ORIGINAL ARTICLE

Optimization of HPLC method for determination of cefixime using 2-thiophenecarboxaldehyde as derivatizing reagent: A new approach

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Abstract The determination of cefixime **1** has clinical and analytical importance due to its broad spectrum antimicrobial activity and stability. Cefixime is a significant member of orally active third generation cephalosporin and has excellent activity against many pathogens. It is for first time that we have developed a new HPLC–DAD method for analysis of imine derivative **3** of cefixime by using reflux method at 100 °C for 50 min without any buffer solution. 2 Thiophenecarboxaldehyde (2TCA) **2** was used first time as a derivatizing reagent for cefixime drug. Furthermore, separation of three components, i.e. drug (cefixime), reagent (2TCA) and derivative was carried out using kromasil 100 C-18 (15 mm × 0.46 mm, 5 μm) column with isocratic elution of methanol: 0.1% aqueous formic acid (70:30 v/v) with flow rate of 1 ml min^{-1} at retention time of 1.8, 2.4 and 3.3 min, respectively; while, total run time was 5 min. The developed method was repeatable with a relative standard deviation (RSD) of 0.81–1.88% for imine derivative. The limit of detection and quantification of imine derivative **3** were obtained within the range of 0.132–0.401 μg ml^{-1} and compared with cefixime drug as 0.30–0.90 μg ml^{-1}, respectively. However, the formation of imine derivative **3** was confirmed by comparing peak height, retention time and spectral changes. The method is rapid, simple, very stable and accurate for the separation and determination of imine derivative **3** of cefixime **1**.

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Peer review under responsibility of King Saud University.

1. Introduction

Cefixime (CFX) **1** [(6R,7R,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], is considered as an important and active member of third generation cephalo-

sporin. The cefixime exists in off white crystals, melts over 250–220 °C and soluble in alcohol (Troy and Beringer, 2005). An orally active cefixime has excellent activity against pathogens such as, Anaerobes, Enterobacteriaceae, gram negative species such as *Escherichia coli*, *klebsiella*, *Haemophilus influenzae*, *Branhamella Catarrhalis*, *Neisseria gonorrhoeae*, *Serratia marcescens*, *Providencia*, *Haemophilus*, and *Meningococcus* including β-lactamase producing strains (Troy and Beringer, 2005; Katzung, 2006; Sayed et al., 2013). Along with its broad spectrum antimicrobial activity and stability, cefixime is considered as most convenient in appropriate dosage for adults as well as pediatrics and widely prescribed among cephalosporin family in Pakistan. Various analytical methods have been reported for analysis of cefixime and other antibiotics after complexation and derivatization with a variety of chemical reagents (Sayed et al., 2013; El-Shaboury et al., 2007; Wani and Patil, 2013; Ahmed et al., 2011; Adegoke and Quadri, 2012; Adegoke and Umoh, 2009). These methods involve spectrophotometric methods (Thakkar and Mashru, 2012; Azmi et al., 2013; Ethiraj et al., 2012; Attimarad et al., 2012; Ramadan et al., 2013; Shah and Kilambi, 2006; Agbaba et al., 1997; El-Wailily et al., 2000), voltammetric method (Rajeev et al., 2010), capillary electrophoresis (Ahemed, 2013), clinical studies (Khan et al., 2008), flow injection spectrophotometry (Abass et al., 2011), HPLC/tandem mass spectrometry (Ronaldo et al., 2001), UPLC/HPLC & IPLC (Ion-Pairing Liquid Chromatography) (Uslu and Ozden, 2013; Manna and Valvo, 2004), alternative continuous infusion method (Hiroyuki et al., 2006) and attenuated total reflectance (ATR) fourier transform infrared (FT-IR) spectroscopy (Kandhro et al., 2013). Among these techniques, UV/Vis spectrophotometry is considered as one of the most widely used technique for determination of cefixime after derivatization (Sayed et al., 2013; El-Shaboury et al., 2007; Wani and Patil, 2013; Ahmed et al., 2011; Adegoke and Quadri, 2012; Adegoke and Umoh, 2009; Thakkar and Mashru, 2012; Azmi et al., 2013; Ethiraj et al., 2012; Attimarad et al., 2012; Ramadan et al., 2013; Shah and Kilambi, 2006; Agbaba et al., 1997; El-Wailily et al., 2000; Rajeev et al., 2010; Ahemed, 2013; Al-Momani, 2001). Furthermore, the case of HPLC is concerned, the literature is available for the determination of cefixime without its derivatization (Falkowisky et al., 1987; Joy et al., 1987; Leroy et al., 1995; Mikawa et al., 1989; Fang et al., 2005; Raj et al., 2010; Khandagle et al., 2011; Da-xing et al., 2013); however, there is no any report on HPLC for the analysis of cefixime after derivatization with any suitable aldehyde. Though, HPLC is much more sophisticated technique as compared to previously reported methods as it provides specific information for all analytes along with corresponding UV/Vis spectra simultaneously, which is very useful tool for the analysis of unknown components of a mixture. Therefore, by keeping in view the need for its analysis and usability of HPLC, we decided to use aldehyde as a derivatizing agent for cefixime and developed a new HPLC method for its analysis.

2-Thiophenecarboxaldehyde (2-TCA) **2** (also known as thiophene 2 carbaldehyde) is an aldehyde compound which is used for condensation process and an intermediate to manufacture pharmaceuticals, aroma compounds and pesticides (Alexandru et al., 2008). Alexandru et al. have utilized the 2TCA for condensation of alkylazulenes, however there is no any report for 2-TCA to be used as derivatizing agent for drug specially cefixime. 2-TCA is a yellow colored liquid having 198 °C boiling point soluble in alcohol and other organic solvents, heterocyclic ring bearing aldehyde which condenses with amine to produce imine derivatives. Generally amine reacts with primary aromatic amine or aliphatic amine to form N-substituted products known as imine derivative and Schiffs base or azomethines reaction may be carried out by heating a mixture of amine and aldehyde in equal molar proportions alone or with a diluent or medium such as acetic acid or alcohol.

The general reaction may be represented as Scheme 1.

Where R' may be alkyl, cycloalkyl, or heterocyclic ring and R'' may be benzene or Aryl ring. Aldehydes and aromatic amines produce the stable colored substances e.g. Schiffs bases from substituted benzaldehyde and amine which are more stable as compared to purely aliphatic amines (C_5–C_{10}) (Raj et al., 2010).

In this work, we report first time the use of 2-thiophenecarboxaldehyde **2** as a derivatizing reagent for cefixime **1** determination and developed a new HPLC–DAD method that could simultaneously separate three components i.e. drug, reagent and imine derivative respectively. Qualitatively, this method is also supported by different other authentic analytical techniques such as UV/Vis, and TLC for characterization of newly developed imine derivative **3**.

2. Materials and methods

2.1. Chemicals and reagents

All reagents and chemicals were of pharmaceutical or analytical grade. The double distilled water used throughout study was obtained from distillation plant all made up of glass. Pure cefixime (CFX) was obtained from Bosch pharmaceuticals (PVT) LTD. (Karachi, Pakistan), 2-thiophenecarboxaldehyde and formic acid were purchased from Aldrich Chemical Company (Milwaukee, WI, USA) and Methanol (HPLC grade) from Fisher Chemicals (Fair Lawn, NJ, USA).

2.1.1. Standards preparation
Stock solution of 100 mg l^{-1} for cefixime drug was prepared freshly after every five days. Working samples were made by diluting the stock solution in appropriate quantity of methanol. However, the (1% v/v) solution of 2-TCA was prepared in methanol solvent in 100 ml of volumetric flask.
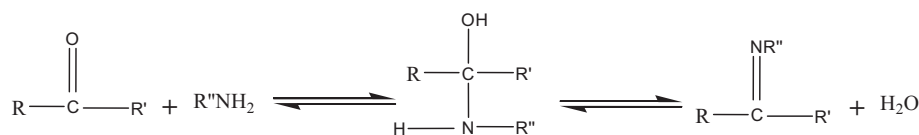
Scheme 1 A representative diagram for general reaction procedure of Schiffs base reaction.

2.2. Derivatization procedure

10 ml of methanolic solution of each reactant (2-TCA and cefixime) was mixed in reflux fitted round bottom flask and refluxed at 90–100 °C on electrical heating mental. No change was observed in freshly added mixture of the above material. During reflux periodically after every 10 min, HPLC detection was performed and remarkable changes observed after 50 min. The mixture was analyzed in HPLC for separation of three components i.e. cefixime, 2-TCA and imine derivative. The synthetic pathway of imine derivative is shown in Scheme 2.

2.3. HPLC analysis of cefixime, 2-TCA and imine derivative

The separation of cefixime, 2-TCA and their imine derivative was achieved in a Spectra system SCM 1000 (Thermo Finnigan, California, USA) liquid chromatograph equipped with a vacuum degasser and a DAD system. A Teknokroma KROMASIL 100 C-18 (15 mm × 0.46 mm, 5 μm) column (Spain) was selected for separation. The mobile phase consisted of methanol (A) and aqueous 0.1% formic acid (B), and all the analysis was carried out within 5 min at isocratic flow of 70:30 (A:B v/v). The flow rate was 1 ml min^{-1} while the injection volume was 20 μl. UV detection was performed at two different wavelengths. Cefixime and 2TCA were detected at 280 nm, and imine derivative was detected at 350 nm.

Software used for data acquisition and evaluation was Chromquest, Version 4.2. Identification and characterization of cefixime, 2-TCA and new imine derivative were based on retention time, peak height, UV spectrum, and TLC. The calibration curve was established by diluting the stock solution of imine derivative in the range of 1–50 μg ml^{-1} that was injected into the HPLC–DAD system sequentially.

2.4. Thin layer chromatographic procedure

A drop (∼1 μl) of cefixime, 2-TCA and imine derivative was spotted on the baseline of silica coated plate with jet formed capillary tube. The sample loaded plate was placed in a TLC chromatojar which already contains saturated mobile phase (methanol:hexane 1:4 v/v). After 15 min of elution time, the height of separated different component was within 1 cm, the plate was taken out carefully from chromatojar and the measurement of eluted distance was carried out in the illumination of D$_2$ Lamp. The value of RF was in the decreasing order of imine derivative, cefixime and 2-TCA, respectively.

2.5. Solvent extraction procedure for imine derivative

About 10 ml of derivatized sample was extracted with 10 ml of n-hexane in separating funnel with extensive shaking (both cefixime and 2-TCA are seldom or poorly soluble in selected solvent). The imine derivative was soluble in hexane and separated out from a mixture by re-extracted with 5 × 2 ml of fresh n-hexane solvent. The collected fraction of n-hexane evaporated using rotary evaporator at 60 °C and the dried contents were re-dissolving with methanol for further analysis on HPLC.

2.6. Validation of HPLC method for derivatization

Newly developed HPLC method for analysis of imine derivative of cefixime was validated according to the ICH interna-
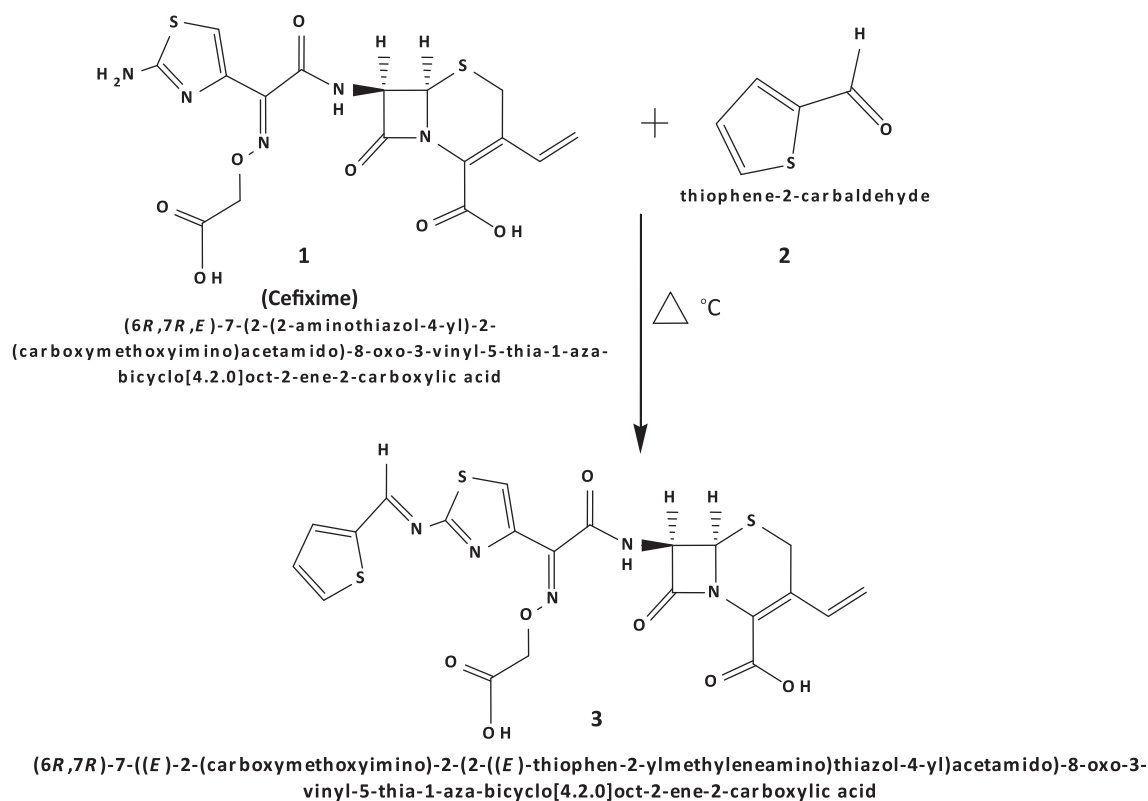
Scheme 2 Synthetic route for the derivatization of cefixime using 2TCA as derivatizing reagent.

tional guidelines (ICH, 2005) for linearity, accuracy, % recovery, sensitivity, precision and stability of solutions.

2.6.1. Linearity

Seven different concentrations of imine derivative in the range of 1–50 μg ml^{-1} were used for calibration and linearity. The linearity of the method was determined by plotting the peak area versus concentration of imine derivative. The slope (m), intercept (b), and the correlation co-efficient (r^2) were determined from the regression analysis.

2.6.2. Accuracy

Accuracy of the developed method was determined by using internal standard addition method. About 5 μg ml^{-1} of pre-analyzed imine derivative solution was taken and three different levels (80%, 100% and 120%) of standard drug (cefixime) were added. The total amount of drug and reagent were estimated by using the proposed methods in triplicate.

2.6.3. Percent recovery measurement

The % recovery was measured by added pure drug (cefixime) with imine derivative solution as

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

where D_t is the total drug concentration after standard addition; D_s is the drug concentration in the imine derivative mixture and D_a is the drug concentration added.

2.6.4. Sensitivity

The sensitivity of proposed method was determined by limit of detection (LOD) and lower limit of quantification (LLOQ) of imine derivative using signal to noise ratio (σ/s) of 3.3 σ/s and 10 σ/s respectively; where σ is the standard deviation of the signal and s is the slope of corresponding calibration curve.

2.6.5. Precision

The precision of the system was determined by injecting the mixture of drug (cefixime), reagent (2-TCA) and imine derivative at least 7 times, that was expressed by repeatability of peak area and retention time of the analytes and determined as the mean standard deviation ($\pm$SD) and the percent relative standard deviation (%RSD) calculated from the data obtained.

To determine the intermediate precision (intraday and interdays), the imine derivative solution was analyzed by three intervals in a day at 08:00, 16:00, and 24:00 h for repeatability and for three successive days for reproducibility. The result was expressed as the mean $\pm$ SD and percent relative standard deviation (% RSD).

3. Results and discussion

### 3.1. Derivatization and HPLC–DAD separation

The derivatization reaction is mostly used to convert an analyte for ease of detection in order to enhance sensitivity, selectivity and stability by any instrumental analytical methods. For separation methods, derivatization is primarily used for
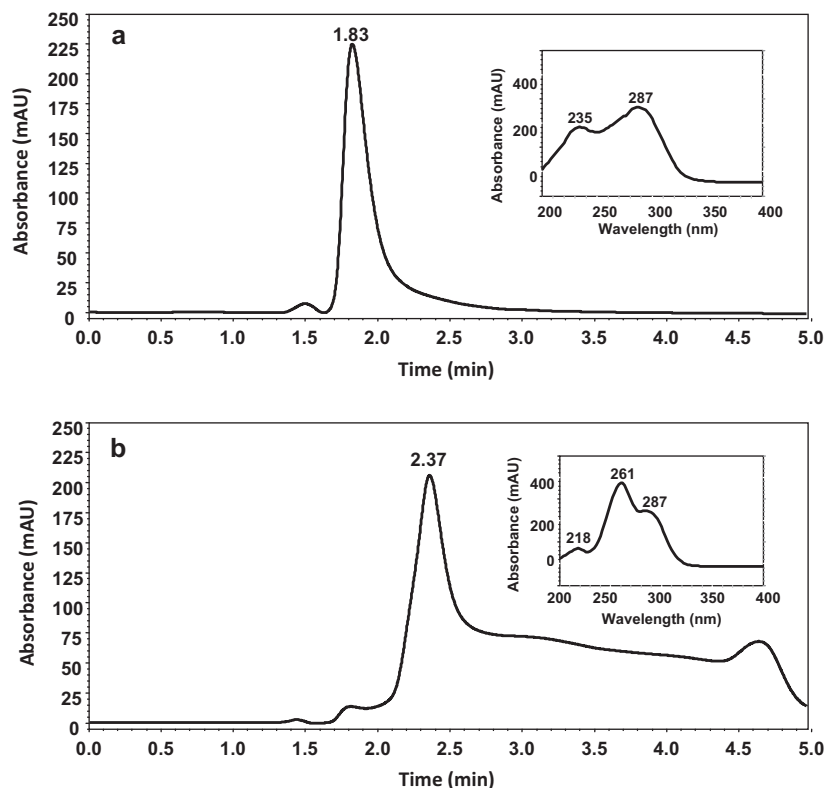
Figure 1 Verification (retention time and UV spectra) and separation of methanolic solution of (a) cefixime (drug) and (b) 2TCA (reagent) by HPLC–DAD.
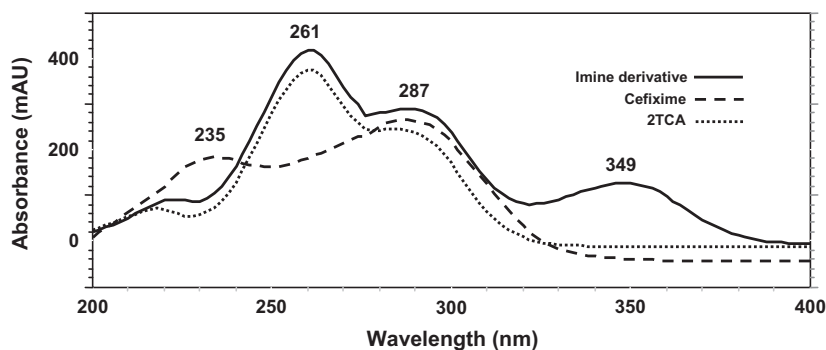
Figure 2 Comparison of UV spectra of imine derivative, cefixime and 2TCA reagent by diode array detector.
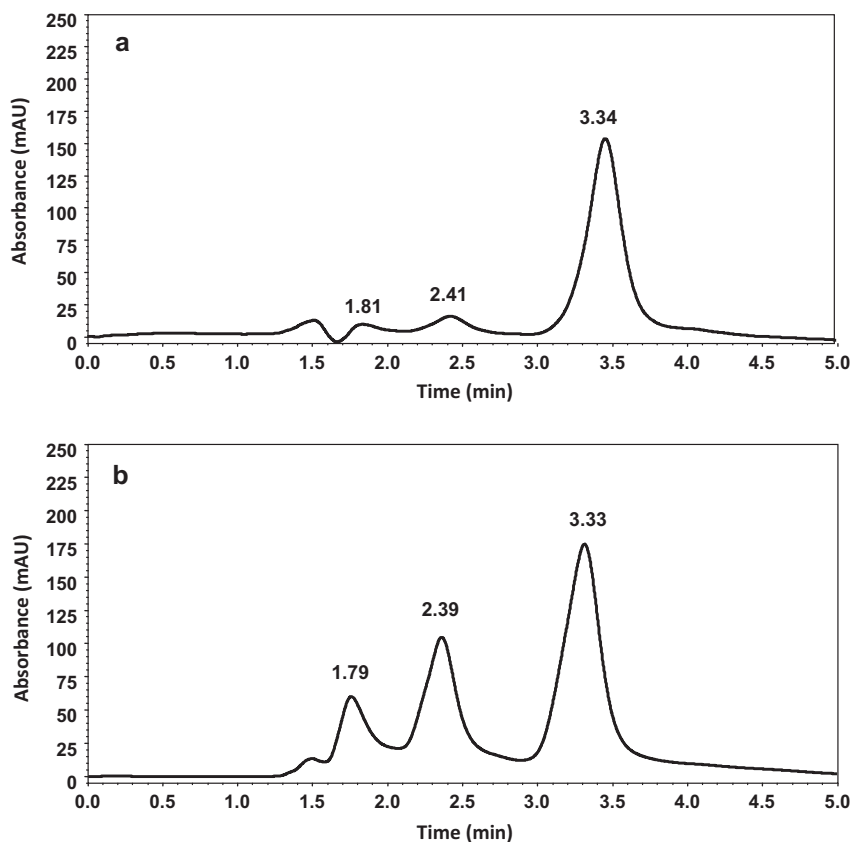

Figure 3 (a) High pressure liquid chromatography separation of three components including cefixime **1** (drug), 2TCA (reagent) **2** and imine derivative **3**, (b) high pressure liquid chromatography separation after internal addition of two components including cefixime **1**, and 2TCA reagent **2** with imine derivative **3**.

modification of analyte functionality in order to enable chromatographic separations. The new product which is generally known as derivative, has similar or closely related structure to the analyte but not the same as the original unmodified compound.

Scheme 2 shows the reaction of 2-thiophenecarboxaldehyde **2** with primary amino group at thiazole ring of cefixime **1** during reflux method and new imine derivative **3** of cefixime was prepared. Before derivatization, the pure form of drug and reagent was analyzed separately by RP-HPLC for the verification of retention time and spectral data respectively. Fig. 1a

and b shows the retention time (1.83 and 2.37 min) as well as UV spectra (λ_{max} 287 nm and 261 nm) of both drug and reagent, respectively. The reaction was initially monitored by HPLC following every ten min of interval and finally complete reaction was analyzed after 50 min. There was no change observed on longer heating and derivative was very stable at room temperature for an extensive time (more than two weeks). The physical appearance of derivatization solution was also changed (from yellow to light green); furthermore, the solution was cooled at room temperature and analyzed in HPLC integrator (Column) for separation of three compo-
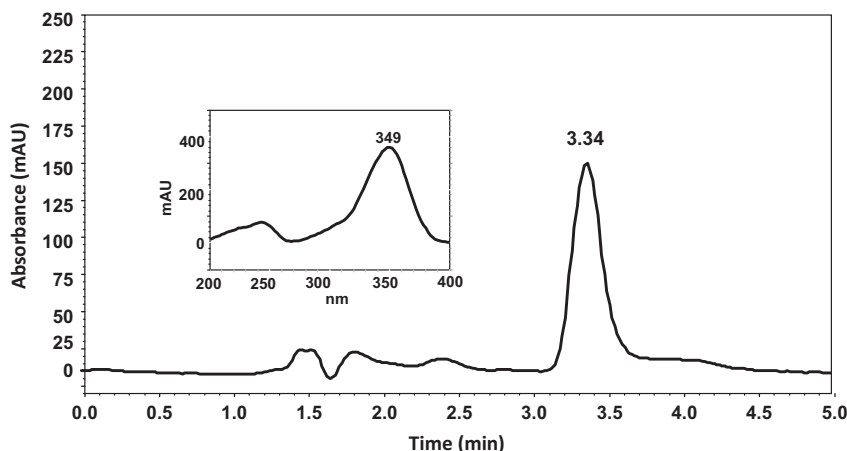
Figure 4 HPLC chromatogram of extracted imine derivative peak and their UV spectra at λ_{max} of 349 nm.
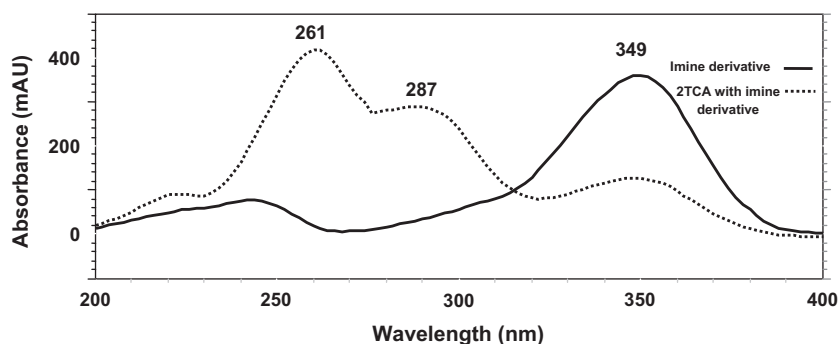
Figure 5 Comparison of UV spectra (at λ_{max} 349 nm) of imine derivative before and after extraction from a mixture by *n*-hexane solvent by DAD detector.

Table 1 Validation parameters for imine derivative analyzed by HPLC method.

| Parameters assessed	Imine derivative		
Linearity	Slope 99385	Intercept 11502	Correlation coefficient 0.998
Percent recovery	80% addition 96.00 ± 0.6	100% addition 99.00 ± 0.8	120% addition 95.00 ± 1.0
Precision ($n = 3$)	Intraday (%RSD) 0.81	Interday (%RSD) 1.88	

nents of a mixture i.e. cefixime, 2-TCA and imine derivative, while the UV spectra of imine derivative were analyzed at 350 nm by diode array detector.

The comparison of UV spectra of three components is shown in Fig. 2, in which λ_{max} of cefixime, 2-TCA and imine derivative are different from each other i.e. 235, 287 nm; 261, 287 nm and 261, 287, 349 nm, respectively. After complete reaction of 2-TCA reagent with cefixime, the displacement of absorption toward a longer wavelength (bathochromic shift) was observed and specific change i.e. 349 nm was observed in the UV spectra of imine derivative (Fig. 2).

Fig. 3a shows the simultaneous separation of three components (cefixime, 2-TCA and imine derivative) by HPLC–DAD with distinctive retention time using isocratic system (70:30) mobile phase (methanol: 0.1% formic acid) at flow rate of 1 ml min^{-1}; however, the internal standard addition method was applied for further confirmation of cefixime and 2TCA in a mixture. Fig. 3b shows the improved height of cefixime and 2TCA with same retention time after internal addition of relative standards and did not have any effect on the height of imine derivative.

However, the further confirmation for preparation of imine derivative was carried out by TLC method using binary mixture of methanol and *n*-hexane (1:4). A good separation with minimal tailing was achieved which revealed that the derivative was eluted for more distance than the other respective compo-
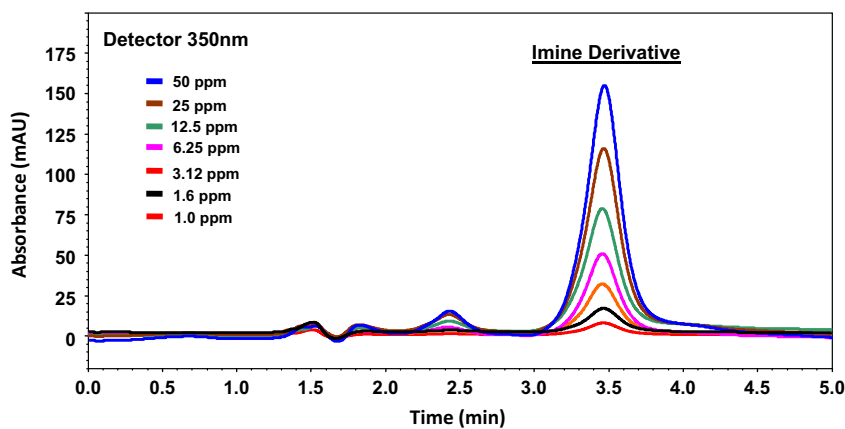
Figure 6 HPLC chromatograph of linear calibration of imine derivative within the concentration range of 1–50 µg mL^{-1}.

Table 2 Sensitivity comparison of proposed method for imine derivative with cefixime drug.

Sensitivity (µg ml^{-1})	Imine derivative	Cefixime drug
Limit of detection (LOD)	0.132	0.30
Limit of quantification (LOQ)	0.401	0.90

nents (cefixime and 2-TCA). The accuracy and precision of the method were done by the repeated performance with diluted concentrations, and the qualitative results were remained unchanged regarding the height and area of components. The proposed method is a significant tool in the confirmation of derivatization and its applications.

3.2. Extraction and separation of imine derivative from a mixture

The extraction of imine derivative from a mixture was carried out by liquid–liquid extraction method. TLC method clearly indicated that, the imine derivative was soluble in *n*-hexane and separated out from a mixture that is why *n*-hexane was used for the extraction of imine derivative in separating funnel with extensive shaking. Hexane was evaporated from collected fraction of imine derivative at 60 °C using rotary evaporator and remaining dried contents were re-dissolved with methanol and analyzed on HPLC. Fig. 4 shows the principal chromatographic separation of extracted imine derivative from a mixture along with the notable UV-spectra at λ$_{max}$ 349 nm, which clearly indicates the formation of new imine derivative of cefixime drug.

Fig. 5 shows the comparison of UV spectra of imine derivative before and after the extraction by *n*-hexane solvent which also gives another evidence for the extraction of imine derivative from remaining cefixime drug and 2-TCA reagent from a mixture.

3.3. Validation of HPLC method for imine derivative

Table 1 shows the summary of validation parameters for imine derivative analysis following the ICH international guidelines

(ICH, 2005) for linearity, sensitivity, % recovery and precision of newly developed HPLC method.

3.3.1. Linearity
The linearity was evaluated for imine derivative up to seven 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 the result was analyzed by linear regression method. A coefficient of regression is tabulated in Table 1 i.e. 0.998 which confirms the method was linear for the determination of imine derivative. Fig. 6 shows the calibration of imine derivative after injection of a series of concentration into HPLC and detected by diode array detector.

3.3.2. Sensitivity
The sensitivity of the proposed method was carried out by calculating the limit of detection and the limit of quantification by serial dilution of imine derivative mixture until the signal-to-noise ratio reached the value of three for LOD and ten for LOQ. The limits of detection and quantification are summarized in Table 2 as 0.1 and 0.4 µg ml^{-1} respectively. Furthermore, Table 2 shows the comparison of LOD and LOQ of imine derivative and cefixime drug which clearly indicates that after derivatization the sensitivity for detection increases as compared to underivatized cefixime.

3.3.3. Percent recovery
Three different percentages i.e. 80%, 100% and 120% of cefixime standard were added during derivatization and recovery was obtained within the range of 95–99% with low percent of standard deviation indicating the high accuracy of the proposed derivatization and analytical method (Table 1).

3.3.4. Precision
An inter-day and intra-day precision for the imine derivative was assessed by injecting the mixture in HPLC and results are summarized in Table 1. For intra-day precision the samples were injected three times within the same day while for the inter-day precision the samples were injected after every day up to three days. Satisfactory results were achieved by calculating RSD for both of the intra-day and inter-day precisions. The low percentage of RSD indicates high measurement of

reproducibility and repeatability in the current experimental condition. These data justified the usability of the method to be stability indicating.

4. Conclusion

In current study, a simple new method has been developed in which 2-thiophenecarboxaldehyde was used first time for derivatization of cefixime and simultaneous separation of three components i.e. Cefixime, 2-TCA and imine derivative was carried out in a very short time by HPLC–DAD. The LOD and LOQ of imine derivative were obtained within the range of 0.132–0.401 µg ml^{-1} consequently; these data were also compared with that of underivatized cefixime, and the results revealed the increased sensitivity and selectivity of derivative. The results were further confirmed by standard addition technique. Imine derivative was confirmed by comparison of peak height, retention time and spectral changes with cefixime drug and 2-TCA reagent and also reconfirmed by extracting out from mixture using n-hexane solvent. Furthermore, HPLC method was validated according to ICH international guidelines which revealed that method was rapid, linear, accurate, sensitive precise, stability indicating and applicable for determination of imine derivative of cefixime.

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