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Validation of An HPLC Method for The Determination of Some B-Lactams Antibiotics Using A Green Analytical Technique

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ABSTRACT

Green analytical chemistry (GAC) primary goal is to develop environmental and health-friendly analytical methods by reduction or replacement of hazardous substances from analytical procedures. In this research, the application of GAC to quantify five β-lactams antibiotics (cefuroxime sodium, cefaclor, ertapenem sodium, ampicillin sodium and sulbactam sodium) by high-performance liquid chromatography (HPLC) using photodiode arrays (PDA) detector was developed. To achieve this, ethanol was used as a green replacement for methanol and acetonitrile, common solvents in chromatographic procedures. Several chromatographic conditions were investigated to accomplish the optimal conditions. The method validation followed the ICH Q2 (R1) guideline, and the developed method was successfully applied for quantifying some pharmaceutical products.

Keywords: Ethanol; Green analytical chemistry; HPLC-PDA; β-lactams antibiotics

INTRODUCTION

β-lactam antibiotics play an essential role in antibacterial treatments. Based on the molecular structures, β-lactam antibiotics can be categorized into subgroups: penicillin, cephalosporins, carbapenem, and monobactam. The β-lactam antibiotics inhibit the activity of penicillin-binding proteins and suspend the cell walls synthesis process, leading to cell lysis (Lima *et al.*, 2020).

Cefuroxime sodium (CFRX) and cefaclor (CFC) are second-generation cephalosporins commonly used in healthcare centers worldwide. Cefuroxime sodium is prescribed for the treatment of various infections: respiratory tract, urinary tract, soft tissue, bone and joint tissues, and central nervous system (Barbour *et al.,* 2009; Fiocchi *et al.*, 2010; G M Salzmann *et al.,* 2007; Vazquez *et al.*, 2011). Cefaclor's clinical prescriptions include otitis media, acute sinusitis, upper and lower respiratory tract infections, pneumonia, and skin infections (Seung-Hyun Jeong *et al.*, 2021).

Ertapenem sodium (ETP) is a carbapenem antibiotic, mainly prescribed for abdominal infections, acute gynecological infections, community-acquired pneumonia, foot infections in diabetes patients, and prophylaxis for colorectal surgery. Unlike the other carbapenems, ertapenem is inactive against *Pseudomonas* or *Acinetobacter* spp. (Joint Formulary Committee, 2022).

Ampicillin sodium (APC) is a penicillin antibiotic mainly prescribed for bronchitis, urinary tract infections, otitis media, sinusitis, uncomplicated community-acquired pneumonia, salmonellosis, and urinary tract infections. Sulbactam sodium (SUL) is a β-lactamase inhibitor, in combination with ampicillin sodium, to maintain the efficiency of the antibiotic (Joint Formulary Committee, 2022).

From a pharmaceutical quality control point of view, reverse-phase high-performance liquid chromatography (RP-HPLC) is the primary technique for the mentioned β-lactams in some Conventional methods and research articles (Briscoe *et al.*, 2012; United States Pharmacopeia Convention, 2020). RP-HPLC is suitable for separating polar, medium-polarity, and even some nonpolar analytes, making it the most popular HPLC mode. In RP-HPLC, the separation depends on the partition coefficients of the analytes between a hydrophobic stationary phase and a hydrophilic mobile phase. For the hydrophilic mobile phase, RP-HPLC typically uses different ratios of methanol (MeOH) or acetonitrile (ACN) with water (Dong, 2019).

Although MeOH and ACN have some remarkable chromatographic properties, such as good solubility in water, low viscosity in aqueous solutions, and low UV cut-off, there has been some

concern about these two solvents about environmental impact and health issues (Yabré *et al*., 2018). According to the International Conference on Harmonization (ICH), acetonitrile (ACN) and methanol (MeOH) are categorized as "Solvents to Be Limited" because these solvents are involved with some health and environmental problems (International Conference on Harmonization of Technical Requirements for registration of pharmaceuticals for human use, 2021). In the presence of enzyme alcohol dehydrogenase in the liver, methanol is converted to format and causes severe health hazards (Ghosh *et al*., 2018).

MeOH toxicity includes retinal edema, ocular lesions, loss of ganglion cells, demyelination of the temporal retina, and necrosis of cells. ACN toxicity includes bradycardia, tachycardia, hypotension, cardiac arrhythmia, cardiac arrest, and death (Joshi & Adhikari, 2019). ACN can be hydrolyzed by strong acid or strong base, which requires considering potential safety hazards (Wang *et al*., 2017).

Regarding these disadvantages of ACN and MeOH, green analytical chemistry (GAC) has received increasing recognition and confirmation (de la Guardia & Garrigues, 2020). One of the principles of GAC is "eliminate or replace toxic solvents" (Gałuszka *et al*., 2013). One strategy to follow this principle is using greener solvents as alternatives to toxic ones. Some solvents have been categorized as green: ethanol, isopropanol, n-propanol, acetone, ethyl acetate, ethyl lactate, and propylene carbonate. Among them, ethanol is the most popular alternative solvent in GAC research. EtOH is less toxic than ACN and MeOH. This solvent has a lower equilibrium vapor pressure, leading to less evaporation and lower exposure portions. When considering chromatographic behaviors as the frame of reference, EtOH is similar to ACN and MeOH (Yabré *et al*., 2018).

In reference to the vital role of β-lactam antibiotics in antibacterial treatment, developing analytical procedures for quantifying CFRX, CFC, ETP, APC, and SUL is critical in pursuing therapeutic efficacy and patient medication safety. Considering the advantage of GAC, this research proposes a green method for the quantification of 5 β-lactam antibiotics by HPLC-PDA. This study investigated several chromatographic conditions to achieve optimal conditions. ICH Q2(R1) guideline (International Conference on Harmonization of Technical Requirements for registration of pharmaceuticals for human use, 2005) was applied to validate the developed method. Finally, the method was used to analyze pharmaceutical products to confirm the method's applicability.

MATERIALS AND METHODS

Chemicals and reagents

All of the studied β-lactam antibiotics reference standards were acquired from Sigma-Aldrich (USA). Cefuroxime sodium powder for injection, cefaclor capsule, ertapenem sodium powder for injection, and the combination of ampicillin sodium and sulbactam sodium in powder for injection were commercial formulations in the Vietnam market. Ammonium acetate and acetic acid were obtained from Merck (Germany). HPLC-grade ethanol was purchased from Scharlau (Spain). HPLC-grade water was prepared using Water Pro PS Polishing Systems (Labconco, USA).

Instruments

The analytical chromatography experiments were performed with a Waters Alliance e2695 separation module and a 2998 PDA Detector (Waters, USA). Elmasonic S100H (Elma, Germany) was used for degassing the mobile phase. The pH was estimated by an Orion Star A221 pH meter from Thermo Fisher (USA).

Preparation of Standard Solutions

Each one of the stock solutions was prepared weekly by solvating an appropriate amount of reference standard in water to obtain the concentration of 1000 µg/mL and stored in the refrigerator at 2˚C until use. To prepare standard solutions, dilute the stock solution in water to obtain the appropriate concentrations.

Preparation of pharmaceutical samples

CFRX powder for injection, CFC capsule powder, and ETP powder for injection (equivalent to 10 mg of each substance) were accurately measured and transferred into separate 100 mL volumetric flasks containing 70 mL of water and ultra-sonicated for 10 min; the 100 mL volume was made up using water, and properly homogenized. The powder for injection containing the combination of APC and SUL (equivalent to 10 mg of APC and 5 mg of SUL) was accurately measured and proceeded as above to prepare the samples.

HPLC initial conditions

The analysis was conducted on a Sunfire C18 (150 x 4.6 mm; 5 µm). The mobile phase was a mixture of ethanol and ammonium acetate buffer at

different pH levels and mobile phase ratios. The flow rate was maintained at 1.0 mL/min. The injection volume was 10 µL. The detection wavelength was 273, 265, and 294 nm for CFRX, CFC, and ETP, respectively, and 210 nm for the simultaneous detection of APC and SUL. Different conditions, such as the pH levels of the mobile phase, the buffer concentrations, the mobile phase ratios, and the column temperatures, were investigated.

Method validation

The developed method was validated based on the ICH Q2(R1) guideline (International Conference on Harmonization of Technical Requirements for registration of pharmaceuticals for human use, 2005).

System suitability

System suitability testing was conducted by data acquisition from six replicate injections of each standard solution. The relative standard deviation of retention time and peak area must be below 2% for each analyte. All peaks must show appropriate symmetry factors (0.8 – 1.2).

Specificity

The specificity of the method was evaluated by comparing the chromatograms of the blank sample, standard sample, and the pharmaceutical sample solution. The discrimination of the method can be confirmed by obtaining positive results when the pharmaceutical sample is compared with the reference solution, coupled with negative when compared with the blank. Furthermore, peak purity testing using the PDA detector must be performed.

Linearity

The linearity testing was conducted by describing the peak area as a function of analyte concentration by linear regression analysis within the appropriate range. Microsoft Office Excel was employed to calculate the statistical results.

Precision

The precision of an analytical method is evaluated based on repeatability and intermediate precision. For repeatability testing, six sample solutions of each surveyed β-lactams were prepared at the quantifying concentration and analyzed at the optimal concentrations on the same day. On the other hand, intermediate precision results are obtained by analyzing samples on different days (inter-day precision) or conducting them on different HPLC apparatuses (inter-apparatus precision). The precision results were expressed as relative standard deviation (RSD%).

Accuracy

The accuracy of an analytical method is evaluated by collecting assay data from samples at the quantifying concentration spiked with standard solutions at different levels (lower, medium, and upper concentration). The differences in peak areas between spiked and unspiked solutions are calculated as the description of the recovery (%).

Application of the method

The optimal chromatographic conditions were applied to quantify CFRX powder for injection, CFC capsule, ertapenem sodium powder for injection and the combination of APC and SUL in powder for injection in the Vietnam market. The preparations of 6 samples for each product were conducted as mentioned above. The content of each substance was calculated by the following formula:

$$\%\ \text{Substance} = \frac{M_w \times A_s}{M_0 \times A_t} \times 100$$

M_w=(g) is the amount of powders equivalent to 5 mg of SUL or 10 mg for the rest of the analytes. M_0= (g) is the amount of powders weighted in experiment; A_S= (mAU × s) is area of standard; A_t= (mAU × s) is area of sample.

RESULTS AND DISCUSSION

Method development for the quantification of CFRX, CFC and ETP

Different chromatographic conditions were investigated to study the effect of mobile phase-related factors and column temperature on chromatographic parameters such as retention time (tR), peak area (S), symmetry factor (As), and capacity factor (k') (Table I).

Effect of pH

The pH level of the mobile phase can affect the ionizing ability of analytes and, therefore, affects on retention time and other chromatographic behaviors. The mentioned β-lactam antibiotics were analyzed separately in different pH values (4.0, 5.0, and 6.0) to assess this effect. In the case of CFRX, increasing pH led to a significant decrease in retention time while maintaining the same symmetry factor. A similar effect of pH on the retention time of ETP was observed, with a slight effect on the symmetry factor. Conversely, the retention time of CFC rose significantly with the increase in pH.

After considering different pH values, 6.0 was chosen for CFRX and ETP, while 4.0 was chosen for CFC for further experiments.

Table I. Experimental results for the chromatographic investigation of cefuroxime sodium (CFRX), cefaclor (CFC), and ertapenem sodium (ETP). The retention time (tR), peak area (S), symmetry factor (As), USP Plate count (N), and K Prime (K') are represented for each analyte at each chromatographic condition.

Condition		Analyte	tR	S	As	N	K'
pH	pH 4	CFRX	20.233	2121296	1.27	11620	12.48
		CFC	10.668	1133849	0.982	11465	6.11
		ETP	24.261	406969	0.99	11488	15.1
	pH 5	CFRX	18.689	1978647	1.24	11400	11.5
		CFC	12.907	1232678	0.98	12254	7.604
		ETP	10.569	442216	1.03	9695	6.05
	pH 6	CFRX	17.155	2020706	1.24	11460	10.4
		CFC	16.945	1114116	1.01	12671	10.23
		ETP	7.705	428700	1.09	8734	4.14
Buffer concentration (mM/L)	15	CFRX	19.691	1693731	1.36	10790	12.12
		CFC	11.231	1109178	0.983	11556	6.49
		ETP	8.427	400656	1.12	8758	4.61
	25	CFRX	17.155	2020706	1.24	11460	10.4
		CFC	10.668	1133849	0.982	11465	6.11
		ETP	7.705	428700	1.09	8734	4.14
	35	CFRX	16.195	2036260	1.205	10860	9.8
		CFC	10.803	1103860	0.988	11451	6.2
		ETP	7.280	413159	1.09	7975	3.85
Ethanol – ammonium acetate ratio	3- 97	CFRX	33.19	45144	1.02	13800	21.14
		CFC	11.231	1109178	0.983	11556	6.49
		ETP	11.029	1215692	1.32	532	6.35
	6- 94	CFRX	17.155	2020706	1.24	11400	10.4
		CFC	24.79	1124343	0.961	13445	15.53
		ETP	7.705	428700	1.09	8734	4.14
	9- 91	CFRX	8.307	2082737	1.2	9070	4.54
		CFC	6.131	1146042	1.01	9663	3.09
		ETP	3.409	303524	1.11	6,566	1.27
Column temperature (°C)	25	CFRX	10.476	2073124	1.19	8042	5.98
		CFC	7.493	1143369	1.01	8458	3.99
		ETP	4.230	303491	1.09	5491	1.82
	30	CFRX	9.342	2063627	1.2	8587	5.23
		CFC	6.908	1129071	1.01	9078	3.61
		ETP	3.771	397654	1.1	6048	1.51
	35	CFRX	8.307	2082737	1.2	9070	4.54
		CFC	6.131	1146042	1.01	9663	3.09
		ETP	3.409	303524	1.11	6566	1.27

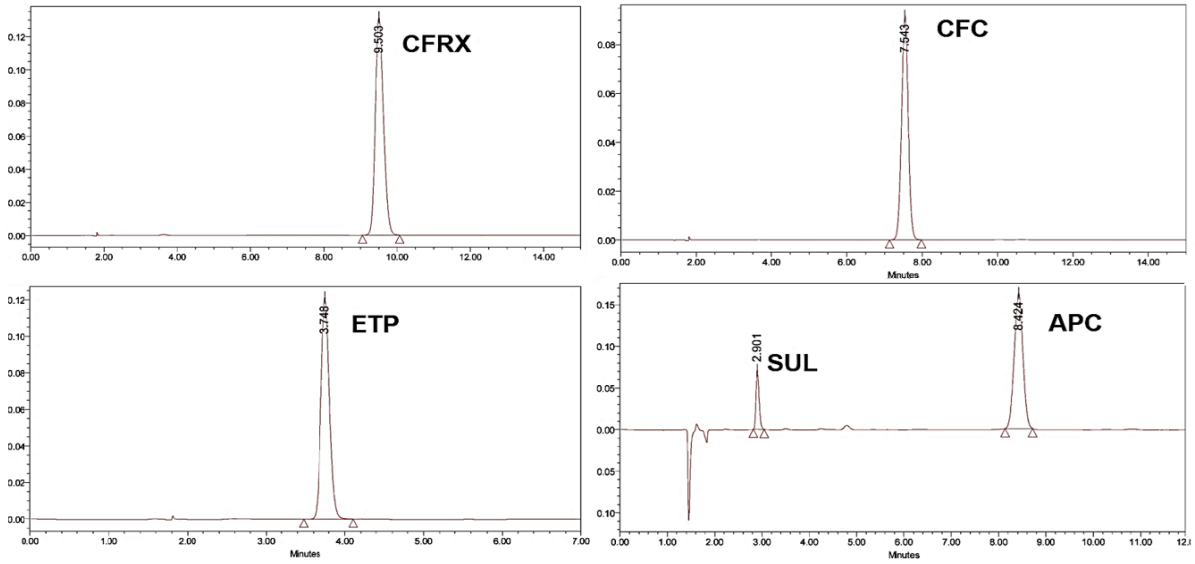

Figure I. Typical chromatograms of cefuroxime sodium (CFRX), cefaclor (CFC), ertapenem sodium (ETP), and the combination of ampicillin sodium (APC) and sulbactam sodium (SUL) at the optimal conditions

Effect of buffer concentration

Ammonium acetate buffer at different concentrations (15.0 mM, 25.0 mM, and 35.0 mM) was tested to find the appropriate condition. Generally, increasing the buffer concentration led to faster eluent of the analytes. However, higher buffer concentration can lead to precipitation in the column. Therefore, ammonium acetate 25 mM was chosen for CFRX and ETP, while 15 mM was chosen for CFC.

Effect of mobile phase ratio

As described in the material and method section, ethanol was used as an alternative to MeOH and ACN. The amount of EtOH investigated was kept under ten percent (3, 6, and 9%) to follow the principle of GAC. Generally, the higher amount of EtOH in the mobile phase led to a faster eluent due to its elution strength. Hence, 9% ethanol in the mobile phase was chosen for further experiments.

Effect of column temperature

Appropriately increasing the column temperature can shorten the eluent due to the reduction of the viscosity of the mobile phase, but the column efficiency and resolution will also decrease. After investigating column temperatures at 25, 30, and 35°C, the appropriate conditions were 30°C for CFRX and ETP and 25°C for CFC.

The results showed that the appropriated chromatographic conditions were: the Sunfire C18 (150 x 4.6 mm; 5 µm) at 30°C for CFRX and ETP, and 25°C for CFC, ethanol and ammonium acetate buffer (9:91, v/v) as eluent in isocratic mode at the flow rate of 1 ml per minute, pH level of the mobile phase was 6.0 for CFRX and ETP, and 4.0 for CFC, the concentration of ammonium acetate buffer was 25 mM/L for CFRX and ETP, and 15 mM/L for CFC, the injection volume of 10 µL, and the detection wavelength was 273, 265 and 294 nm for CFRX, CFC and ETP, respectively (Figure I).

Method development for the simultaneous detection of APC and SUL

As APC was formulated in combination with SUL to enhance the antibacterial effect, it is critical to develop an assay method for the simultaneous detection of APC and SUL. pH, buffer concentration, and column temperature had a slight effect on the retention time of both analytes (Table II). The mobile phase ratio significantly affected the retention time of both analytes, as the increase in EtOH content led to a decrease in retention. After considering the chromatographic results, the appropriate conditions were: the Sunfire C18 (150 x 4.6 mm; 5 µm) at 30°C, the mobile phase contained ethanol and ammonium acetate 25 mM/L (pH=5.0) (9:91, v/v), the flow rate of 1 mL per minute, the injection volume of 10 µL, and the detection wavelength of 210 nm. An exemplary signal chromatogram with the appropriate retention was observed (Figure I).

Table II. Experimental results for the simultaneous investigation of ampicillin sodium (APC) and sulbactam sodium (SUL). The retention time (tR), peak area (S), symmetry factor (As), USP Plate count (N), and K Prime (K') are represented for each analyte at each chromatographic condition.

Condition		Analyte	tR	S pic	As	N	Rs	K'
pH	pH 4	APC	12.673	22173	1.02	13090	35.3	7.45
		SUL	3.361	34888	1.15	8270		7.45
	pH 5	APC	14.549	887987	0.99	12600	35	8.70
		SUL	3.257	137180	1.14	11000		1.17
	pH 6	APC	21.601	876311	1.03	12990	43.01	13.4
		SUL	2.967	137242	1.03	11400		0.98
Buffer concentration (mM/L)	15	APC	13.513	907889	1.02	10781	31.7	8.01
		SUL	3.236	139546	1.24	9916		1.16
	25	APC	14.328	920673	1.02	12258	34.8	8.55
		SUL	3.257	131836	1.17	11273		1.17
	35	APC	14.038	835666	1.04	11649	34.3	8.36
		SUL	3.051	122362	1.14	10374		1.03
Ethanol – ammonium acetate ratio	3- 97	APC	No peak observed after 30 minutes of running time					
		SUL	4.906	144928	1.21	10972	-	2.27
	6- 94	APC	14.328	920673	1.02	12258	34.8	8.55
		SUL	3.257	131836	1.17	11273	34.8	1.17
	9- 91	APC	7.313	931976	1.04	9556	22.7	3.88
		SUL	2.695	147689	1.2	9348	22.7	0.8
Column temperature (°C)	25	APC	8.672	925898	1.02	8348	22.5	4.78
		SUL	2.968	147210	1.18	8115	22.5	0.98
	30	APC	7.961	936084	1.04	8854	22.6	4.31
		SUL	2.818	147514	1.18	8734	22.6	0.88
	35	APC	7.313	931976	1.04	9556	22.7	3.88
		SUL	2.695	147689	1.22	9348	22.7	0.79

Table III. System suitability results for the assay of cefuroxime sodium (CFRX), cefaclor (CFC), ertapenem sodium (ETP) and the combination of ampicillin sodium (APC) and sulbactam sodium (SUL)

Analyte	t_R (% RSD)	S (% RSD)	N	As
CFRX	0.135	1.329	8615	1.198
CFC	0.329	0.181	8454	1.01
ETP	0.097	0.211	5794	1.136
APC	0.072	0.600	9793	1.007
SUL	0.108	1.030	9089	1.275

Method validation

System suitability

System suitability testing was conducted to scrutinize the applicability and reproducibility of the chromatographic system. Relative standard deviations of retention time (tR) and area (S) were less than 2% for each analyte (Table III). The symmetry factors (As) were in range (0.8 – 1.2) for all peaks. Therefore, the proposed methods met the system suitability requirements guided by the literature.

Specificity

The specificity testing (Figure II) can be observed from the peak purity analysis that there are no coeluting peaks at the retention time of CFRX, CFC, ETP, APC, and SUL that interfere with the peaks of analytes. This result indicated that each analyte's peak was pure, which confirmed the method's specificity.

Linearity

The linear ranged from 0.01 to 0.25 mg/mL for SUL and 0.02 to 0.5 mg/mL for the rest of the analytes. Within the concentration range, the regression equations of each analyte have the appropriate correlation coefficient ($R^2 > 0.9995$). The ANOVA calculated for each analyte showed no linear deviation statistically ($p< 0,05$) since the F calculated was lower than F critical.

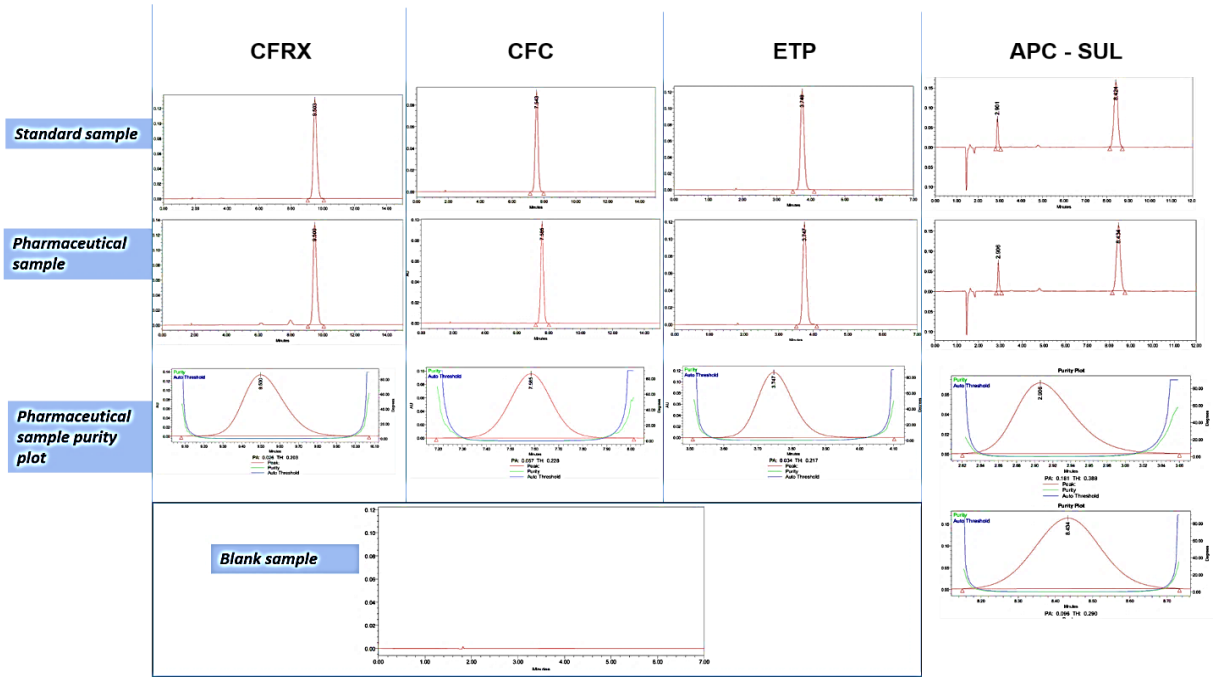

Figure II. Selectivity results were obtained by chromatographical comparison of the blank sample, the standard and the pharmaceutical samples of cefuroxime sodium (CFRX), cefaclor (CFC), ertapenem sodium (ETP), and the combination of ampicillin sodium (APC) and sulbactam sodium (SUL). Peak purity assessment of pharmaceutical samples was conducted using the PDA detector.

Table IV. Linearity assessment results of cefuroxime sodium (CFRX), cefaclor (CFC), ertapenem sodium (ETP), and the combination of ampicillin sodium (APC) and sulbactam sodium (SUL) at the chosen chromatographic conditions

Parameters	**CFRX**	**CFC**	**ETP**	**APC**	**SUL**
Range (mg/mL)	0.02 – 0.5	0.02 – 0.5	0.02 – 0.5	0.02 – 0.5	0.1 – 0.25
Regression equation	y=21354x + 22272	y =13032x – 26511	y=10223x – 80116	y=20692x + 19492	y=6518x + 2388.6
Correlation coefficient (R^2)	0.9999	0.9999	0.9997	0.9997	0.9998
Number of data points (n)	6	6	6	6	6

Hence, the proposed method's linearity was validated within the chosen ranges.

Precision and accuracy

The method's precision was confirmed by showing the RSD% below 2% for all the analyzed β-lactam (intra-day and inter-day). For the accuracy evaluation, overall recovery was 98.00 – 102.0%, and RSD% was less than 2.0% for all analytes.

Application to pharmaceutical products

Several pharmaceutical products in the Vietnam market were analyzed using the proposed method to confirm the method's applicability in practical analysis. To follow the principles of GAC, the preparations of pharmaceutical samples used water as a diluting solvent, as mentioned in the materials and methods section.

In powder for injections containing 750 mg CFRX, the average CFRX content was 100.30%. The average CFC found in capsules containing 500 mg CFC was 100.87%. In ertapenem sodium powder for injection containing 1 g of ETP, the average ETP found was 99.05%. In the case of the combination of APC and SUL in powder for injection product, the content found was 100.17% and 100.90% for APC and SUL, respectively.

In comparison with the assay methods described in the United States Pharmacopeia (United States Pharmacopeia Convention, 2020),

the proposed procedures bring significant advantages. First, the C18 column was used in the developed method as the stationary phase for the separation. The C18 column is one of the most commonly used stationary phases in analytical laboratories due to its robust performance for analyzing a wide variety of compounds. Thus, the wide availability of the C18 column makes the developed method more applicable. Second, ethanol was used as a green organic solvent instead of acetonitrile and methanol. The small proportion of ethanol (not exceeding 10%) in the mobile phase assures occupational health and environmental friendliness without compromising chromatographic performance.

CONCLUSION

Considering the popularity of HPLC in the industry and the rise of green analytical chemistry, in this work, we proposed a green, simple, and economical method to quantify cefuroxime sodium and cefaclor by using a low percentage of ethanol in the mobile phase. Ethanol is more accessible to dispose of and less hazardous than acetonitrile and methanol, which are generally applied organic solvents in HPLC. Moreover, the analysis is short, leading to a minor artifact of organic waste, showing the eco-friendliness of the method.

The effects of different pH levels of the mobile phase, buffer concentrations, mobile phase ratios, and column temperatures on chromatographic results were investigated elaborately and modified to achieve the appropriate conditions. For method validation, the proposed green HPLC method achieves all the requirements guided by the literature. The developed method was successfully employed to quantify some pharmaceutical products containing CFRX or CFC, affirming the method's applicability.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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