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ORIGINAL

Method Development for Determination of Antibiotic Drugs Using Newly Prepared *p*-Morpholinomethylcalix[4]arene Mesoporous Silica-Based HPLC Column

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Abstract

Mesoporous materials are described by their relatively high surface areas and pore volumes. They possess uniform channels within nanometer range. These materials have numerous applications in catalysis, separation and many other fields. The qualitative and quantitative determination of antibiotic drugs, i.e., ciprofloxacin and cefixime has clinical and analytical importance due to their broad spectrum of antimicrobial activity and stability. Both antibiotic drugs are orally active and have excellent activity against different pathogens. It is for the first time that we have developed an analytical method for the simultaneous analyses of both drugs using a newly developed *p*-morpholinomethylcalix[4]arene (*p*-MC4) mesoporous silica-based HPLC column (15 × 3 mm I.D.). Furthermore, separation of these two components was carried out using isocratic elution of methanol and 0.1% aqueous formic acid (70:30 v/v) with flow rate of 1 ml min^{-1} at retention time of 2.71 and 4.21 min and retention factor 1.85 and 1.19 for ciprofloxacin and cefixime, respectively; while total run time was 5 min. The developed method was repeatable with a relative standard deviation (RSD) of 0.90–2.08% for antibiotic drugs. The limits of detection and quantification of ciprofloxacin and cefixime were obtained within the range of 0.152–0.801 and 0.40–1.23 µg mL^{-1}, respectively. The method is highly applicable, rapid, simple, very reproducible and accurate for the separation and determination of antibiotic drugs.

Keywords HPLC column · Calix[4]arene · Antibiotics · Method development · ICH international guideline

Introduction

Cefixime [(6*R*,7*R*,*E*)-7-(2-(2-aminothiazol-4-yl)-2-(carboxymethoxyimino)acetamido)-8-oxo-3-vinyl-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid] and ciprofloxacin [1-cyclopropyl-6 fluoro-1, 4-dihydro-4-oxo-7-(1piperazinyl)-3 quinolone carboxylic acid] [1, 2] are considered as most appropriate when provided in recommended dosage for adults as well as in pediatrics and are widely prescribed antibiotic drugs in Pakistan. Chemical structures of cefixime and ciprofloxacin are shown in Fig. 1. Both drugs are orally active and have excellent activity against pathogens such as Gram-negative (*E-coli, klebsiella, H. influenza, branhamella cattarrhalis, Neisseria gonorrhoeae, Salmonella, Shigella, Campylobacter and Pseudomonas*) [3–6] and Gram-positive (*Streptococcus, Pneumoniae and Enterococcus faecalis*) organisms [5, 6].

Besides their enormous applications, there is a great need for their identification and determination in a vast range of samples. These samples may include: pharmaceutical samples in the form of tablets and syrups, physiological samples (dealing with physical and chemical processes of living organisms and their parts), blood samples including human plasma, urine samples, wastewater samples (human and veterinary pharmaceuticals that reach the environment by excretion and by improper disposal of unused medications) and process samples (due to handling error during manufacturing process); for this, a large number of analytical procedures to detect the presence of drugs in pharmaceutical

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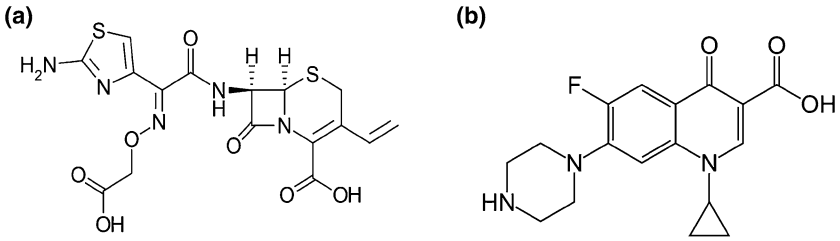


Fig. 1 Chemical structures of **a** cefixime and **b** ciprofloxacin drugs

and physiological samples have been developed. For example, several methods including fluorometry, voltammetry, FTIR spectrometry, chemiluminescence, flow injection analysis and capillary electrophoresis have been used for individual or simultaneous determination of drugs [7–16]. Among them, several reports failed to point out unresolved issues of precision, time consuming and loss of compounds of interest.

So far, HPLC is generally the method of choice for different drugs determination and separation, due to the required time and efficient analysis. However, several HPLC methods with a variety of columns have been applied for individual as well as simultaneous determination of antibiotic drugs [17–19]. Nevertheless, some of these columns are easily available but due to problems associated with their less precision, low accuracy, higher retention times and higher limits of detection and quantification, there is a great need to develop novel, cheap, reliable and highly efficient materials for HPLC columns. In this regard, in the present study, authors decided to take step towards development of mesoporous-based material that can be used as stationary phase to separate/identify two types of antibiotic drugs.

Currently, there has been increasing interest in the synthesis of mesoporous silica materials with macrocyclic compounds such as dendrimers, calix[*n*]arene based dendrimers, cyclodextrins (CDs) or crown ethers and calix[*n*]arenes [20–24]. Mesoporous-based silica gel materials are applied in different fields of analytical as well as material chemistry such as drug delivery systems, purification of water from toxic metals and nanoparticle analysis. From last decade, the synthesized mesoporous silica materials have been used as stationary phase in high-performance liquid chromatography (HPLC) column [25, 26] and solid phase extraction methods [27]. Calixarenes are often used as mobile phase additives and chemically bonded stationary phase for HPLC [28–30].

Numerous earlier works revealed the distinct efficiency of calix[4]arene-based stationary phases in reverse-phase chromatography as a result they exhibit potential applications in HPLC [26]. Fatih and his coworkers utilized the calix[4] arene bonded HPLC column for preconcentration and determination of Cu^{2+}, Pb^{2+}, Cd^{2+} and Hg^{2+} from industrial water. Erdemir and Yilmaz have prepared an HPLC column after modification of silica gel with 1, 3-alternate-calix[4] arene for separation of aromatic amines, phenols and drugs.

In this work, we report synthesis of *p*-MC4 bonded mesoporous silica material, its characterization by FTIR, SEM and CHNS techniques and its application as stationary phase of HPLC for a simple, sensitive, accurate and fast HPLC method for the simultaneous estimation of cefixime and ciprofloxacin antibiotic drugs. The proposed method was validated in compliance with ICH guidelines.

Experimental Work

Materials and Methods

All the solvents were of HPLC grade which were procured from Aldrich Chemical Company (Milwaukee, WI, USA) and Fischer (Fair Lawn, NJ, USA) suppliers. The Sigma-Aldrich (Saint Louis, MO, USA) supplied the silica resin (40–63 µm particle size). Compounds (**1–4**) were synthesized according to earlier reported methods [26]. Ciprofloxacin HCl (99.4%) and cefixime (99.5%) were arranged from a local pharmaceutical industry and were used as reference standard without further purification.

Instrumentation

Elemental analyses of calix[4]arene and its derivative (*p*-morpholinomethylcalix[4]arene) was performed by CHNS elemental analyzer (model Flash EA, 1112, 20090; Rodano, Milan, Italy) and Gallenkamp apparatus with a sealed capillary was used to determine related melting points. The FT-IR (Thermo Nicolet AVATAR 5700) spectrum of synthesized mesoporous silica was recorded in the range from 4000 to 400 cm^{-1} using KBr pellets. Electronic scanning of each compounds were performed using scanning electron microscope (model SEM-JSM-6380) instrument.

A quaternary pump HPLC-DAD spectra system SCM 1000 (model Q Grade-A 0024752 Thermo Finnigan) with vacuum degasser and rheodyne manual injector system (20-µL loop) was used for the seperation and quantitative analysis of antibiotic drug. The *EZChrom Elite* software was used for data acquisition and analysis.

Synthesis of *p*-Morpholinomethylcalix[4]arene (3) Appended Silica (4)

The synthesis of all compounds as shown in Scheme 1 from 1 to 4 was performed according to the previously reported methods [26] while the control of *p*-morpholinomethylcalix[4]arene (**4**) on silica was: a 10 g of silica (40–63 µm particle size) was dried in vacuum followed by adding 1 M (mol L^{-1}) solution of $SiCl_4$ in dry THF. Triethylamine (3 mL) was added to mixture for deprotonation of the OH group on the surface of silica. Resultant cloudy mixture was placed at room temperature for 18 h, after that the solvent was removed with the help of a rotary evaporator. The resultant white powder was then mixed with *p*-morpholinomethylcalix[4]arene (1.5 g) and dissolved in 50 mL of THF. To a resultant mixture 10 mL of triethylamine were added and refluxed for 60 h. The modified silica was filtered and then washed through different solvent such as THF (150 mL), methanol (100 mL), water (100 mL) to remove impurities and unreacted *p*-morpholinomethylcalix[4]arene. The maximum amount of ligand **3** immobilized onto the silica was 0.35×10^{-4} mmol g^{-1}.

Column Packing

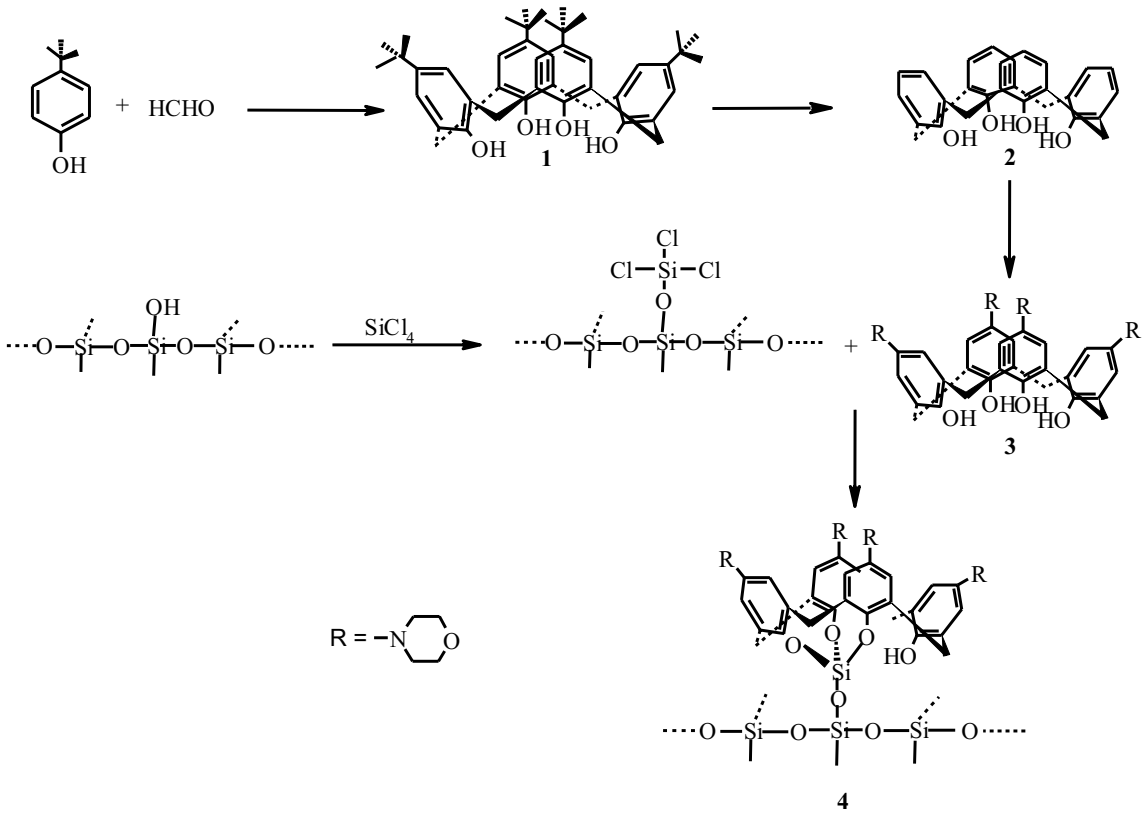
The stainless steel column with internal diameter of 3 mm and length of 15 cm was selected for column packing. Initially, the empty column was washed carefully with suitable reagents (DI water, aqueous HCl and NaOH solution, and methanol) for removing any environmental impurity. The column was packed using ultra-high-pressure column packing pump (Lab Alliance Model CP) at high and constant pressure in advanced research laboratory. The filled column was washed with 0.01 M HCl and ultra-pure water to remove any possible impurity [26, 27].

Standards Preparation

Reference stock solutions (100 mg L^{-1}) for each drug (ciprofloxacin and cefixime) in the mobile phase were freshly made and filtered through 0.45-µm filter after every 5 days. Working samples for calibration were prepared by diluting the stock solution in appropriate quantity of methanol.

Sample Preparation

Ciprofloxacin and cefixime tablets were used for the HPLC determinatioin. Each film-coated tablet contained cefixime



Scheme 1 Synthetic approach for *p*-morpholinomethylcalix[4]arene (**3**) appended silica (**4**)

hydrochloride USP equivalent 400 mg and ciprofloxacin hydrochloride USP equivalent 500 mg. About five tablets of each drug were weighed and finely powdered. An amount of the powder equivalent to both drugs (400 and 500 mg, respectively) for determination was weighed into volumetric flasks (50 mL) followed by addition of approximately 30 mL of mobile phase in each flask. After that, the samples were sonicated for 15 min and then solutions were diluted with mobile phase up to 50 mL volume, then mixed filtered for use.

HPLC Determination of Ciprofloxacin and Cefixime Drugs

The separation of ciprofloxacin and cefixime was achieved in an HPLC spectra system (SCM 1000, Thermo Finnigan, California, USA) equipped with a vacuum degasser and a DAD system. Identification and characterization of both drugs were based on retention time, and UV spectrum. The calibration curve was established by diluting the stock solution of each drug standards in the range of 1–50 μg mL^{-1} that were injected into the HPLC-DAD system sequentially. Before analysis, each standard and sample was filtered carefully by passing 5 ml into 0.45 μm filter paper and inject into HPLC chromatographic column.

Method Validation

ICH international guideline [31] was followed for the method validation of newly developed HPLC column. The method development for determination of cefixime and ciprofloxacin drugs was followed by linearity, accuracy, % recovery, sensitivity and precision of respected drug solutions.

Linearity

Six different concentrations of each drug in the range of 1–50 μg mL^{-1} were used for calibration and linearity. By plotting the peak area versus concentration of each drug, the linearity of the method was determined. However, the slope (m), intercept (b), and the correlation co efficient (R^2) were determined from the regression analysis.

Accuracy

The developed method was validated for its accuracy using internal standard addition method. About 5 μg mL^{-1} of pre-analyzed drug solution was taken and three different levels (80, 100 and 120%) of standard drug (ciprofloxacin or cefixime) were added. The total amount of sample was calculated by performing experiments in triplicate.

Percent Recovery

The % recovery was measured by adding pure drugs (ciprofloxacin and cefixime) with sample solutions, i.e.,

$$\%\ \text{recovery} = \left(\left(D_t - D_s\right)/D_a\right) \times 100$$

where D_t is the total drug concentration after standard addition, D_s is the drug concentration in sample solution and D_a is the added drug concentration.

Sensitivity of Developed Method

The sensitivity of proposed method was determined by calculating the lower limit of detection (LLOD) and lower limit of quantification (LLOQ) of each drug by signal-to-noise ratio (r/s) of 3.3 r/s and 10 r/s, respectively; where s is the slope and r is the standard deviation of the signal.

Precision

The precision of the system was determined by injecting both drugs (ciprofloxacin and cefixime) seven times, that was determined by repeatability of area of peak and retention time of the analytes and determined as the percent relative standard deviation (%RSD) and mean standard deviation (± SD) calculated from obtained data.

To determine the intermediate precision (intraday and interdays), each drug solution was analyzed by three intervals in a day at 8, 16, and 24 h for repeatability for three continuous days for reproducibility. The result was articulated as mean ± SD and percent relative standard deviation (%RSD).

Specificity

By injecting placebo as well as reference solutions, the specificity of the HPLC method was determined. However, no other peak was observed at same retention times of both drugs indicating the absence of interfering substances.

Results and Discussion

Characterization of the Stationary Phase

Synthesis and characterization of *p*-morpholinomethylcalix[4]arene bonded resin was carried out according to the previously reported methods [26, 27] using FT-IR (Fig. 2) and SEM (Fig. 3) techniques. The results achieved using these techniques were in agreement with that of our previously published work [27], i.e., suggesting the appearance of characteristic bands of compound **3** (i.e., the band at 3135 and 2937 cm^{-1} for aromatic and aliphatic -CH stretching along with aromatic C=C band at 1603 cm^{-1})

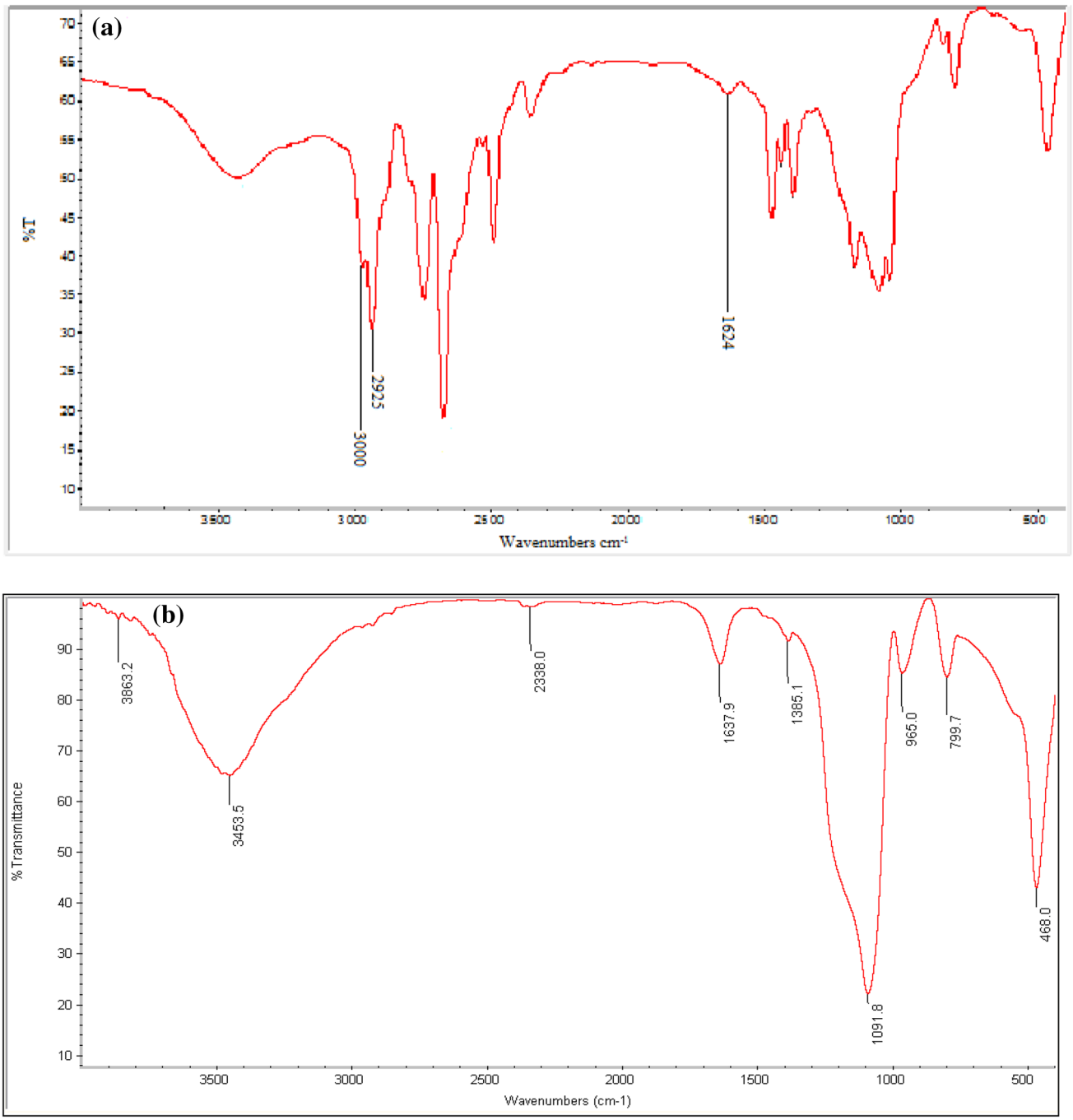


Fig. 2 FT-IR spectra of **a** immobilized and **b** pure silica

in the spectra of immobilized resin (Fig. 2a); however, no such bands are observed in the spectra of pure silica (Fig. 2b). Similarly, SEM spectra were recorded for the pure and immobilized resin. Figure 3 shows the surface of pure silica (Fig. 3a) is smooth as compared to that of the immobilized silica (Fig. 3b-d) which shows the attachment of calixarene moiety (**3**) with the creation of troughs and crests on the surface of silica. Figure 3a–d represents SEM micrographs of (a) pure resin (without immobilization) is focused at the dimension of 100 µm, 100×, similarly (b) is immobilized resin is focused at same dimension as that of (a), i.e., 100 µm, 100×, for comparison purpose; while Fig. 3c, d is also the same immobilized resin but at more close focus, i.e., at 50 µm, 400× and 100 µm, 550× to justify more clarity of immobilization from close look of the same particles.

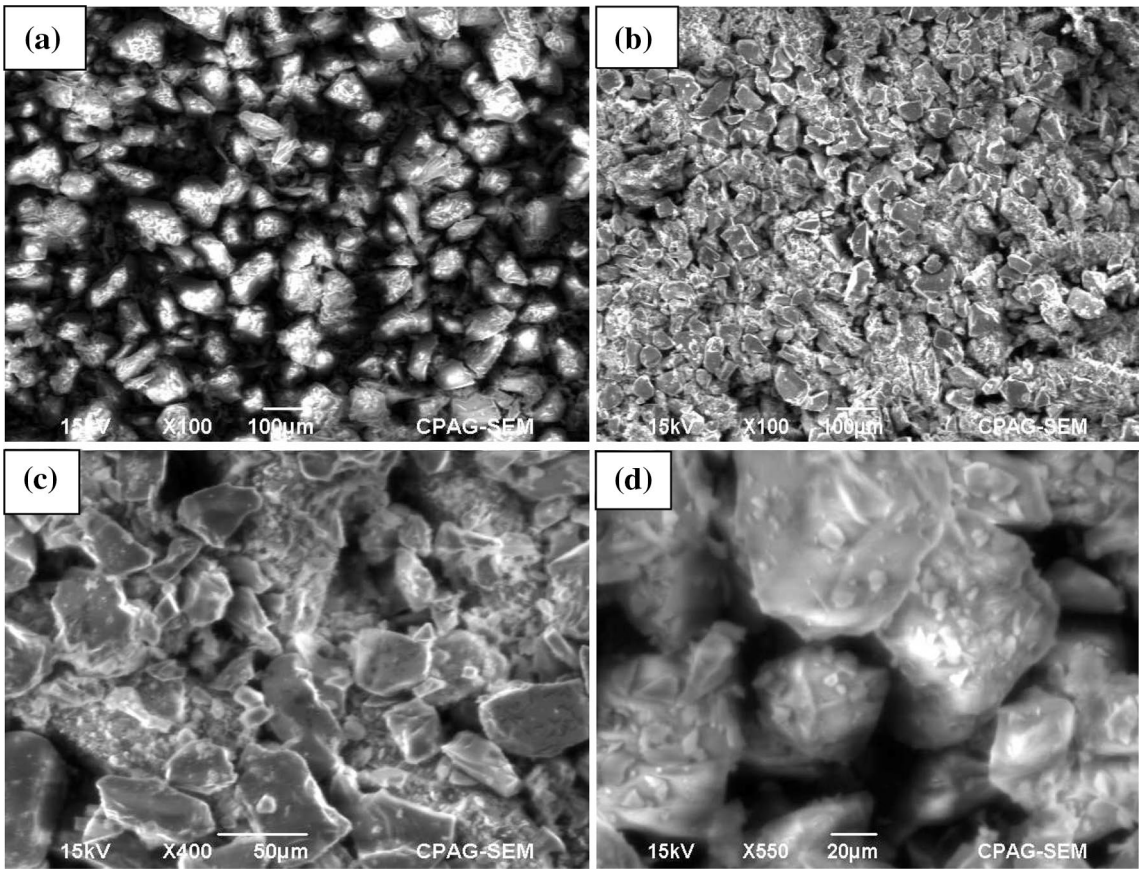


Fig. 3 SEM micrographs of **a** pure resin (15 kV, 100 µm, 100×), **b** immobilized resin (15 kV, 100 µm, 100×), **c** immoblized resin (15 kV, 50 µm, 400×), and **d** immoblized resin (15 kV, 100 µm, 550×)

Validation of HPLC Method

Table 1 shows the summary of validation parameters for both antibiotic drugs analysis following the ICH international guidelines for linearity, sensitivity, % recovery and precision for method development on newly prepared HPLC column [31].

Linearity

The linearity was evaluated for both antibiotic drugs up to six concentrations in the range of 1–50 µg mL^{-1}. Calibration curve was plotted by getting average peak area ($n=3$) against the concentration of derivative and linear regression method was used for result analysis. The regression coefficient of both drugs is tabulated in Table 1, i.e., 0.998 and 0.999, which confirms that the method is linear for the

Table 1 Assessment of method development parameters for two antibiotic drugs (ciprofloxacin and cefixime) on newly prepared HPLC column

Parameters assessed	Ciprofloxacin			Cefixime		
Linearity	*M*	*C*	R^2	*M*	*C*	R^2
	99,385	11,502	0.998	27,885	19,307	0.999
Sensitivity (mg/L)	*LOD*	*LOQ*		*LOD*	*LOQ*	
	0.132	0.401		0.4	1.23	
Precision ($n=5$, %RSD)	Inter-day	Intra-day		Inter-day	Intra-day	
	1.980	1.101		1.823	0.803	
% Addition	80%	100%	120%	80%	100%	120%
% Recovery (±RSD)	95.0 ±0.6	97.0 ±0.9	99.8 ±1.2	96.0 ±0.8	98.0 ±0.9	101.0 ±1.2

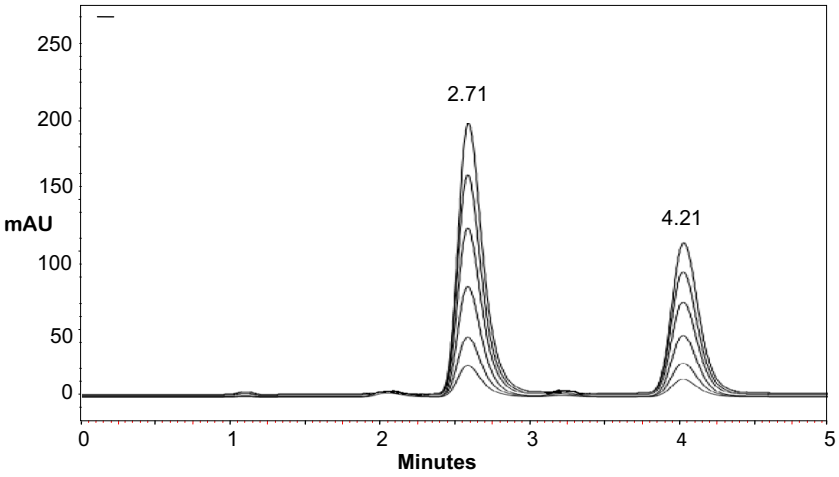


Fig. 4 Simultaneous separation and calibration of two different antibiotic drugs (ciprofloxacin and cefixime) on newly developed HPLC column

determination. Figure 4 shows the separation and calibration of each drug after injection a series of concentration (ranged from 1 to 50 mg L^{-1}) into newly prepared HPLC column and detected by diode array detector.

Sensitivity of Method

To evaluate the sensitivity of the proposed method, lower limit of detection (LLOD) and the lower limit of quantification (LLOQ) was calculated by serial dilution of respected drugs separately until the signal-to-noise ratio reached the value of 3 for LLOD and 10 for LLOQ. Consequently the limits of detection and quantification for ciprofloxacin and cefixime are summarized in Table 1 as 0.132–0.401 and 0.40–1.23 µg mL^{-1}, respectively.

Percent Recovery

For percent recovery experiment, the sample was treated with 80, 100 and 120% of each drug, i.e., ciprofloxacin and cefixime standard. The recovery was obtained within the range of 95–99% with low value of standard deviation. The results revealed that proposed analytical method (Table 1).

Precision

To assess the precision for each antibiotic drug, inter-day and intra-day experiments were conducted and results are tabulated in Table 1. The samples were injected three times within the same day for intra-day precision; while, the samples were injected after every day up to 3 days for the inter-day precision. Adequate results were obtained by calculating RSD for both of the intra and inter-day precisions. The low percentage of RSD indicates high measurement of reproducibility and repeatability in the current experimental condition. These data justify the usability of the newly prepared HPLC column and developed method to be stability indicating.

Conclusion

In the current study, a simple new method has been developed for *p*-morpholinomethylcalix[4]arene (*p*-MC4) based HPLC column, which was used first time for simultaneous separation of two different antibiotic drugs such as ciprofloxacin and cefixime. The separation was carried out in a very short time by HPLC–DAD. The LLOD and LLOQ of both drugs were obtained within the range of 0.132–0.401 and 0.40–1.23 µg mL^{-1}, respectively. Furthermore, HPLC method was validated according to ICH international guidelines, which revealed that method is rapid, linear, accurate, sensitive precise and applicable for the determination of antibiotic drugs.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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