

**Original Article**

## HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE SIMULTANEOUS ESTIMATION OF CEFOPERAZONE AND SULBACTAM IN RAT PLASMA AND ITS IMPORTANCE IN THERAPEUTIC DRUG MONITORING

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### ABSTRACT

**Objective:** To study the Therapeutic drug monitoring and pharmacokinetics of marketed antibiotics formulation by developing a sensitive and specific Bioanalytical Chromatographic method.

**Methods:** In the present study, we developed a rapid, sensitive and selective chromatographic method for simultaneous estimation of Cefoperazone (CEF) And Sulbactam (SAL) in male Wistar rat plasma. A novel liquid phase extraction method has adopted the preparation of plasma sample preparation. The CEF and SAL were eluted on a peerless Basic C18 (25 cm; 4.6 mm x 5 µm) column maintained at controlled environmental conditions. The gradient mobile phase comprised of 10 mmol ammonium acetate and acetonitrile. A UV detector was set at 250 nm and retention times for CEF and SAL were approximately 5.6 and 14.2 min, respectively. The proposed HPLC method was validated according to the US FDA guidelines with respect to the linearity, accuracy, precision, detection and quantitation limits, robustness and specificity.

**Results:** Calibration curves of CEF and SAL were linear across the concentration range of 600-1000 and 6-10 µg/ml, with correlation coefficients ( $r^2$ )>0.9977 and ( $r^2$ )>0.9987, respectively. The limits of detection for CEF and SAL were 70.48 and 0.35 µg/ml, respectively. Additionally, CEF and SAL were stable in plasma for at least 24 h when stored at room temperature and 2-8 °C.

**Conclusion:** The developed chromatographic method was effectively utilized to measure the plasma CEF and SAL concentrations in a pharmacokinetics study after intravenous injection to the healthy male Wistar rats.

**Keywords:** Cefoperazone, Sulbactam, Pharmacokinetics study, Liquid phase extraction and HPLC

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### INTRODUCTION

Antibiotics are chemically synthesized antimicrobial molecules regularly prescribed over the times for the prevention and treatment of various infectious diseases. Additionally, now day's antibiotic and/or antibiotics combinations (e. g. cefoperazone/sulbactam; sulfamethoxazole/trimethoprim and amoxicillin/clavulanate) are also frequently used to treat severe infections such as abdominal, urinary tract infections and respiratory disorders [1, 2]. Appropriate dosing of the antibiotics is of utmost significance for clinical management. Besides, attaining the correct therapeutic level is of high importance for avoiding the development of antimicrobial resistance. Additionally, it also affects the drug distribution, metabolism and clearance. Therefore, guided management with therapeutic drug monitoring (TDM) helps to maximize the efficiency of the antibiotic and similarly to reduce adverse side effects, likewise enhancing the clinical outcomes. By knowing these consequences, for effective antibiotic TDM, in the present study bio-analytical method was developed and validated for commonly prescribed antibiotics combinations of cefoperazone (CEF) and sulbactam (SAL) [3-5].

Various active analysts and researchers took numerous efforts to develop the analytical method for the simultaneous estimation of the CEF/SAL in the biological matrix. A high-performance liquid chromatography (HPLC) [6-10], high-performance thin-layer chromatography (HPTLC) [11, 12] gas chromatography (GC), liquid chromatography/tandem mass spectrometry (LC-MS/MS) [13] and ultraviolet-visible spectroscopy (UV-VIS) [14, 15] spectrophotometric method have been developed and explored for simultaneous estimation of the antibiotics combinations. Among the available analytical techniques, HPLC found to be a helpful tool for quality control and assessment of antibiotics because of its simplicity, few processing requirements and low cost. Additionally,

it also has been commercially used to develop a chromatographic fingerprint for various synthetic, semi-synthetic antibiotics molecules and formulations.

CEF is chemically (6R,7R)-7-[[[2R)-[[[(4-Ethyl-2,3-dioxo-1-piperazinyl)carbonyl]amino] (4-hydroxy phenyl) acetyl] amino]-3-[[[1-methyl-1H-tetrazol-5-yl]thio]methyl]-8-oxo-5-thia-1-azabicyclo-oct-2-ene-2-carboxylic acid. CEF is a third-generation cephalosporin antibiotic routinely prescribed for *Pseudomonas* bacterial infections [15]. Additionally, it also used for the treatment of respiratory infection, urinary tract infection and female genital tract infection. It is a white color crystalline powder with a log P value of -0.74 and two ionizable groups (pKa = 2.55 and 9.55). Therefore, CEF exists mainly as a mono-anionic at a physiological pH. Different analytical techniques have been well documented in the scientific literature for determining the CEF in the various matrix such as reverse phase HPLC [7-10], HPTLC [11, 12] and UV spectrophotometry [14, 15]. SAL is a semi-synthetic beta-lactamase inhibitor and is chemically (2S, 5R)-3,3-dimethyl-4,4,7-trioxo-4λ6-thia-1-azabicyclo[3.2.0] heptane-2-carboxylic acid. It is administered in combination with β-lactam antibiotics (e. g. penicillin and cephalosporin) to inhibit β-lactamase [15]. It is a small molecule (233.24 g/mol) with a log P value of -0.92 and freely soluble in water (48.5 mg/ml). Several analytical techniques have been reported to determine the SAL in combination, including HPLC [7-10], HPTLC [11, 12], LC-MS/MS [13] and UV spectrophotometry [14, 15] for the quantitative determination of SAL in the pharmaceutical dosage form, bulk drug and plasma.

To the finest of our information, no efforts have been made to analyze and detect the CEF and SAL combinations in plasma using a suitable chromatographic method. Therefore, in the present study, we developed and validated a bio-analytical method for the simultaneous detection of CEF and SAL in male Wistar rats.

Additionally, the method utilizes the efficient and less time-consuming isolation process i.e. liquid phase extraction (LPE) for simultaneous detection of CEF and SAL. Furthermore, the developed method was validated using the Food and Drug Administration (FDA), United States (US) guidelines [16].

## MATERIALS AND METHODS

### Chemical and reagent

CEF and SAL were as obtained as a gift sample from Emcure Bhosari, Pune. Cefoperazone-Sulbactam injection (1:0.5) purchased from a local medical shop, Pune. HPLC grade ammonium acetate and acetonitrile were obtained from Merck Specialties Private Limited, Mumbai, India. Ultrapure water was obtained from a Milli-Q Apparatus (Millipore, Barnstead). Normal Saline (Eurolife®) was obtained from Eurolife Healthcare, Pune. Syringe filters (0.22 µm), filter papers (0.45 µm) and ethylenediamine tetraacetic acid (EDTA) tubes were obtained from Merck Specialties Private Limited, Mumbai, India. Male Wistar Rats (200-220 gm) were purchased from Agharkar Research Institute, Pune.

### Animals

Healthy Wistar male rats weighing 200-220 g were procured from Agharkar Research Institute, Pune. Obtained rats were maintained in single polypropylene cages at a steady temperature (25±1 °C) and humidity (45-55 %) with 12 h dark-light cycles. The rats were fasted but supplied with free access to water overnight previous to the start of the study. All animal procedures were implemented as per the Guidelines for the Care and Handling of Laboratory Animals of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) and sanctioned by the Animal Ethics Committee of Bharati Vidyapeeth Deemed to be University, Poona College of Pharmacy registered with CPCSEA (Reg. No. 1703/PO/Re/S/01/CPCSEA).

### Chromatographic system

The method was developed and validated on an LC-4000 Series Low-pressure gradient HPLC system equipped with a quaternary pump (PU-2080+), online degasser (PU-4180), HPLC column oven (HCO-02) and coupled with a dual-wavelength absorbance detector (UV-4075). Data acquisition and processing were carried out using Chrom NAV 2.0 software (Chromatography Data System (CDS)). Chromatographic separation was performed on a Peerless basic C18 (25 cm x 4.6 mm x 5 µm Peerless Silica 3 µm particle size) analytical column equipped with a guard column (Column Saver; 0.5µm/10pk). Chromatographic separation was performed at ambient temperature (25 °C±2 % and 45 %±2 % humidity). Gradient elution was performed using mobile phase A consisting of 10 mmol Ammonium Acetate, and mobile phase B consisting of acetonitrile. The gradient was initiated at 0 % mobile phase B upon injection and increased up to 100 % by scouting gradient curve over 20 min. The column was re-equilibrated at 5 % mobile phase A for five minutes prior to each injection. Single wavelength detection was utilized at a UV absorbance of 250 nm and the flow rate was set at 1 ml/min.

### Preparation of stock solutions, working solutions and calibration standards

CEF (1000 µg/ml) and SAL (100 µg/ml) standard were prepared with normal saline. Subsequent working standards were prepared to contain 600/6, 700/7, 800/8, 900/9 and 1000/10 µg/ml of CEF and SAL by serial dilutions with Millipore water.

### Sample preparation

#### Liquid phase extraction

Liquid phase extraction (LPE) was performed as a method described by Veillette *et al.*, (2016) with few modifications [17]. Briefly, the 50 µl of rat plasma was added carefully to 800 µl acetonitrile and subjected to the vortex (Spinix vortex shaker, Tarsons) for five minutes. The obtained mixture was centrifuged (Eppendorf AG 22331, Hamburg) at a constant speed (8000 RPM) for five minutes. After centrifugation supernatant was carefully pipette out and filtered through a 0.22 µm syringe filter. Obtained filtrate (20 µl) used as such for the HPLC analysis.

### Extraction efficiency

The extraction efficiency of CEF and SAL from rat plasma was estimated at CEF concentrations of 600, 700 and 800 µg/ml and at SAL concentrations of 6, 7 and 8 µg/ml. The extraction efficiency was calculated by co-relating the CEF and SAL drug standards with the peak area of extracted plasma samples (three samples at individual concentration level).

### Linearity

The linearity of the method was established by a five-point concentration calibration curve, achieved by spiking a series of standard mixtures of CEF (600-1000 µg/ml) and SAL (6-10 µg/ml) into rat plasma, extracting both drugs by LPE and analyzing by triplicate injections. Calibration curves for spiked plasma samples were then acquired by plotting the peak areas against their respective concentrations. Linear least-squares regression was applied, and the slope, intercept, correlation coefficient (r), and coefficient of determination (R<sup>2</sup>) was determined.

### Recovery and precision

Analytical recovery and precision were assessed by the analysis of five separately prepared samples of the low (LLOQ), medium, and high drug concentration spiked plasma samples. Intra-day data of the five separately prepared samples was collected over a single run, while inter-day data of another five separately prepared five samples were collected over four separate days. Recovery was calculated using the following **Equation 1** and expressed in terms of percentage. Intra-and inter-day precision expressed as the coefficient of variation.

$$\text{Recovery} = \frac{\text{mean measured concentration}}{\text{known spiked concentration}} \times 100. \text{ Eq. 1}$$

### Specificity/selectivity

The selectivity/specificity of the analytical method was investigated by confirming the complete separation and resolution of both the analytes in the standard solution and spiked rat plasma samples. Moreover, the retention times of endogenous plasma components were compared with those of CEF and SAL to assess for interference.

### LOD and LOQ

The limit of detection (LOD) and the limit of quantification (LOQ) were well described by the US FDA guidelines for bio analytical method validation [16]. The LOD and LOQ are estimated by the signal to noise method.

### Stability of CEF and SAL in the extraction fluid

The stability of CEF and SAL in the LPE extraction fluid was determined at room temperature (25 °C±5) as well as refrigerated at (2-10 °C) conditions for up to 24 hr (i.e., over a period greater a typical daily run).

### Analysis of marketed product

There are several crucial challenges such as low sample volume, tedious sample preparation variable detection limits and development of a robust analytical method to perform TDM in wide patient groups. CEF and SAL are having a large volume of distribution and mainly excreted via bile and urine, respectively particularly, the pharmacokinetics of CEF is highly variable and thus need close TDM during the treatment period [18, 19]. Additionally, the pharmacokinetic data in pediatric patients are limited and thus it makes TDM and dose adjustment more challenging [20]. By knowing this scenario, the pharmacokinetic profile of the marketed CEF/SAL combination was studied using the developed analytical method. Marketed CEF/SAL combination (1:0.5) was administered to male Wistar rats (n=4) via intravenous injection. Blood samples were collected from retro-orbital puncture after 0.5 h of administration. Blood samples were collected in the EDTA tubes and centrifuged at 8000 RPM for five min to collect the plasma. Collected plasma samples were kept at 2-10 °C until analysis. The concentrations of both the drugs in plasma samples were quantified using the developed analytical method.

## RESULTS AND DISCUSSION

Chromatographic separation of CEF and SAL in the biological fluid was performed using reverse phase HPLC method. Initially, various mobile phases were explored to gain maximum response for CEF and SAL. Chromatographic methods for the separation of antibiotic combination in biological solutions have used inorganic buffers in the mobile phase(s) to maintain low pH. Thus, we initiated method development with a combination of phosphate buffer (pH 2.5) and acetonitrile. Various ratios of phosphate buffer and acetonitrile were investigated. The response of low pH mobile phase was much more selective and sensitive for SAL whereas it was opposing for CEF. Therefore, by considering the pKa values of CEF (pKa 3.4) and SAL (pKa 2.86) the mobile phase composition was modified to 10 mmol

ammonium acetate (pH 5) and acetonitrile. The response of the modified mobile phase (high pH) was much more selective and sensitive for both the compounds. Other chromatographic conditions, such as the sample injection volume and flow rate, column saturation time, total run length were also optimized to give accurate, precise and reproducible retention time, proportioned peak shape and better separation for both drugs. The method was optimized to analyze both drugs with no interference from plasma components. The retention time (R<sub>i</sub>) for CEF and SAL was 5.6 and 14.2 min for the 10 mmol ammonium acetate (pH 5) and acetonitrile mobile phase at controlled environmental conditions. The run time of the chromatogram was 20 min. The distance between the peaks was 8.6 min. The peak shape was sharp and more symmetrical. The optimized chromatographic conditions are shown in table 1.

**Table 1: Linear regression data, LOD, and LOQ for the antibiotics using the proposed HPLC method (n=3)**

Parameters	CEFO	SAL
Slope	210.64	152116.8
Intercept	0.6949	1.521
Correlation coefficient (r)	0.99779	0.99826
Coefficient of determination (R <sup>2</sup> )	0.9865	0.9965
Calibration Range (µg/ml)	600-1000	6-10
LOD(ppm)	70.4871787	10.35527949
LOQ(ppm)	213.5975112	1.076604516

\*LOD = Limit of detection, LOQ = Limit of quantification

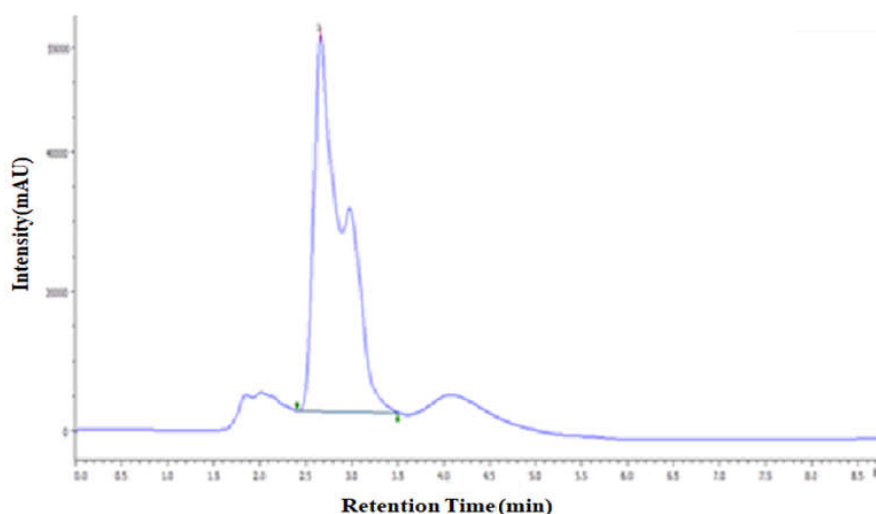
### Preparation of working standard solution

Accurately weighed CEF (100 mg) and SAL (10 mg) were transferred to 100 ml volumetric flask and a sufficient amount of saline water was added to attain the concentration of 1000 µg/ml CEF and 100 µg/ml SAL. The prepared standard solution was diluted further to obtain the CEF and SAL in the concentrations range of 600-1000µg/ml and 6-10 µg/ml, respectively.

### Plasma extraction sample preparation

To establish an efficient method for the extraction of CEF and SAL from plasma, several purification methods were explored. Initially, the various extraction solvents such as trichloroacetic acid (1-15 %), methanol, 10 % per chloric acid, ethyl acetate, chloroform and dichloromethane were used for sample preparation. These processes were proved to be labor-intensive, prone to emulsion formation, and consumed relatively large volumes of high-purity solvents with expensive disposal requirements and thus did not

appear an attractive one for use with a large number of samples. By using these high-purity solvents the resulting chromatogram of blank plasma (no drug) showed numerous peaks that would interfere with the quantization of this drug at 250 nm. Also, recoveries for both CEF and SAL were below 90 % with this method. The highly polar nature (log P ≤ 1) and solubility characteristics of CEF and SAL (CEF is in sodium salt form which is freely soluble in water and SAL is freely soluble in water and slightly soluble in ethanol and dilute acids) suggested that the use of a liquid phase extraction method to separate drug from plasma components using a polar solvent such as acetonitrile/10 mmol ammonium acetate eluent would not only be reasonable, but more appropriate and applicable. Until it was determined that the 800 µl of Acetonitrile maximized the extraction of both components while minimizing the elution of unwanted serum constituents. When blank serum (no drug) was extracted using LPE, the resulting chromatogram exhibited a vastly improved baseline, free of interfering plasma components at a detection wavelength of 250 nm (fig. 1).



**Fig. 1: Chromatogram of blank rat plasma**

### Specificity

Specificity of the method was confirmed by the complete baseline separation of all analytes, as well as the absence of interfering peaks

at the retention times of CEF (4.2 min), SAL (14.2 min) in both a spiked sample (fig. 2) and in a sample from a rat 0.5 h after receiving a 1.5 g intravenous dose of injection (1:0.5g Cefoperazone-Sulbactam) (fig. 3).

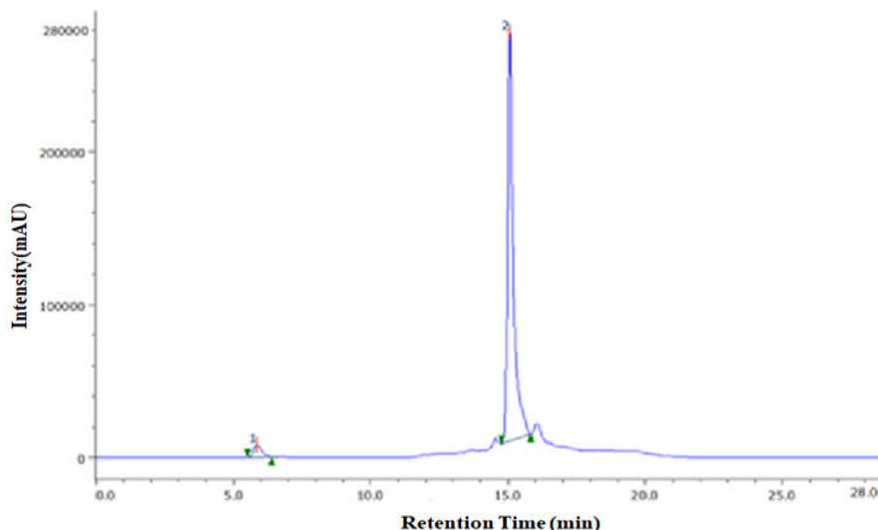


Fig. 2: Chromatogram of spiked plasma containing 600:6 µg/ml, CEF and SAL, resp

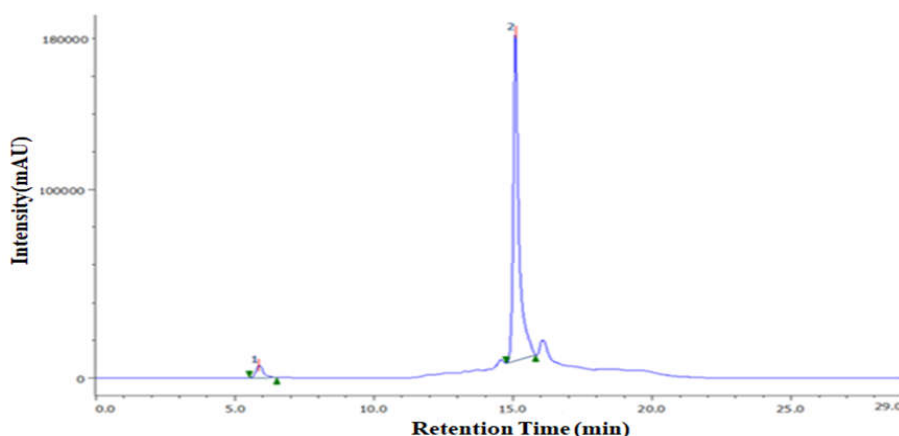


Fig. 3: Chromatogram after receiving a 1:0.5 g dose of formulation, (i.e. 1.0 g CEF and 0.5 g SAL)

### Linearity, LOD and LOQ

Table 1 shows the parameters of the calibration curves, as well as the LOD and LOQ for the developed method. The linearity of measurement was evaluated by analyzing standard solutions of CEF and SAL in and the range of 600-1000 µg/ml and 6-10 µg/ml for both drugs respectively and calibration plot was constructed. The plots revealed that the residual values of each standard sample used to build the calibration curve were scattered randomly about zero, and exhibited no trends of expansion with respect to concentration. As a result, it was determined that the standard curves for CEF and SAL were adequately described by least-squares linear regression analysis over the ranges studied.

LOD and LOQ of CEF and were determined by the calibration curve method. Detection limits were determined as a signal/noise ratio being at least five. Analyte peak should be identifiable, discrete, and reproducible with a precision of 20 % and accuracy of 80-120 %. The lower limit of quantification was 70.48 µg/ml for CEF (S/N>5) and 0.35µg/ml for SAL (S/N>5), respectively.

### Extraction yield, recovery and precision

The data for the extraction yield of CEF and SAL from plasma, as well as the mean intra-and inter-day recovery and precision data are summarized in table 2. At all concentrations, the extraction yield was 90% or greater, and the variation in precision was less than 5%.

### Stability

The stability of CEF and SAL in the LPE extraction fluid was determined at temperatures ranging from -20 °C to room temperature. We sought to ascertain that there was sufficient stability of both CEF and SAL in the LPE eluent fluid to enable the analysis of large batches of samples under either refrigerated (2-8 °C) or room temperature (20-25 °C) conditions. Results demonstrated that samples eluted from LPE by using 800 µl Acetonitrile were stable, defined as no reduction in peak areas and no detection of new peaks when stored for 24 h at either refrigerated (2-8 °C) or room temperature. The results are summarized in table 3.

Table 2: Extraction yield and intra-and inter-day recovery and precision (n=3)

Known Conc (µg/ml)	Extraction yield (mean±SD)	Intra-day Recovery (%)	Intra-day Precision (% CV)	Inter-day Recovery (%)	Inter-day Precision (% CV)
<b>CEFO</b>					
600	90.9±3.05	95.03	0.38	87.39	0.45
700	91.1±2.01	97.43	0.44	89.34	0.43
800	90.31±1.01	93.21	0.39	86.33	0.34
<b>SAL</b>					
6	100±1.127	103.23	1.03	98.30	1.23
7	99.98±4.27	101.21	2.34	97.56	2.34
8	98.67±3.23	100.03	1.75	86.87	1.45

\*conc = concentration, SD = standard deviation and CV=coefficient of variation

Table 3: Stability of CEF and SAL by liquid phase extraction at two different storage conditions (n=3)

Known conc (µg/ml)	Initial conc (µg/ml) (mean±SD)	Conc at 24 h storage at room temperature (µg/ml) (mean±SD)	Conc at 24 h storage 2-8 °C (µg/ml) (mean±SD)
<b>CEFO</b>			
600	540±0.23	538±0.21	541±0.23
700	630±1.05	632±2.09	630±0.45
800	720±1.23	721±1.29	718±2.23
<b>SAL</b>			
6	6.03±0.23	6.83±0.23	6.70±1.35
7	6.99±0.21	6.89±0.21	7.86±1.54
8	7.89±0.36	8.89±1.36	7.86±1.38

\*conc = concentration, SD = standard deviation

#### Application of the method

To assess the clinical applicability in a pharmacokinetic analysis, the developed method was used to quantify the concentrations of CEF and SAL in plasma samples obtained from healthy male Wistar rats. A demonstrative chromatogram from a collected plasma sample is shown in fig. 2. Additionally, there were no endogenous interfering peaks in the chromatogram. A non-compartmental model was adopted for pharmacokinetic analysis. The three

pharmacokinetic parameters such as AUC<sub>(0-∞)</sub>, C<sub>max</sub> and t<sub>1/2</sub> were calculated for CEF and SAL at 0.5 h after an intravenous administration of the marketed formulation. The summary of the pharmacokinetic analysis is shown in table 4. This highlights the importance of the liquid phase extracting technique in bio-analytical sample preparation. Briefly, the developed bio-analytical method was found to be more sensitive as compared to previously published methods and acts to be more appropriate for antibiotics TDM and clinical assessment.

Table 4: Pharmacokinetic parameters of CEF and SAL in male wistar rats following an intravenous injection administration (n=4)

Pharmacokinetic parameters	CEFO (mean±SD)	SAL (mean±SD)
C <sub>max</sub> (µg/ml)	2070.15±40.30	13.31±2.62
AUC <sub>(0-∞)</sub> (µg/ml/h)	132261.05±32.35	1610.26±89.23
t <sub>1/2</sub> (h)	24.42±0.12	2.44±1.01

C<sub>max</sub>: maximum plasma concentrations; AUC<sub>(0-∞)</sub>: area under the plasma concentration–time curve during the 8-h dosing interval and t<sub>1/2</sub>: terminal half-life.

#### DISCUSSION

A novel, precise and robust bioanalytical method was developed for the simultaneous estimation of CEF and SAL in plasma samples obtained from healthy male Wistar rats using a peerless Basic C18 column with a gradient mobile phase comprised of 10 mmol ammonium acetate and acetonitrile. Single wavelength detection was utilized at a UV absorbance of 250 nm and the flow rate was set at 1 ml/min. The method is highly specific as there was no interference observed between chromatograms of blank, standard and sample. This method highlights the importance of the liquid phase extracting technique in bio-analytical sample preparation. Good linearity with the coefficient of correlation 0.99 indicated that the proposed method was linear within the range of 600 to 1000 ppm, 6 to 10 ppm respectively for CEF and SAL. The method was found to be accurate as the extraction values observed for CEF and SAL was well within the range of 90-100% as shown in table 2 for intra and inter day. As the stability of CEF and SAL at different storage conditions were found to be 2.9% and 3.1% w/w respectively as shown in table 3. According to the method described herein, the pharmacokinetics of CEF and SAL were

determined at 0.5 h after an intravenous administration to the four healthy male wistar rats found the more sensitive and selective method than previously published methods [21-24] as shown in table 4. The LOD and LOQ values showed the method was sensitive for the simultaneous estimation of CEF and SAL at low concentrations also. The method is validated as per the US FDA guidelines.

#### CONCLUSION

This article explains a simple, rapid and cost-effective method for the simultaneous quantification of CEF and SAL concentrations in rat plasma using HPLC coupled with UV-visible spectroscopy. Additionally, the method involves a simple and novel liquid phase extraction technique to process the bio-analytical samples. The method presents several key advantages for assaying the drug combination with superior accuracy and high sensitivity. This chromatography technique was effectively applied for a pharmacokinetic study of CEF and SAL combination following intravenous administration in male Wistar rats. The ease of the method makes it more appropriate for regular therapeutic drug

monitoring and clinical pharmacokinetic investigations of antibiotics and its combinations.

#### FUNDING

Nil

#### AUTHORS CONTRIBUTIONS

All authors have contributed equally.

#### CONFLICTS OF INTERESTS

The authors declare that there are no conflicts of interest.

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