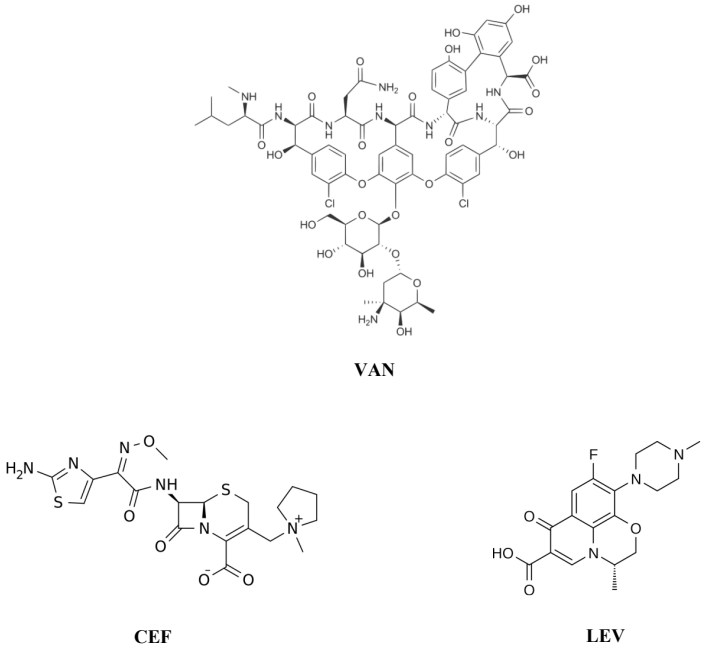

Figure 1: Chemical Structures of Vancomycin (VAN), Cefepime (CEF) and Levofloxacin (LEV).

To the best of our knowledge, this is the first HPLC method that deals with the simultaneous separation and determination of the three antibiotics, CEF, VAN and LEV in human plasma despite their synergistic action. As such, the present chromatographic method is supposed to be introduces a simple, very rapid, reproducible and sensitive method for the determination of the three drugs in human plasma.

Experimental

Apparatus

Acquity UPLC instrument with GmbH® Reprosil BDS C18 column (5 µm, 150 x 4.60 mm), TUV detector, sample manager-FTN, Quaternary solvent manager and connected to a PC computer loaded with Acquity Software.

Materials and Reagents

All solvents and reagents were HPLC analytical grade including ethanol and acetonitrile (POSH S.A., Poland), potassium dihydrogen phosphate and ortho-phosphoric acid (Merck, USA). Cefepime (Smithkline Beecham ((GSK)), Egypt), vancomycin (ONE PHARMA MEDICS MYLAN, Egypt) and levofloxacin (Amoun, Egypt) standard solutions of 200 µg/mL were prepared by dissolving 0.01 mg of each pure drug in 50 ml of the mobile phase. Mobile phase was a freshly prepared of EtOH and mixture of 95% (0.025M potassium dihydrogen phosphate adjusted to pH 3.50 using ortho-phosphoric) : 5% acetonitrile, filtered and degassed using 0.45 µm membrane filter. The human plasma was kindly provided by Zagazig University Hospital and was tested to be drug and disease-free. The plasma was kept frozen before use and was then stored either at −4 °C between uses or at −20 °C for freeze–thaw cycle stability studies.

Procedures

Preparation of Standard Calibration Curves

Appropriate mixed dilutions of CEF, VAN and LEV standard stock solutions were done in 10 mL volumetric flasks to get final concentrations of 0.78125, 1.5625, 3.125, 6.25, 12.5, 25, 50 and 100 µg/mL for cited drugs. A 10 µL of each mixture was then injected into the column, and the chromatogram was obtained at 220 nm. A graph was plotted as concentration of drugs against response (peak area). Regarding validated quality control (QC) samples, concentrations of 2.50, 10, and 20 µg/mL were selected as low (LQC), medium (MQC), and high (HQC) levels, respectively.

Human Plasma Samples Preparation

Calibration curve and validated QC samples at concentrations of 2.50, 10, and 15 µg/mL in plasma were prepared. Aliquots of 200 µL plasma samples and different drug mixture volumes ranging from 100 up to 200 µL were added into 10 mL centrifuge tubes and vortexed for 1 min. The mixture was then precipitated with methanol (total volume: 2 mL). After vortexing for 1 min, the samples were centrifuged at 5000 rpm for 15 min. Aliquots of 10 µL of the supernatant was filtered through 0.45-µm PTFE syring filters (Membrane Solutions, USA) and injected directly into the HPLC system for analysis.

Results and Discussion

Optimization of Chromatographic Conditions

All chromatographic conditions are illustrated in Table 1. Spectroscopic analysis of the three drugs in the range of 200–400 nm showed that CEF and VAN have UV absorbance maxima (λmax) at 235, 280 nm, respectively, while LEV has two absorbance maxima at 227 and 292 nm as depicted in Figure 2. It was necessary to undergo an additional full scan on the Acquity Software to determine which wavelength range is the most appropriate for the simultaneous determination of the drug mixture. As depicted in Figure 3, three wavelength ranges of 220, 254 and 280 nm were used for the trials scan and it was found that 220 nm achieved the best area under peak appearance and value for the three drugs. Therefore, the chromatographic detection was performed at 220

nm as the appropriate wavelength using a TUV detector. The method was performed on a GmbH® Reprosil BDS C18 column (5 µm, 150 x 4.60 mm). Furthermore, under several trials of mobile phase optimization regarding its composition ratio and pH, it was observed that the optimized mobile phase was determined as a gradient mixture of EtOH: 0.025M KH2PO4 adjusted to pH 3.50 using ortho - phosphoric acid in the following sequence, (0: 100, v/v) after 6 min (30 : 70, v/v) then after 2 min (0 : 100, v/v) at ambient temperature with a flow rate 1 mL/min. Under these conditions, CEF, VAN and LEV can be separated and eluted at 2.90 ± 0.04, 3.53± 0.02 and 5.42 ± 0.05 minutes, respectively as illustrated in Figure 3A. Also, the mixture determination in plasma didn't show the matrix interference effect, as the human plasma was eluted separately at 1.29 minute in correspondence with the migration times of the three drugs (Figure 4). However, in all cases, the optimum mobile phase showed symmetrical peaks ($0.85 < T < 1.20$), capacity factor < 10, resolution > 2, and theoretical plates > 2000 which are in agreement with the CDER values recommendation [40]. Table 2 shows all system suitability parameters of the proposed RP-HPLC method for simultaneous determination of the three drugs in both pure and plasma samples.

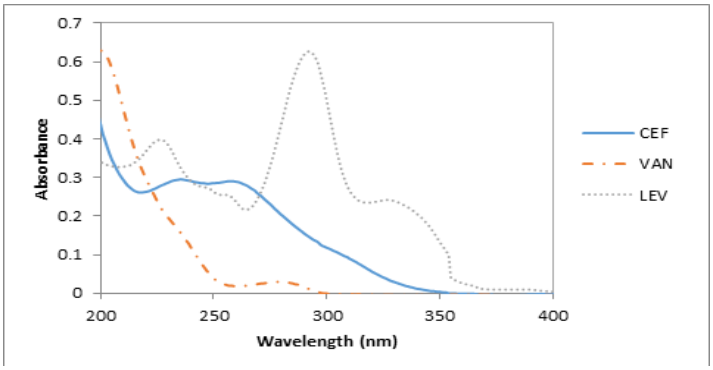

Figure 2: Overlain Spectra of 1 µg/ml Cefepime (CEF___), Vancomycin (VAN-.-.-) and Levofloxacin (LEV.....) at Maximum Wavelengths of 235, 280 and 227 & 292 nm, Respectively.

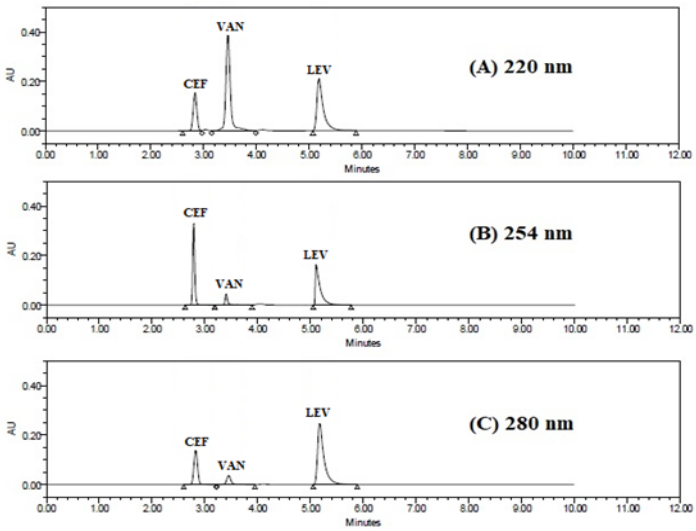

Figure 3: HPLC Chromatogram of 12.50 µg/mL mixture of cefepime (CEF), vancomycin (VAN) and levofloxacin (LEV) using GmbH® Reprosil BDS C18 column (5 µm, 150 x 4.60 mm) column and a gradient mobile phase of EtOH : 0.025 M KH2PO4 adjusted to pH 3.50 using ortho - phosphoric acid (0 : 100, v/v) after 6 min (30 : 70, v/v) then after 2 min (0 : 100, v/v) at different wavelengths (A) 220 nm, (B) 254 nm and (C) 280 nm. Other chromatographic conditions are stated in Table 1.

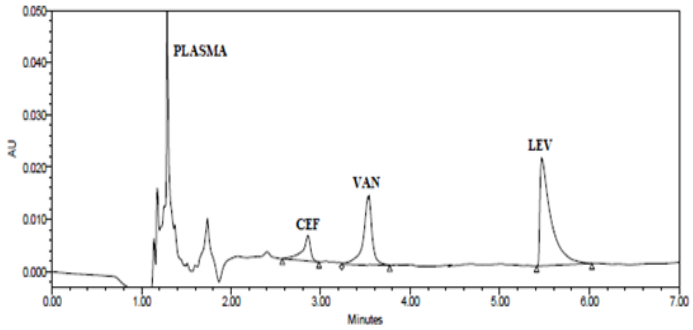

Figure 4: HPLC Chromatogram of 2.50 µg/mL Mixture of Cefepime (CEF), Vancomycin (VAN) and Levofloxacin (LEV) Spiked in Plasma Using the Same Conditions in Figure 3.

Table 1: Chromatographic Conditions for the Proposed Method.

Parameters	Conditions
Column	GmbH® Reprosil BDS C18 column (5 µm, 150 x 4.60 mm)
Mobile phase	Gradient, EtOH : 0.025 M KH_2PO_4 adjusted to pH 3.50 using ortho - phosphoric acid (0 : 100, v/v) after 6 min (30 : 70,v/v) then after 2 min (0 : 100,v/v)
UV detection, nm	220
Flow rate, mL/min	1
Injected volume, µL	10
Temperature, °C	Ambient 25

Table 2: System Suitability Parameters Comparison for Cefepime (CEF), Vancomycin (VAN) and Levofloxacin (LEV) in Both Pure and Plasma Samples.

Parameters	Pure sample			Plasma sample			Reference values [40]
	CEF	VAN	LEV	CEF	VAN	LEV	
Capacity factor, k'	1.25	1.72	3.10	1.19	1.71	3.16	Accepted k' value (1-10)
Peak asymmetry (Tailing factor, T)	0.99	1.10	1.19	0.85	0.93	1.20	Accepted T value $\leq$ 2
Therotical plates, N	4512	6216	8627	3815	5548	7660	Accepted N value > 2000
Resolution, Rs	---	3.43	8.74	---	3.63	8.78	Accepted value > 2
Selectivity (Separation factor, α)	---	1.37	1.80	---	1.43	1.84	

Method Validation

The method was validated according to the Food and Drug Administration guidelines for bioanalytical methods validation [41, 42].

Linearity

Different six concentrations of the drug mixture were specified for linearity studies. The calibration curves obtained by plotting peak area against concentration showed linearity in the concentration range of 0.781-25 µg/mL for CEF, 3.125-100 µg/mL for VAN and 1.562-50 µg/mL for LEV (Table 3). Also, Linear regression equations for CEF, VAN and LEV were found to be y = 3639.6 x + 2984.6, y = 11456 x - 8134.2, and y = 7890.1 x - 6485.2, respectively, while the regression coefficient values (r) were 0.9999 for the three drugs, indicating a high degree of linearity (Figure 5).

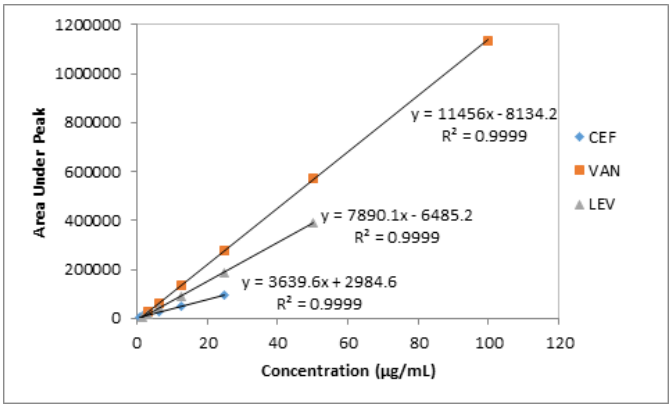

Figure 5.: Calibration Curves for Authentic Mixture of Cefepime (CEF), Vancomycin (VAN) and Levofloxacin (LEV) using the Proposed HPLC Method.

Table 3: Results of Analysis for Cefepime (CEF), Vancomycin (VAN) and Levofloxacin (LEV) in Pure Form Using the Proposed Method.

Parameters	CEF				VAN				LEV			
	Taken µg/mL	Found µg/mL	Recovery %	Accuracy %	Taken µg/mL	Found µg/mL	Recovery %	Accuracy %	Taken µg/mL	Found µg/mL	Recovery %	Accuracy %
	0.781	0.792	101.45	1.45	**3.125**	3.15	101.02	1.02	**1.562**	1.53	98.12	-1.87
	1.562	1.584	101.42	1.42	**6.25**	6.23	99.71	-0.28	**3.125**	3.07	98.47	-1.52
	3.125	3.179	101.75	1.75	**12.50**	12.73	101.91	1.91	**6.25**	6.15	98.44	-1.55
	6.25	6.177	98.84	-1.15	**25**	24.66	98.65	-1.34	**12.50**	12.48	99.87	-0.12
	12.50	12.36	98.89	-1.10	**50**	50.28	100.56	0.56	**25**	25.09	100.37	0.37
	25	25.08	100.33	0.33	**100**	99.46	99.46	-0.53	**50**	50.95	101.90	1.90
Mean			**100.44**	**0.44**			**100.22**	**0.20**			**99.53**	**-0.46**
±SD			1.31				1.17				1.46	
±RSD			1.31				1.17				1.47	
±SE			0.53				0.47				0.59	
Variance			1.73				1.37				2.14	
LOD (µg/mL)			0.19				0.17				0.21	
LOQ (µg/mL)			0.63				0.56				0.70	

Accuracy and Precision

The accuracy of the method was determined by investigating the recoveries of CEF, VAN and LEV at concentration levels covering the specified range (3 replicates of each concentration) either in pure form or in spiked plasma. From the amount of the drug estimated, the percentage recovery was calculated, and the results shown in Tables 3 and 4 indicate excellent recoveries for all drugs.

The precision of the method was evaluated according to intra-day and inter-day precision using validated QC samples at concentrations of 2.5, 10, and 20 µg/mL in pure form. Intra-day precision was evaluated in terms of both standard deviation (SD) and coefficient of variation (CV%), regarding 3 replicate determinations using the same solution containing pure drugs at the first day of analysis. The SD and CV% values (varied from 0.29 to 2.19) revealed the high precision of the method. For inter-day reproducibility, the day-to-day SD and CV% values were also in the acceptable range of 0.32–1.35. These results show that the proposed method has an adequate precision in simultaneous determination of the three drugs (Table 5).

Table 4: Result of Analysis of Spiked Cefepime (CEF), Vancomycin (VAN) and Levofloxacin (LEV) in Plasma Samples Using the Proposed HPLC Method.

Parameters	CEF				VAN				LEV			
	Taken µg/mL	Found µg/mL	Recovery %	Accuracy %	Taken µg/mL	Found µg/mL	Recovery %	Accuracy %	Taken µg/mL	Found µg/mL	Recovery %	Accuracy %
	1	1.01	101.39	1.39	**1**	1.01	101.41	1.41	**2.50**	2.53	101.39	1.39
	2.50	2.49	99.77	-0.23	**2.50**	2.50	100.35	0.35	**5**	4.97	99.47	-0.53
	5	4.91	98.29	-1.71	**5**	4.90	98.19	-1.81	**10**	10.11	101.13	1.13
	10	9.80	98.07	-1.93	**10**	10.05	100.54	0.54	**15**	15.20	101.42	1.42
	15	14.75	98.38	-1.62	**15**	15.19	101.32	1.32	**20**	20.28	101.41	1.41
Mean			**99.31**	**-0.82**			**100.62**	**0.36**			**100.96**	**0.96**
±SD			1.29				1.32				0.84	
±RSD			1.30				1.32				0.83	
±SE			0.58				0.59				0.37	
Variance			1.68				1.76				1.076	

Table 5: Intra- & Inter-Day Precision and Stability Results Using 3 Quality Control Samples of Cefepime (CEF), Vancomycin (VAN) and Levofloxacin (LEV) Using the Proposed Method.

	Drugs	Concentrations (µg/mL)	Found concentration	Mean ± SD	CV (%)	Accuracy %
Intra-day runs (n=3)	CEF	2.50 10 20	2.48 10.06 20.05	99.40 ± 1.03 100.68 ± 1.40 100.27 ± 1.67	1.03 1.39 1.67	-0.59 0.68 0.28
	VAN	2.50 10 20	2.502 10.06 20.06	100.18 ± 1.20 100.64 ± 0.89 100.33 ± 0.29	1.19 0.89 0.29	0.18 0.64 0.33
	LEV	2.50 10 20	2.503 10.05 19.97	100.49 ± 0.60 100.54 ± 2.19 99.81 ± 1.14	0.60 2.18 1.14	0.49 0.55 -0.19
Inter-day runs (n=3)	CEF	2.50 10 20	2.502 10.01 19.82	100.18 ± 1.18 100.22 ± 1.35 99.13 ± 1.05	1.18 1.34 1.05	0.19 0.22 -0.86
	VAN	2.50 10 20	2.52 9.91 20.05	101.04 ± 0.97 99.18 ± 1.29 100.28 ± 1.27	0.96 1.30 1.27	1.05 -0.82 0.28
	LEV	2.50 10 20	2.501 10.13 19.92	100.06 ± 1.25 101.44 ± 0.32 99.61 ± 1.32	1.25 0.32 1.32	0.07 1.44 -0.38
Freeze thaw cycle	CEF	2.50 10 15	2.503 10.05 15.09	100.35 ± 1.52 100.53 ± 1.07 100.66 ± 1.15	1.52 1.07 1.14	0.35 0.55 0.66
	VAN	2.50 10 15	2.49 9.96 15.20	99.61 ± 0.97 99.66 ± 1.13 101.37 ± 0.63	0.98 1.13 0.62	-0.37 -0.32 1.37
		2.50 10 15	2.502 9.91 15.05	100.29 ± 1.69 99.12 ± 1.25 100.35 ± 1.98	1.68 1.26 1.97	0.29 -0.87 0.36

Selectivity and Specificity

The selectivity of the method was checked by injecting the solutions of CEF, VAN and LEV into the column separately, where 3 sharp peaks were obtained at retention times of 2.90 ± 0.04, 3.53± 0.02 and 5.42 ± 0.05 minutes, respectively, and these peaks were not obtained for the blank solution. Also, the specificity studies revealed that the presence of human plasma didn't show any kind of interference with the sharp and well-resolved peaks of the three drugs (Figure 4).

Robustness

The robustness of the methods was evaluated by making deliberate subtle changes (± 0.05) in the flow rate, mobile phase composition ratio and temperature keeping the other chromatographic conditions constant. The changes effect was studied on the basis of percent recovery and standard deviation of all drugs. Table 6 shows that the changes had negligible influences on the results as revealed by small SD values (≤ 1.52).

Table 6: Results of the Robustness for the Determination of 12.50 µg/mL Cefepime (CEF), Vancomycin (VAN) and Levofloxacin (LEV) Using the Proposed Method.

	CEF			VAN			LEV		
	Mean recovery ± SD	CV (%)	Accuracy %	Mean recovery ± SD	CV (%)	Accuracy %	Mean recovery ± SD	CV (%)	Accuracy %
Flow rate 1.45 mL (- 0.05)	100.72 ± 1.06	1.05	0.72	100.19 ± 1.03	1.03	0.19	99.54 ± 0.74	0.75	-0.46
Flow rate 1.55 mL (+ 0.05)	100.33 ± 1.05	1.05	0.33	100.36 ± 1.29	1.29	0.36	99.97 ± 0.44	0.44	-0.03
EtOH : Buffer 30.5 : 69.5	100.73 ± 1.06	1.05	0.73	100.26 ± 1.13	1.13	0.26	100.30 ± 0.96	0.96	0.30
EtOH : Buffer 29.5 : 70.5	100.87 ± 1.20	1.19	0.87	100.28 ± 1.17	1.17	0.28	99.52 ± 0.77	0.77	-0.48
Temp 24.5 °C (- 0.05)	100.85 ± 1.17	1.16	0.85	100.18 ± 1.02	1.02	0.18	100.28 ± 0.92	0.92	0.28
Temp 25.5 °C (+ 0.05)	99.94 ± 1.52	1.52	-0.06	99.92 ± 0.79	0.79	-0.08	99.56 ± 0.70	0.70	-0.44

Limits of Detection (LOD) and Limits of Quantification(LOQ)

For determining the LOD and LOQ, the method based on the signal-to-noise ratio (3:1 for LOD & 10:1 for LOQ) was adopted. LODs were reported to be 0.19, 0.17 and 0.21 µg/mL, while LOQs were calculated to be 0.63, 0.56 and 0.70 µg/mL for CEF, VAN and LEV, respectively (Table 3). These results show that the proposed method is highly sensitive and applicable for pharmacokinetic and bioequivalence studies, where detection of small concentrations in plasma is required.

Analysis of Human Plasma

The proposed method was also applied for determination of CEF, VAN and LEV in human plasma samples by applying protein precipitation method. Retention times of CEF, VAN and LEV in plasma sample and the other system suitability parameters were also pretty similar to those of pure one (Table 2).

Also, the plasma chromatogram (Figure 4) confirms the specificity of the method in clinical studies as the plasma peak (eluting at 1.29 minute) is not interfering but well separated from the other three peaks of cited drugs. Calibration curves of the spiked plasma were also found to be linear over the clinical range of 1–15 µg/mL for CEF and VAN and 2.50-20 µg/mL for LEV. The mean recoveries for drugs mixture in plasma were in the acceptable range of 99.31, 100.62 and 100.96 % for CEF, VAN and LEV, respectively, according to the FDA guidelines (Table 4).

Furthermore, stability studies were conducted by applying plasma freeze–thaw cycles at −20 °C (over three days) using the validation QC samples of concentrations 2.50, 10 and 15 µg/mL in plasma, and the results are summarized in Table 5. The SD and CV% values (varied from 0.62 to 1.97) show that the spiked drug plasma samples are highly stable, indicating the high suitability of the method in pharmacokinetic studies regarding the three drug mixture in biological samples.

Conclusion

The presented method was developed and validated for rapid simultaneous estimation of cefepime, vancomycin, and levofloxacin within 6 min. The results obtained indicated that the proposed method is rapid, accurate, selective, robust, and reproducible. This analytical method can be also adequate and useful for the clinical estimation of the three drugs in human plasma samples of Acquired Pneumonia Patients according to the FDA guidelines in respect of pharmacokinetic and bioequivalence studies that would be useful in therapeutic drug monitoring.

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Conflict of interest

The authors declare that there is no conflict of interest in the manuscript.

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