Original Article
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Stability indicating RP-HPLC and spectrophotometric methods for simultaneous estimation of sodium benzoate and cefdinir in the presence of its degradation products. Application to blank subtraction method

Bozunma ürünlerinin mevcudiyetinde sefdinir ve sodyum benzoatın eş zamanlı ölçümü için RP-HPLC ve spektrofotometrik yöntemler gösteren stabilite. Boş çıkarma yöntemine uygulama

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Abstract:
Objectives: Empower 3 software is important in modeling, optimization, and reducing the time of manual calculation of related substance by subtracting the baseline of a blank chromatogram from the unknown sample automatically, so the major objective of the developed method is to introduce a new, selective and economic HPLC and spectrophotometric methods for simultaneous estimation of sodium benzoate (SDB) and cefdinir (CFR) in the presence of its degradation products.
Materials and Methods: Chromatographic separation is optimized in a Hichrom C18 column (15 × 0.46 cm,) 5 µm particle size or equivalent, using a binary gradient consisting of solution A (0.1% tetramethylammonium hydroxide solution (pH 5.5) with 0.1M EDTA (1000:0.4 v/v)) and solution B (0.1% tetramethylammonium hydroxide solution (pH 5.5): acetonitrile: methanol : 0.1M EDTA (500:300:200:0.4v/v) using injection volume 10 µL for RP-HPLC with a wavelength equals to 254 nm and flow rate 1.0 mL/min. Two ecofriendly spectrophotometric methods were successfully utilized for resolving the spectral overlap of drugs.
Results: Method A, the first derivative of ratio spectra spectrophotometric method (1stDD) where CFR was determined at two wavelengths 283.5 nm, 313.4 nm and SDB was determined at 216.7 nm, 235.5 nm. Method B, ratio subtraction method (RSM) is performed to overcome the interference between CFR and the preservative SDB. The UV spectrum of the laboratory mixture is divided by that of CFR (20µg/mL) as a divisor then subtracting the amplitudes in the plateau region at 250-315 nm (the constant) from that ratio spectrum. The zero-order spectra of SDB were obtained at 225 nm via multiplying the resulted ratio spectra by the divisor (CFR), zero order of CFR has been estimated at a wavelength value of 283 nm after multiplication the divisor by the obtained constant.
Conclusion: The optimized method was adjusted and validated and could be easily utilized by quality control laboratories and for laboratory-prepared mixtures.
Keywords: Empower 3 software, RP-HPLC-UV, Spectrophotometric methods, Blank subtraction method, Cefdinir, Sodium Benzoate.

Öz:
Hedefler: Empower 3 yazılımı, bilinmeyen numuneden boş bir kromatogramın taban çizgisini otomatik olarak çıkararak ilgili maddenin manuel olarak hesaplanmasında modelleme, optimizasyon ve sürenin azaltılmasında önemli bir rol oynar, bu nedenle geliştirilen yöntemin ana görevi, bir bozunma ürünlerinin varlığında sefdinir (CFR) ve sodyum benzoatın (SDB) eşzamanlı tahmini için yeni, doğru, kesin ve ekonomik HPLC ve spektrofotometrik yöntemler.
Materyaller ve Metotlar: Kromatografik ayırma, bir Hichrom C18 kolonunda (150 mm × 4.6 mm, 5 µm partikül boyutu veya eşdeğeri) 1.0 mL/dk akış hızında, A solüsyonundan (%0.1 tetrametilamonyum hidroksit solüsyonu) oluşan ikili bir gradyan kullanılarak optimize edilir (pH5.5) 0.1M EDTA (1000:0.4 v/v)) ve solüsyon B (%0.1 tetrametilamonyum hidroksit solüsyonu (pH5.5): asetonitril: metanol : 0.1M EDTA (500:300:200:0.4v/v) ile) RP-HPLC için enjeksiyon hacmi 10 µL ve 254 nm'de UV tespiti kullanılarak İlaçların spektral örtüşmesini çözmek için iki çevre dostu spektrofotometrik yöntem başarıyla uygulandı. CFR 283.5 nm, 313.4 nm'de ve SDB 216.7 nm, 235.5 nm'de belirlendi Yöntem B: İkili karışımın spektrumunu standart spektruma bölerek CFR ve SDB arasındaki girişimi çözmek için oran çıkarma yöntemi (RSM) kullanılır. sonra bölen olarak CFR (20µg/mL) 250-315 nm'de (sabit) plato bölgesindeki genlikleri bu oran spektrumundan çıkarmak. SDB'nin sıfır dereceli spektrumları, elde edilen oran spektrumlarının bölen (CFR) ile çarpılmasıyla 225 nm'de elde edildi ve ayrıca sıfır dereceli CFR, bölen sürekli elde edilen ile çarpıldıktan sonra λmax 283 nm'de tahmin edildi.
Sonuç: Önerilen yöntem ICH yönergelerine göre doğrulanmıştır ve kalite kontrol ve laboratuvarda hazırlanan karışımlara kolaylıkla uygulanabilir.
Anahtar Kelimeler: Empower 3 yazılımı; RP-HPLC-UV, Spektrofotometrik yöntemler, Boş çıkarma yöntemi, Sefdinir, Sodyum Benzoat.

INTRODUCTION

Application of blank subtraction method in related substance and degradation products have been widely applied in the quality control lab in the pharmaceutical industries to optimize, achievement and decrease number of experimental trials of chromatographic system for manual calculation by using the empower software. Empower 3 software PDA can subtract mobile phase effects to a standard or sample. The possibility of blank subtraction is useful when the application is affected by gradient runs, in which least one of the solvents contains a UV-absorbing compound, system peaks and contaminants in the mobile phase. Blank subtraction removes the chromatographic tool from the data set, resulting in a 3D chromatographic scheme corrected to the baseline. In fact, this 3D chromatogram is the difference between the blank and the standard or the sample at the specific wavelengths. Blank baseline subtraction procedure improves the chromatogram scheme in these ways; Baseline closer to 0 AU, no additional peaks, less drift and peaks are easier to integrate.[1]
Cefdinir (CFR) in Omnicef capsules and powder for granules suspension is referred to for the treating of patients with minor to major infections resulting from micro-sensitive strains. CFR, is an extended group of semi-synthetic cephalosporins. Cefdinir compound A is a combination of four isomers called cefdinir open ring lactones a, b, c and d. Its molecular formula is $C_{14}H_{15}N_5O_6S_2$ and molecular weight is 413.43. The molecular formula for cefdinir related compound B is $C_{14}H_{14}N_4O_4S_2$ and molecular weight is 366.41. (See Figure 1(a-m)) for CFR and their related substances.[2] Sodium Benzoate (SB) is chemically known as sodium benzenecarboxylate ($C_7H_5NaO_2$), (Figure. 1(n)). The ingredient is used as excipient; treatment of hyperammonemia due to urea cycle disorders; treatment of non-ketotic hyperglycinemia.[3]
CFR and SDB are formally announced in the European and British Pharmacopeias which illustrated chromatographic method for CFR and a titration one for SDB.[3] While USP prescribed chromatographic method for each.[4] Some new articles were published for determination of cefdinir and its impurities using LC/MS methods,[5-9] HPLC method.[10-11] Only one HPLC & UPLC methods had been reported for simultaneous quantitation of CFR and SDB in their dosage forms, but this method is not indicated for the

determination of impurities and there is no spectrophotometric method for simultaneous optimization of the laboratory mixture of CFR and SDB in their dosage forms,[12] TLC,[13,14] spectrophotometric.[15-21]
The novelty of the proposed method is lying in its ability to detect, identify and separate all related substances to CFR while the previous published methods cannot separate most of related substances in CFR. Besides, it overcomes the overlapping of the binary mixture by using spectrophotometric method without the need of sophisticated application. The unique advantage of the proposed method is the reduction of waste time in manual calculation of impurities through the application of blank subtraction method in which Empower PDA software can subtract the effects of the mobile phase on a standard or sample and peaks will be easier to integrate. Therefore, the main objective of this method is to identify and separate cefdinir and sodium benzoate in the presence of cefdinir's degradation products using blank subtraction method and solving the interference of the binary mixture using simple spectrophotometric method.

EXPERIMENTAL AND REAGENTS

CFR and SDB were provided by hikma pharmaceutical industries (HPI) which is located in Beni-Suef governorate, Egypt. Reference standards of related cefdinir (compound A and B) were purchased from USP store (USA, Rockville, MD). All HPLC- and analytical grades were purchased from (Fisher Scientific, USA).

Instrumentation and Data Processing

Waters UHPLC System (waters corporation, USA), equipped with LC quaternary pump with PDA detector, autosampler and quaternary solvent management, it has the potential for multiple uses and flexibility to move from HPLC and UPLC and provided with Empower™ 3 Software for processing methods.

UV 1900 (Shimadzu- Japan) provide with UV probe (2.7.1) software for processing data.

Chromatographic Method

Chromatographic separation of CFR and its related substances with the preservative SDB are accomplished using a binary gradient consisting of solution A (0.1% tetramethyl ammonium hydroxide solution (pH 5.5) with 0.1M EDTA (1000:0.4 v/v)) and solution B (0.1% tetramethyl ammonium hydroxide solution (pH 5.5): acetonitrile: methanol : 0.1M EDTA (500:300:200:0.4v/v) with Hichrom C18 column (15 × 0.46 cm, 5 µm or equivalent, 150 Å pore size) at flow rate 1.0 mL/min, using injection volume 10 µL for RP-HPLC and wavelength at 254 nm with Auto Sampler Temperature 4.0°C, Column Temperature 40.0°C and gradient program following the scheme: (i) 0–8 min: 95% (A), 5% (B) isocratic; (ii) 8–28 min: 75% (A), 25% (B); (iii) 28–43 min: 50% (A), 50% (B)) and (iv) 43–64 min: 95% (A), 5% (B).

Solution Preparations

A) For laboratory prepared mixture

Cefdinir solution: Accurately weigh about 10 mg CFR WS, transfer to 100 mL volumetric flask, add some solvent, dissolve by sonication, allow to cool and made up to the mark with solvent .
Sodium Benzoate Solution: Accurately weigh about 10 mg SDB, transfer to 100 mL volumetric flask and proceed similarly.
Resolution solution: 10.0 mL of both stock solutions of the above prepared drugs are pipetted into 100 mL volumetric flask, completed to the mark with solvent and the chromatogram is drawn as displayed in Figure. 2 (a).

B) For Related Substances

Solution (1): Weigh about 14.2 g of anhydrous dibasic sodium phosphate, transfer to 1000 mL flask and dissolve in deionized water.

Solution (2): Weigh about 27.2 g of monobasic potassium phosphate and dissolve in 2.0 L of deionized water.
Buffer preparation: Combine the suitable quantities of solutions 1 and 2 (about 2:1) to get a solution having the pH value of 7.0 ± 0.1.
Solvent preparation: Dilute 8 mL of tetramethylammonium hydroxide (25 % in water) to 2000 mL with deionized water; adjust pH to 5.5 ±0.1 using diluted appropriate acid.
Resolution solution preparation:
Stock solution (A): Weigh accurately about 10.0 mg of USP related cefdinir compound A, transfer quantitatively to 250 mL volumetric flask using an appropriate volume of solvent, sonicate till complete dissolution then complete to volume with solvent to get a concentration of 0.04 mg/mL.
Stock solution (B): Weigh accurately about 10.0 mg of USP related cefdinir compound B and proceed similarly to stock solution (B) to get a concentration of 0.04 mg/mL.
Working resolution solution: Weigh accurately about 37.5 mg of CFR, convey quantitatively to 25 mL volumetric flask using 10 mL of buffer. Sonicate till dissolution, complete to volume using buffer. Posteriorly, add 5.0 mL from each solution of related compounds (A&B) and make up to the mark with solvent. Mix well and filter through 0.45 µm nylon membrane, then inject in the HPLC. The HPLC chromatogram is presented in Figure. 2 (b, c)
Cefdinir standard solution preparation: Weigh accurately about 37.5 mg of CFR in 50 mL volumetric flask and complete to volume with buffer. Of the resulting solution, an aliquot of 2 mL is diluted to 100 mL with solvent, mix and filter through the nylon membrane, then inject in the HPLC. The HPLC chromatogram is showed in Figure 2 (d).
Sample solution preparation: Constitute Omnicef as directed in the product label & leaflet. Transfer accurately about the equivalent to 150 mg of Cefdinir in 100 mL volumetric flask using 30 mL buffer and complete to the mark with solvent. Similarly, mix, filter and inject. Figure 2 (e, f).

Calculations:
Identify any peak for solvent and placebo by processing method to be excluded.
Consider any peak that present in the chromatogram other than solvent, placebo, known impurities and cefdinir as unknown impurities.
The percentage of each impurity in the cefdinir portion of the oral suspension powder can be calculated by the below equation:

Result = (Pt / PS) x (Ds / Dt) x (100/F) Where,
Pt = peak area of individual impurity in the test solution
PS = peak area of CFR standard solution
Ds = actual concentration of CFR (mg/mL)
Dt = nominal concentration of cefdinir in the test solution (mg/mL)
F = FRR (factor of relative response)

Procedures

Derivative ratio method (DD^1)

The absorption spectra of CFR were divided by a dvisor of SDB (5 µg/mL) and the first derivative values caused by ratio spectra were registered. The linear curve for maximum and minimum amplitudes at 283.5 nm and 313.4 nm are established versus the congruous concentrations of CFR to calculate the regression equation. Alternatively, the scanned absorption spectra of SDB were divided by a CFR devisor (10 µg/mL) and the first derivative (D^1) were stored. As well, the linear curve for maximum and minimum amplitudes at 216.7 nm and 235.5 nm were dotted against the congruous concentrations of SDB to construct the regression equation.

Ratio subtraction method

The spectra of the bilateral mixture are divided by the advisor of CFR (10 µg/mL), then the amplitudes were subtracted in the plateau region at λ 250-315 nm (the constant) from that ratio spectrum. The zero order spectra of SDB were resolved via multiplying the resulted ratio spectra by the divisor (CFR). The conc of SDB was computed through the congruous regression equation at 225 nm.

RESULTS AND DISCUSSION

Methods development and optimization

Blank subtraction

Before you decide to proceed 3D blank baseline subtraction in empower 3 software, consider whether you are having these types of issues with your chromatography: Incapable to properly integrate the standard or sample due to small noise peaks or a drifting noisy baseline. The blank chromatogram includes characteristics that are worth subtracting (for example, small noise peaks). The blank chromatogram does not change from run to run and 3D blank baseline subtraction does not improve the signal-to-noise ratio of the signal. Blank baseline subtraction removes only the background signal and may increase the noise. After that, select alter sample in the sequence or sample set, then labeling the blank injection with special mark as "B", open the method set used to obtain the data, then press the top of derived channels and select create new derived channel. In the first tab, "first (only) channel", press the channel drop-down list and select "DAD", on the second tab, choose the operator "-" and "DAD" from the channel drop-down list and check box form injection labeled and write down "B", write a name for the new derived channel "Blank Subtraction". press ok, edit the processing method and change channel from "DAD" to "Blank Subtraction", then save method set and process the data with the method set, the blank chromatogram will subtract from sample automatically,[22] as clarified in Figure.2 (g).

Detection of wavelengths

Various wavelengths are checked and scanned at (200 – 400 nm) for 20 µg/mL of each of the mixture members of both pure CFR and SDB drugs and in their dosage forms to accomplish best selectivity wavelength at 254 nm with minimum noise. Figure 3.

Optimization of temperature and flow rates

To achieve the best resolution and separation, many trials were performed on column temperatures at (35, 40, and 45ºC), in addition to changes in flow rates (0.7, 1.0 and 1.3 mL/min); the flow rate of 1.0 mL/min and 40ºC were the best couple for system with good selectivity.

Stationary phase

Preparatory experiments had been performed by trying various columns with different lengths and particle sizes, including Thermo® C18 column (15 × 0.46 cm, 5.0 µm), Agilent ZORBAX -C18 column (15 × 0.46 cm, 5.0 µm) and Hichrom C18 column (0.46 x 15 cm, 5.0 µm, 150 Å pore size) and the last column is the best better selectivity and resolution for peaks of all impurities.

Optimization of gradient program

The binary gradient program is experimented using various system : (1) (i) 0–2 min: 20% (B), 80% (A); (ii) 2–20 min: 30% (B), 70% (A); (iii) 20–35 min: 50% (B:A), (iv) 35–55 min: 80% (A), 20% (B). (2) (i) 0–2 min: 10% (B), 90% (A); (ii) 2–20 min: 30% (B), 70% (A); (iii) 20–35 min: 50% (A,B), (iv) 35–55 min: 90% (A), 10% (B). The followed binary gradient proved as the best system for selectivity and resolution : (i) 0–8 min: 95% (A), 5% (B) isocratic; (ii) 8–28 min: 75% (A), 25% (B); (iii) 28–43 min: 50% (A), 50% (B)) and (iv) 43–64 min: 95% (A), 5% (B).

Derivative ratio method (DD^1)

The method was verified for simultaneous estimation of the compounds to resolve the interference in binary mixtures. DD^1 spectrophotometric method was established to increase the selectivity of the analysis of CFR without interference from SDB. For adjustment the DD^1 method, many concentrations of the SDB as a divisor were tried including 1, 2, 4, and 5 µg/mL of SDB and optimum results were achieved on applying 5 µg/mL of SDB as a divisor. The obtained ratio spectra are distinguished as per the used wavelength, and DD^1 values showed good selectivity at the maximum 283.5 nm and the minimum 313.4 nm (Figure 4). For estimation of SDB in the presence of CFR, many concentrations of CFR are tried including, 2, 5, 10, and 15 µg/mL of CFR, and the best results were achieved when using CFR as a divisor with concentration of 10 µg/mL. The obtained ratio spectra were recorded for maximum and minimum amplitudes at 216.7 nm and 235.5 nm, respectively (Figure 5).

Ratio subtraction method

This method was selected for the estimation of binary mixtures in which the spectrum of one component is more extended than that of the other one. It was applied to solve the overlapping spectra of the mixture of CFR and SDB to get the extended (SDB) in zero order. The method involves dividing of the spectrum of the mixture in the zero-order by divisor of CFR (10 µg/mL). The resulted ratio spectrum is a new graph that represents in plateau region. By subtracting this constant (plateau in 250-315 nm), after that multiplying the new graph by the divisor, the original spectrum of SDB in the mixture can be obtained at 225 nm. Thus, the interference of CFR was removed (Figure 6). Also, the same procedure is repeated to gain the extended (CFR) in the zero order by subtracting the constant which is found in the plateau region (205-230 nm) and multiplying the new graph by the divisor of SDB (5 µg/mL) consequently, the zero-order spectrum of CFR is gained at λ_{max} 283 nm. These data are represented in (Figure 7).

METHOD VALIDATION

The proposed methods have been achieved by following the guidelines of ICH recommended for method validation[23].

Linearity and range

linearity of related substances is evaluated in the range (0.003-0.075 mg/mL) and (0.002-0.050 mg/mL) for CFR and SDB respectively, with correlation coefficient of regression > 0.9999, Y-Intercept of Level 100% response of CFR equal to 0.4 % and (0.005-0.0025 mg/ml) for CFR and SDB for spectrophotometric methods with correlation coefficient of regression > 0.999 . After running each preparation in triplicate, the RSD % of peak area of 3 injections for each level ≤ 2.0%. All the parameters of the regression analysis of the developed methods were presented in Table 1.

Limit of detection and quantitation

The quantitation limit refers to the lowest quantity of analytical material in a sample that can be quantified with appropriate accuracy. The obtained results for limit of detection and limit of quantitation are shown in Table 1.

Precision

System Precision (Repeatability)

The related substances results for six preparations were tabulated and the average of the 12 preparations with RSD % were calculated as listed in Table 1.

Method Precision

Method precision was evaluated by analyzing three different concentrations of the studied drugs, each in triplicate on different days, performed by different analysts and different equipments and %RSD was calculated for both methods, see Table 2 for ruggedness related substances results.

Specificity

Selectivity

If interference is observed (due to placebo, blank, diluent, etc...), it must not exceed 2.0% of the main peak target concentration limit. Placebo preparation was proceeded as under test preparation and (Figure 8 a) confirmed that the API doesn't interfere with the placebo and solvent.

Forced Degradation

The forced degradation of CFR was performed under different acid, base, oxidative, thermal, photolytic and neutral conditions. In order to establish the stability indicating capability of the related substances test method for Omnicef, standard solution of cefdinir was subjected separately to the above mentioned conditions. All degradants of CFR are well resolved and didn't show any interference with CFR peaks. CFR peak was found to be pure under all forced degradation conditions, since that the peak purity angle match for CFR under all conditions was found to be less than the purity threshold as displayed in Table 3 and Figure 8.

i) Acid hydrolysis

Transfer 10 mL of the standard stock solution → 3mL of 1 N HCl, sonicate for 20 min. and mix well, store in room temperature for 2 hours → Add 50 mL solvent → Complete to 100 mL volumetric flask with solvent.

ii) Basic hydrolysis

Applied on 10 mL standard stock solution → 3mL of 1 N NaOH, sonicate for 20 min. and mix well, store in room temperature for 2 hours → Add 50 mL solvent → Complete to 100 mL volumetric flask with solvent.

iii) Oxidation

Transfer 10 mL of the standard stock solution → 3mL of 30 % H_2O_2, sonicate for 20 min. and mix well, store in room temperature for 2 hours → Add 50 mL solvent → Complete with solvent to 100 mL volumetric flask.

iv) Thermal decomposition

Applied on 10 mL of the standard stock solution → heat for 2 hours at 85 °C in water bath → Add 50 mL solvent → Complete to 100 mL volumetric flask with solvent.

v) Light decomposition

Applied on 10 mL of the standard stock solution → Keep in sunlight for 2 hours → Add 50 mL solvent → Complete to 100 mL with solvent.

CONCLUSION

Efficient and novel stability indicating HPLC and spectrophotometric methods have been validated and developed for simultaneous quantification of the CFR and SDB in the presence of its degradants. As for the chromatographic method, it was developed and validated as per ICH guidelines using blank subtraction method on Empower PDA software that reduce waste time in manual calculation of impurities.

Two spectrophotometric methods were developed, the first derivative of ratio spectra spectrophotometric method (1^{st}DD) and the second is ratio subtraction method (RSM) were utilized for resolving the interference between CFR and SDB. Based on peak purity results which have obtained from selectivity and forced degradation analysis, we can confirm that the proposed method is selective and sensitive, and it can be used as stability indicating one for assay and related substance for CFR in QC labs. The proposed method

was demonstrated to achieve shorter time, high sensitivity and save cost of analysis and consumable reagents.

Declaration of Competing Interest

The authors declare that, they have no conflicts of interest

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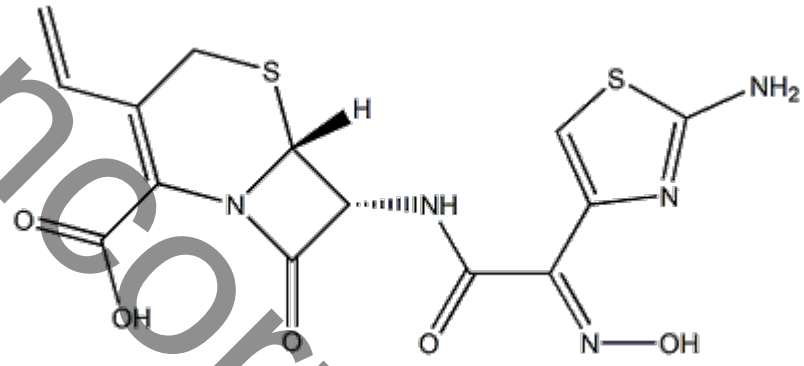

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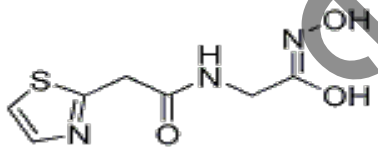

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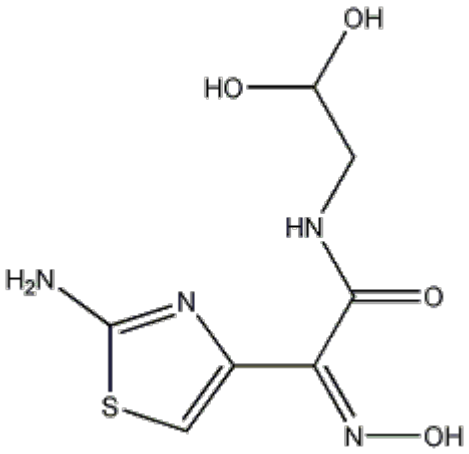
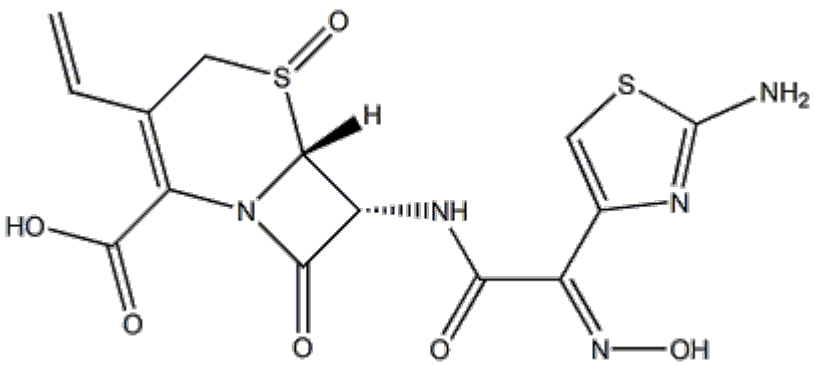


(c)



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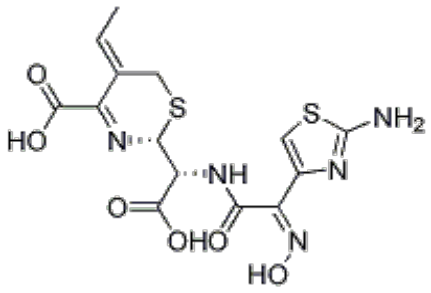

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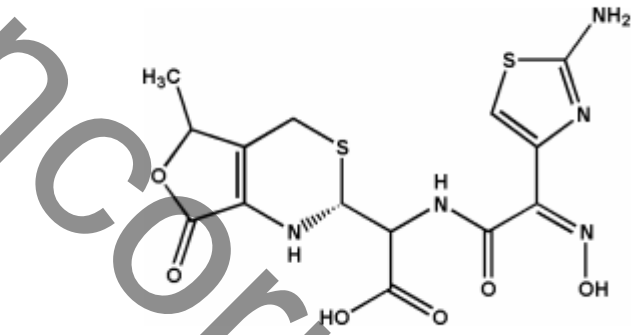

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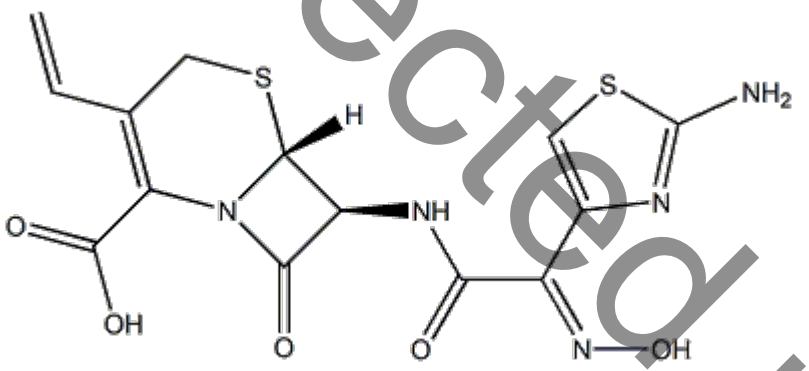

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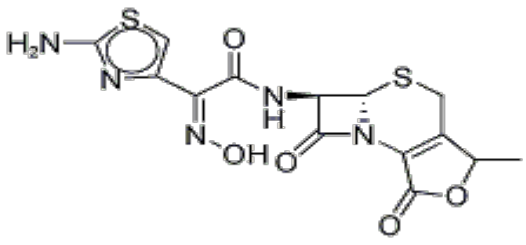

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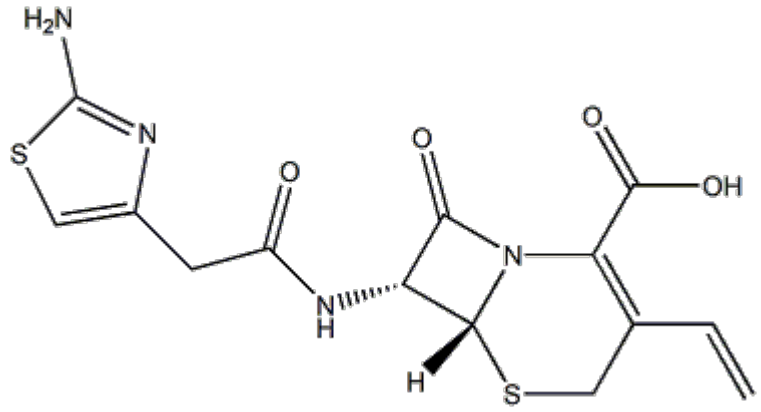

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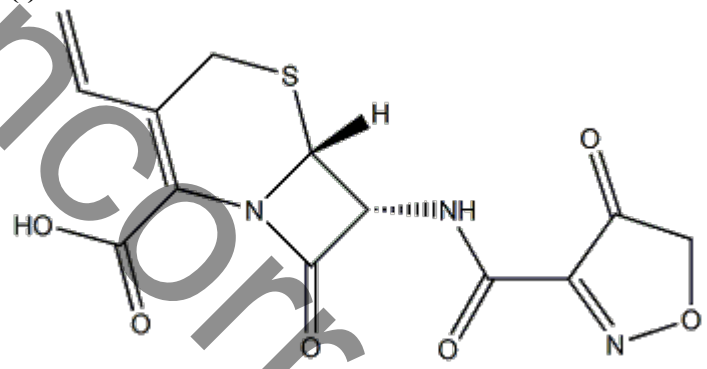

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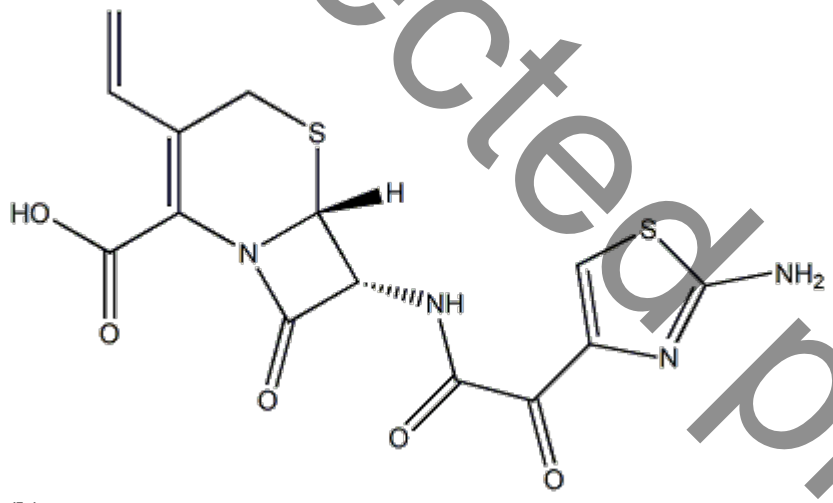

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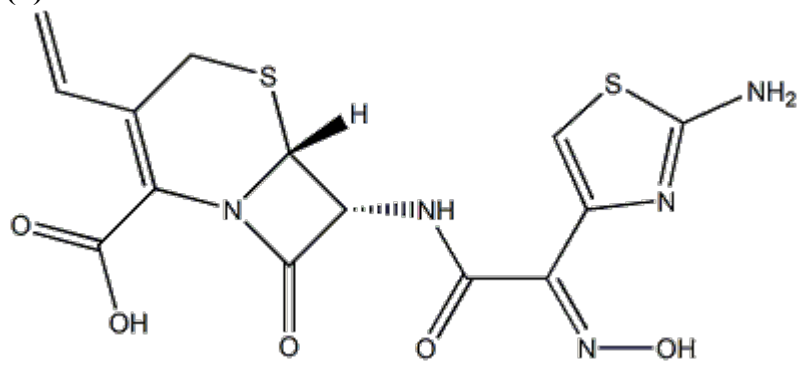

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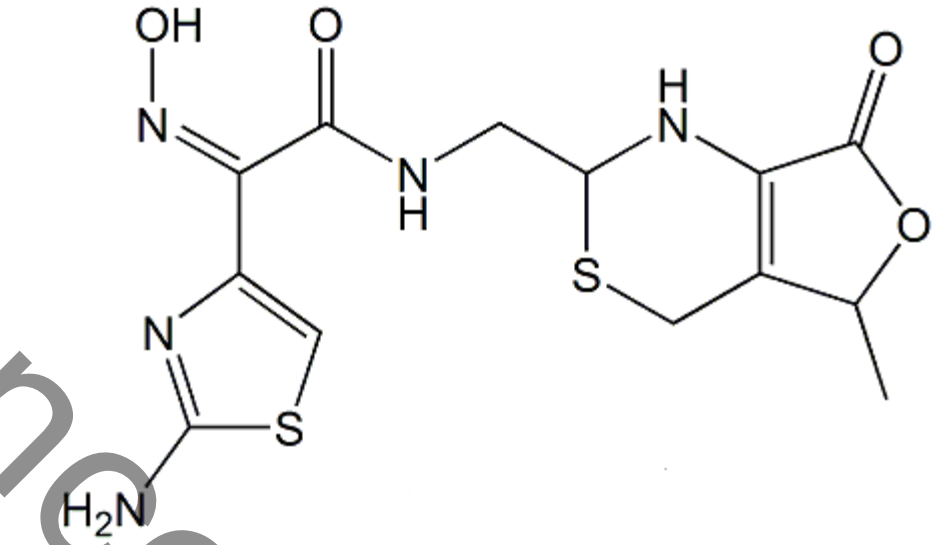

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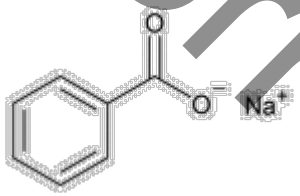
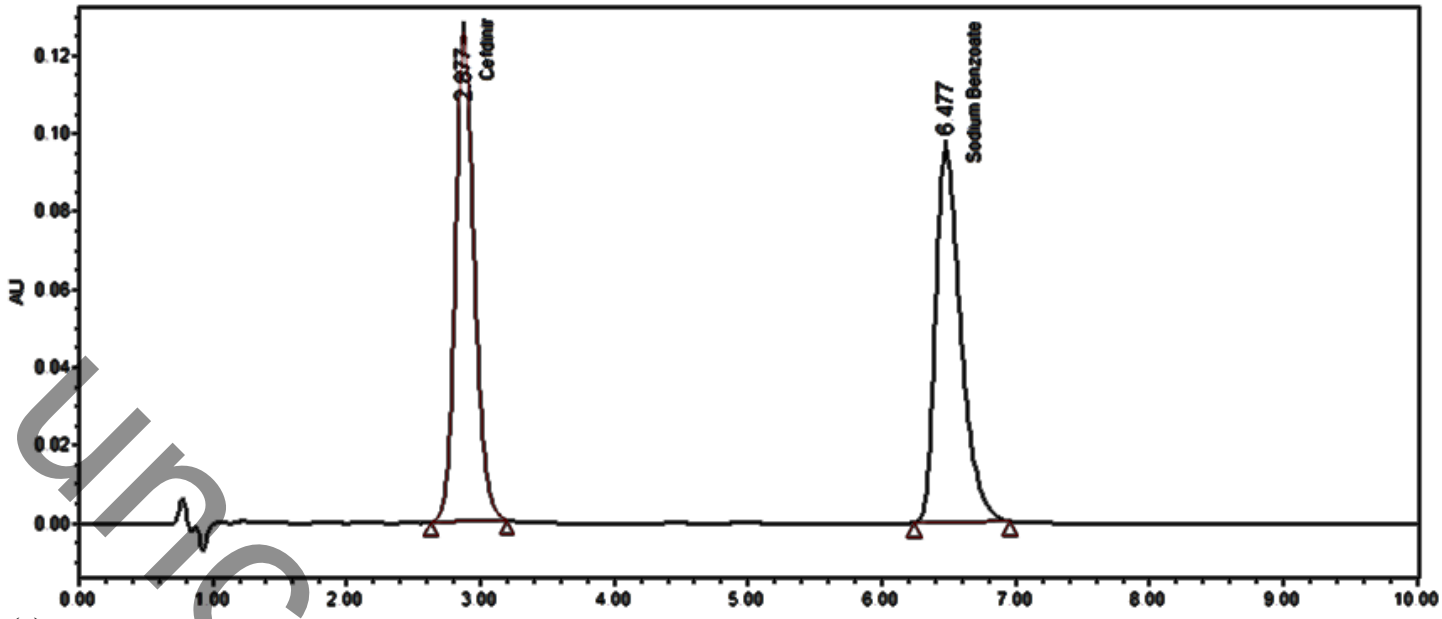
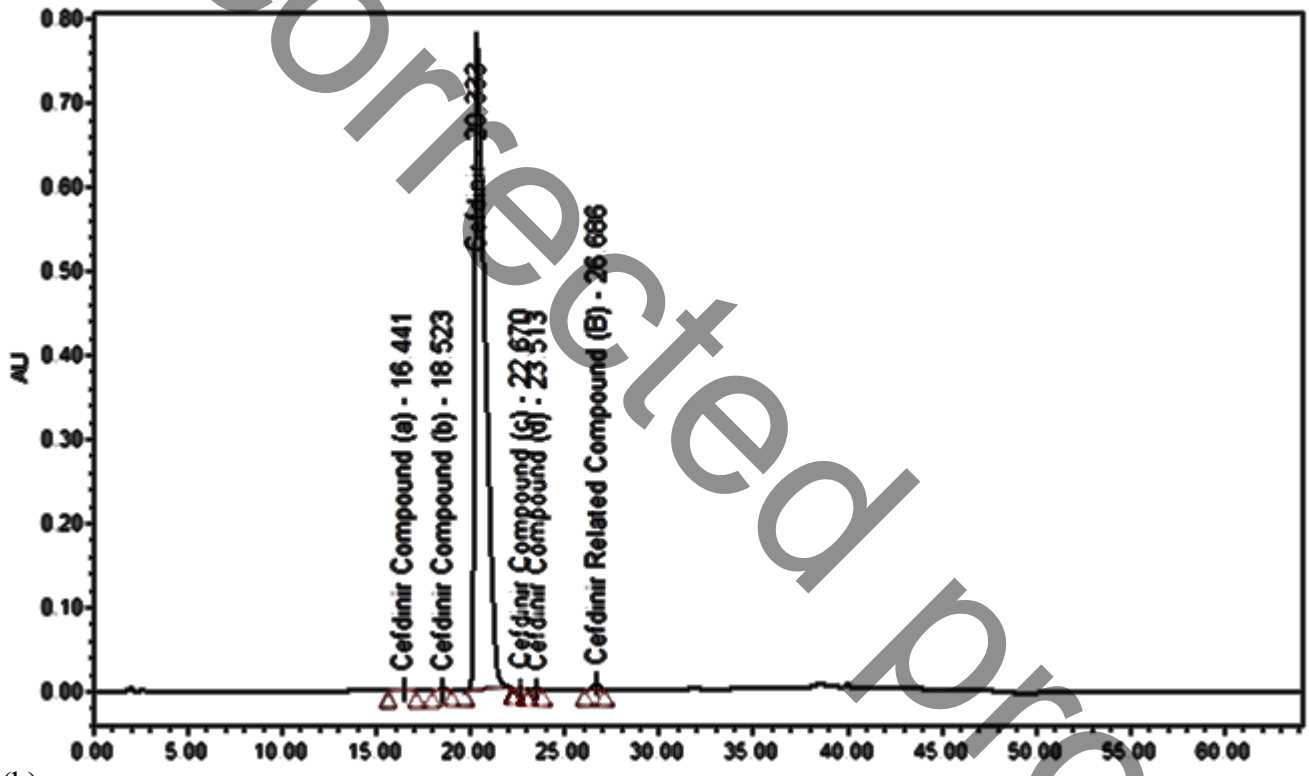


(n)

Figure 1. Chemical structures of (a-m) CFR and their related substances, and (n) SDB.



(a)



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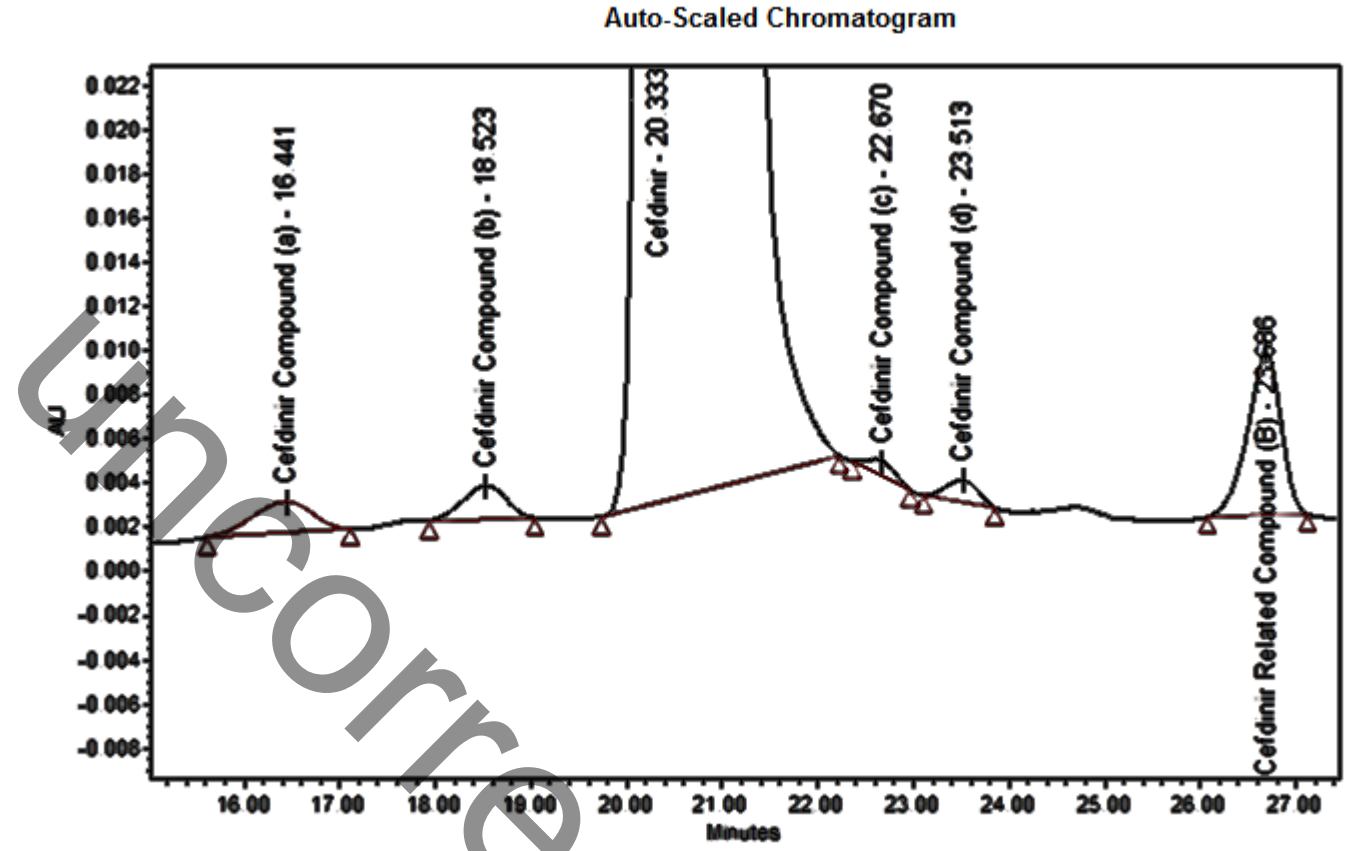

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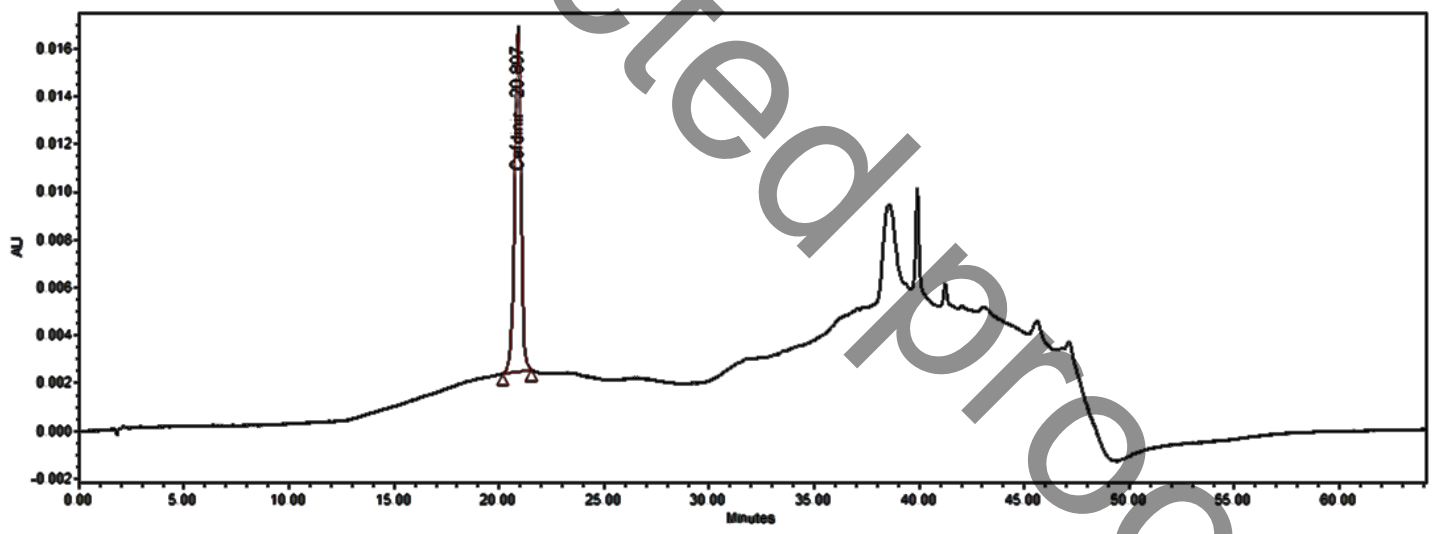

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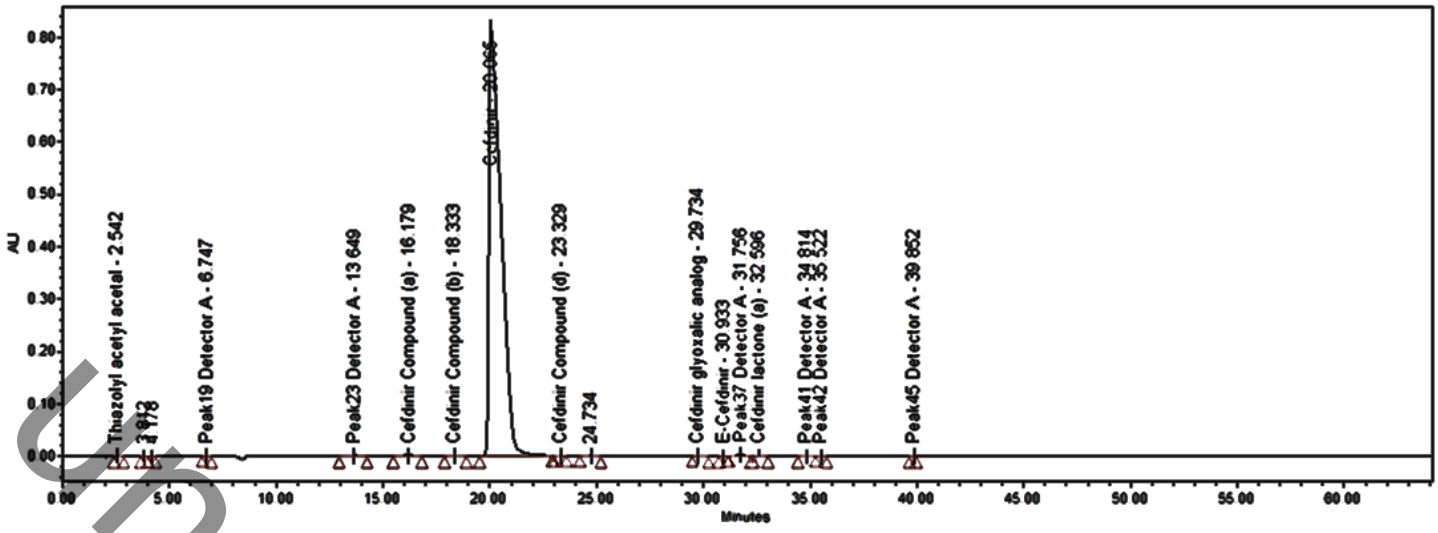

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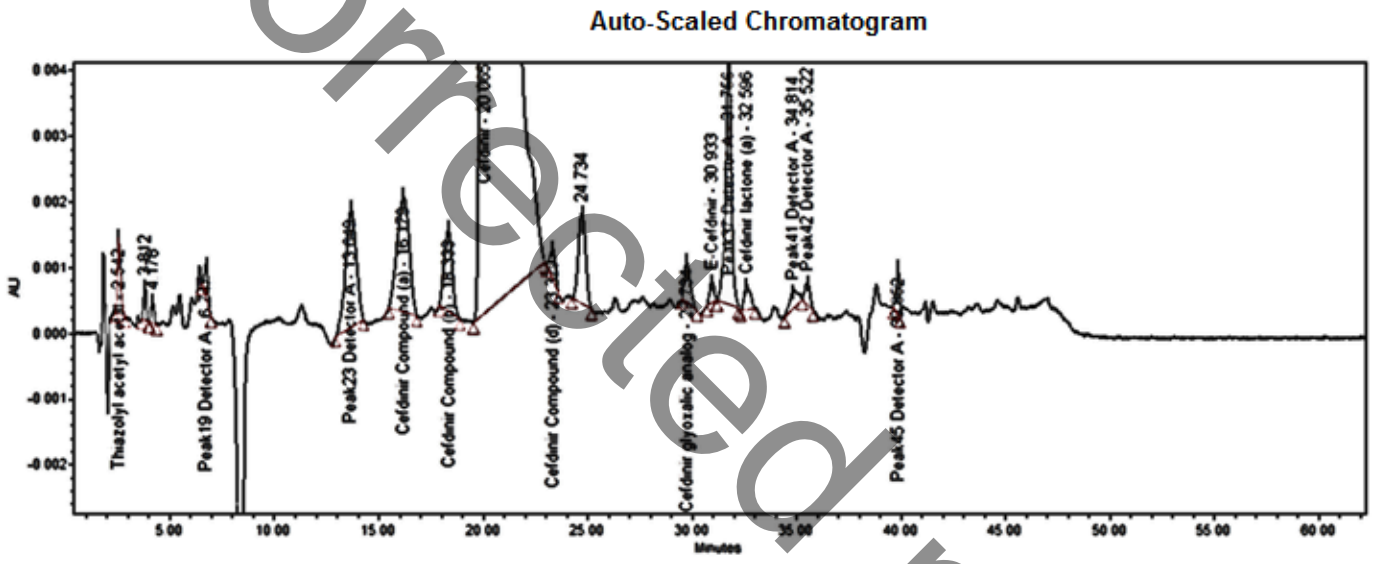

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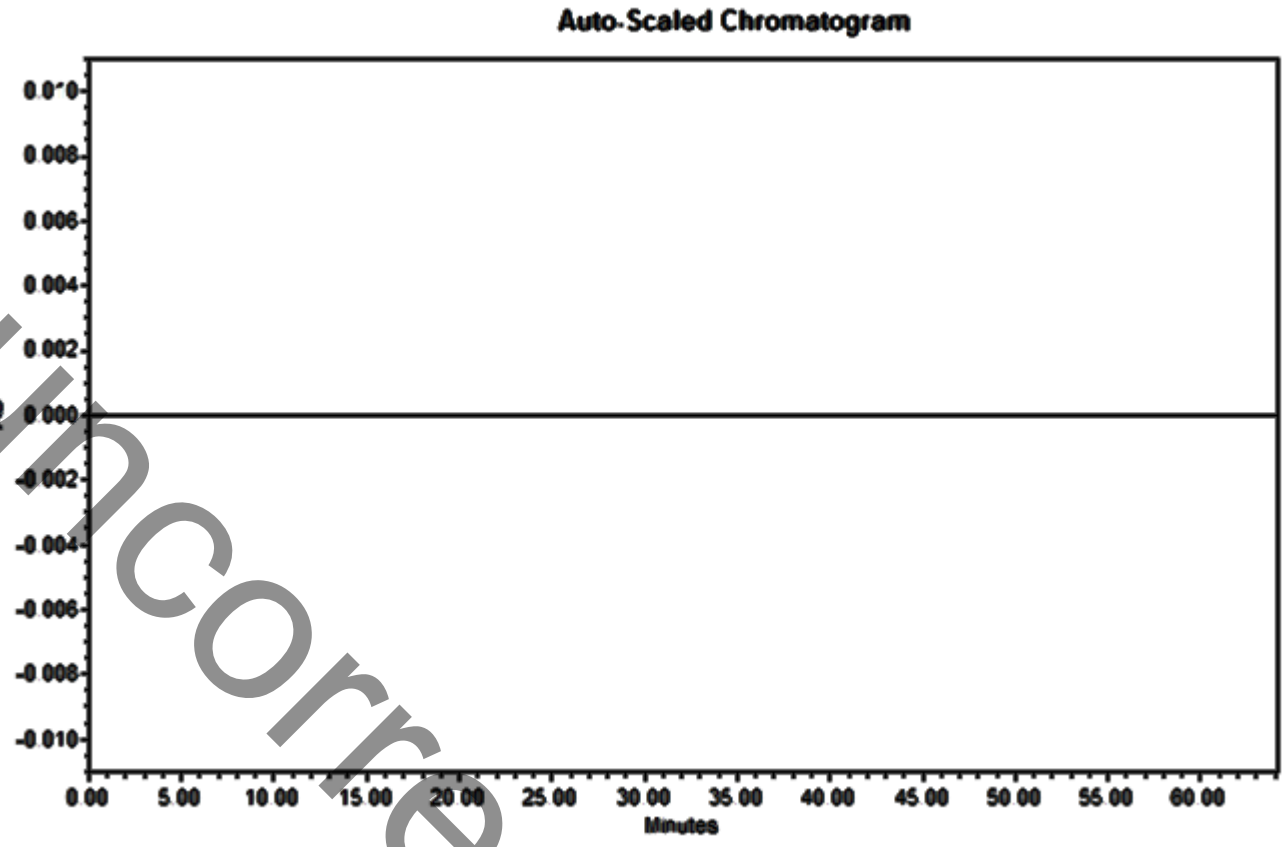


(g)

Figure 2. HPLC chromatograms of (a) 10 µg/mL of laboratory prepared mixture of CFR and SDB, (b, c) 1500 µg/mL of system suitability solution of (CFR and related compound (A, B)), (d) 15 µg/mL of standard solution of (CFR) , (e,f) Sample solution of Omnicef., and (g) Blank subtraction.

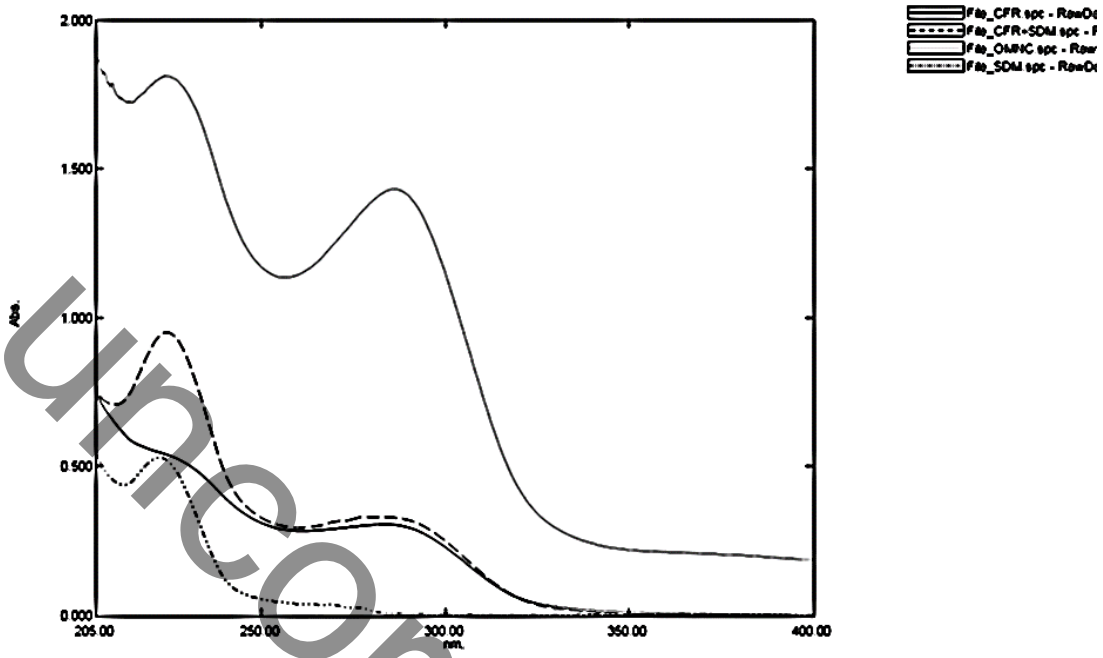
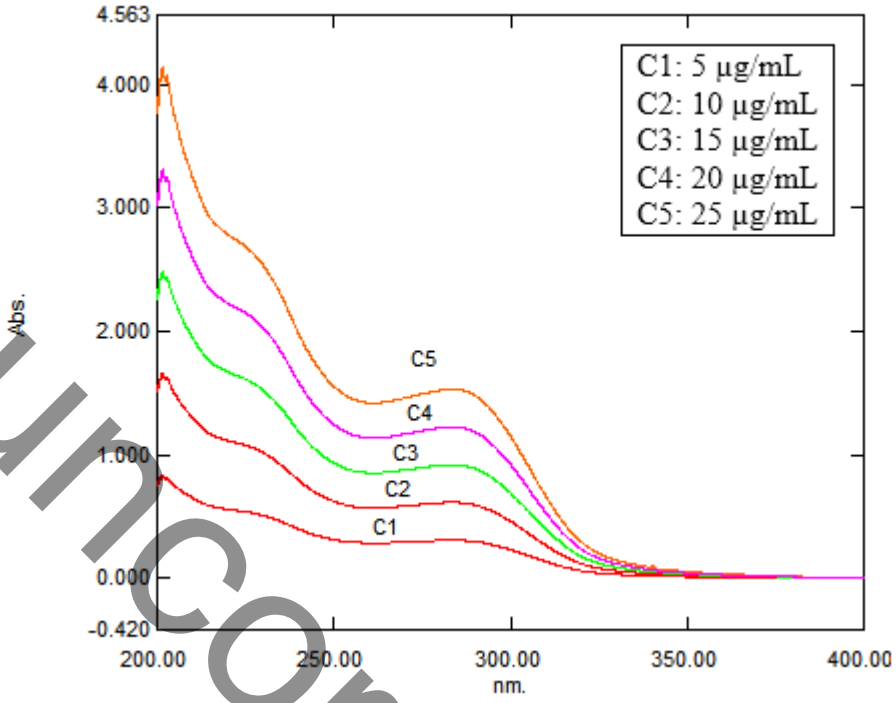
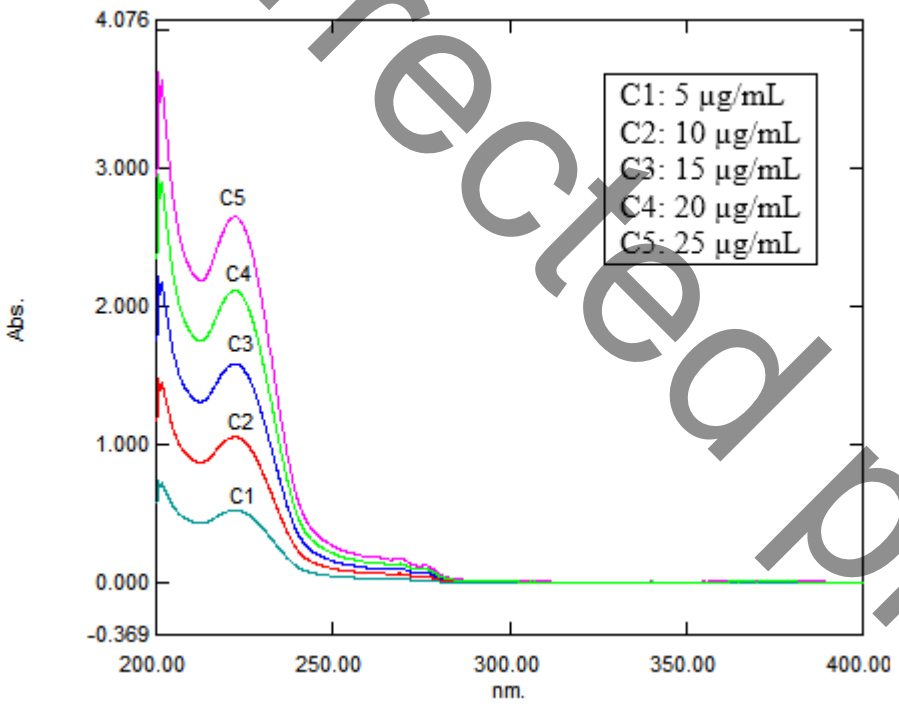


Figure 3. Zero order absorption spectra of 20 µg/mL of each of CFR, SDB, Mix of CFR and SDB and Omnicef 100 mg/5ml using solvent as blank.





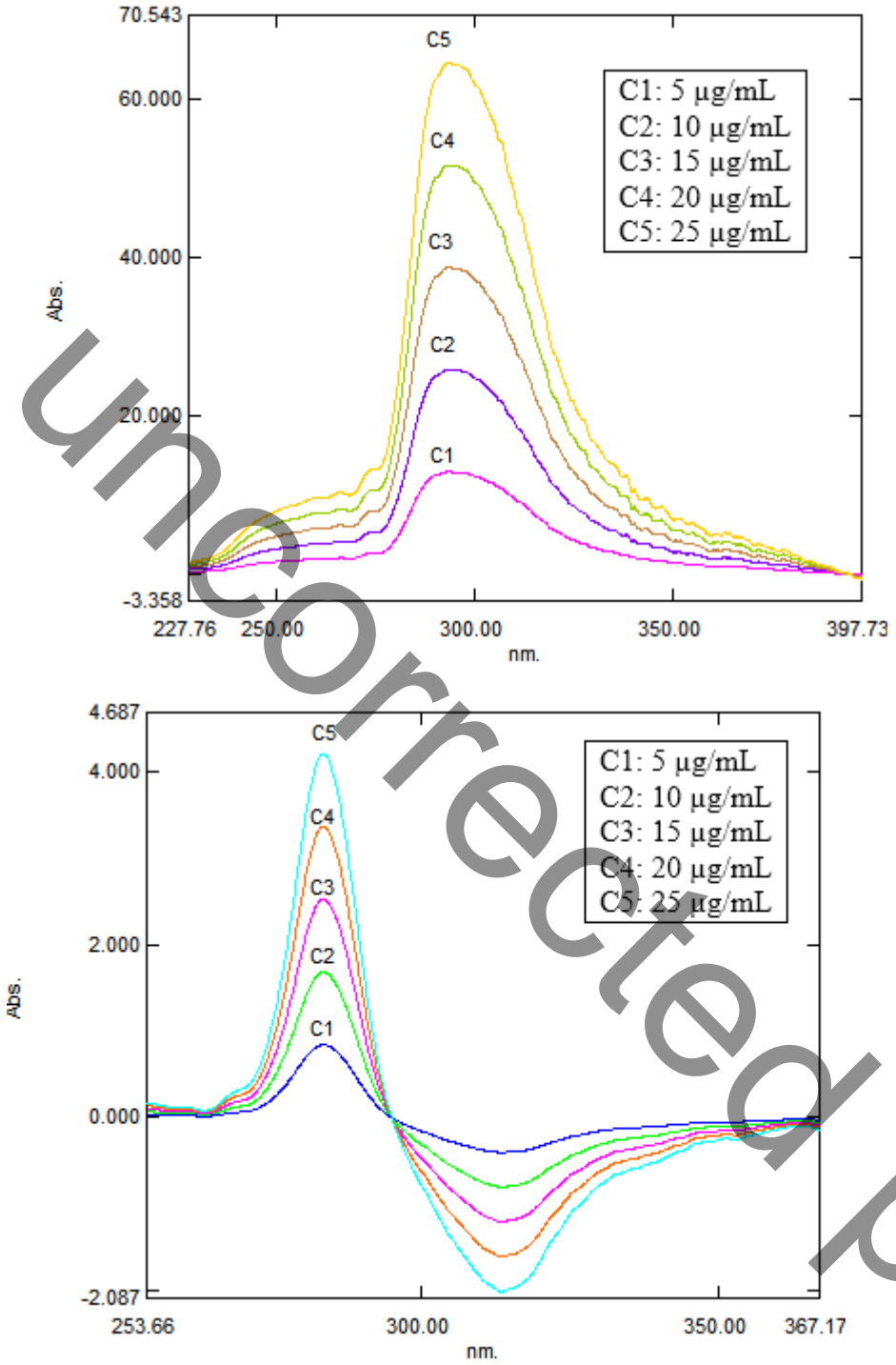


Figure 4. Ratio spectra and first derivative of the ratio spectra of standard solution of CFR using 5 µg/mL of SDB as a divisor and solvent as a blank.

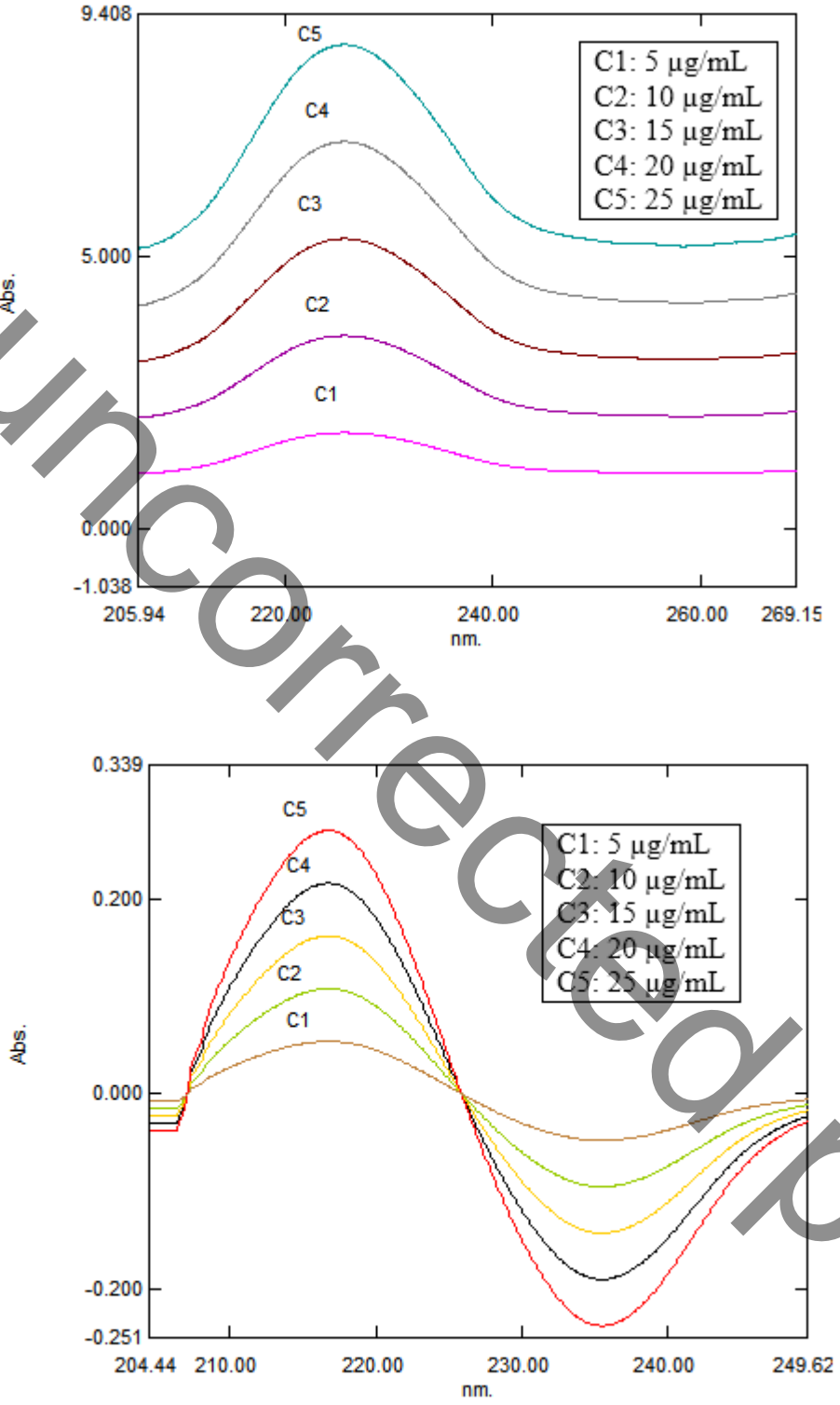
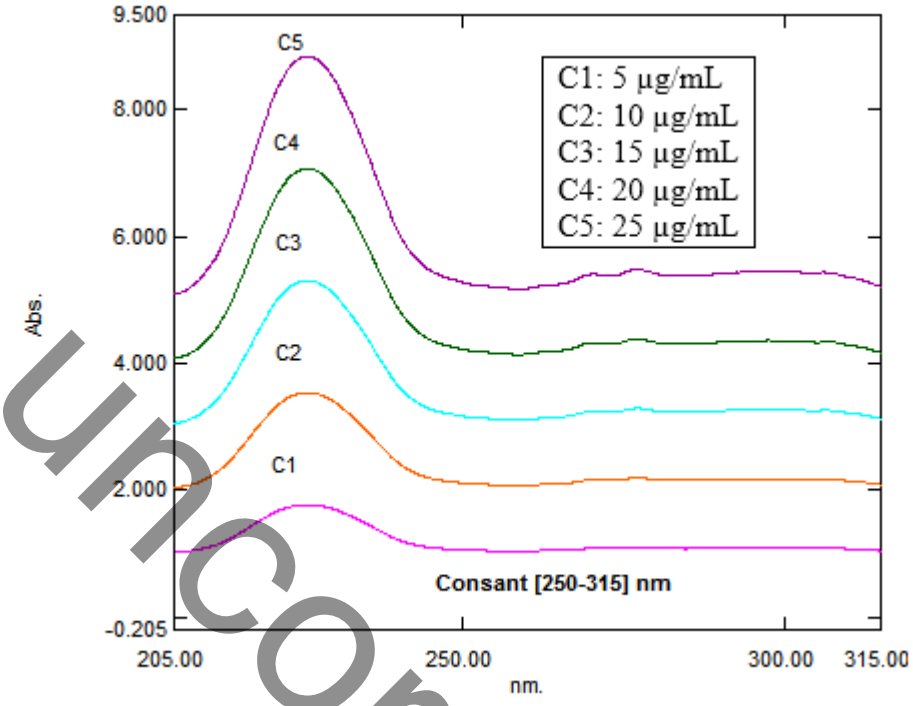
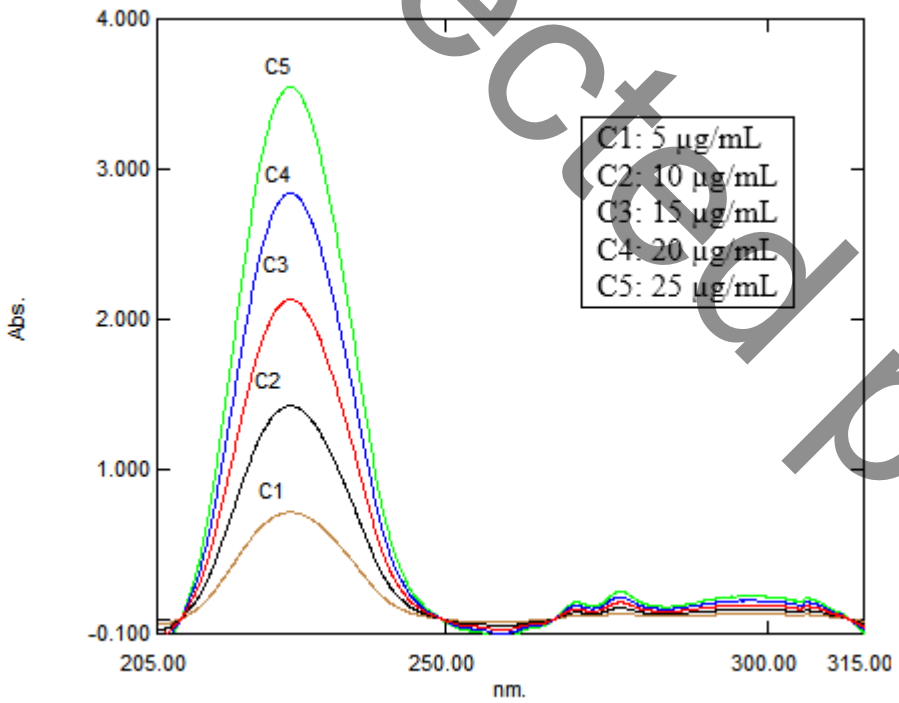


Figure 5. Ratio spectra and first derivative of the ratio spectra of standard solution of SDB using 10 µg/mL of CFR as a divisor and solvent as a blank.



(a)



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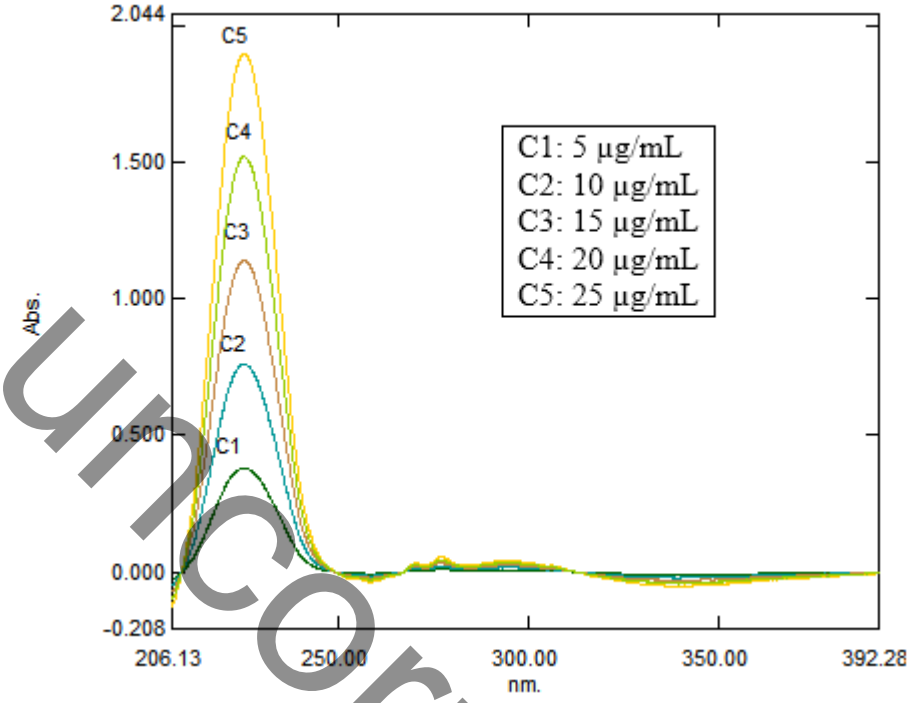
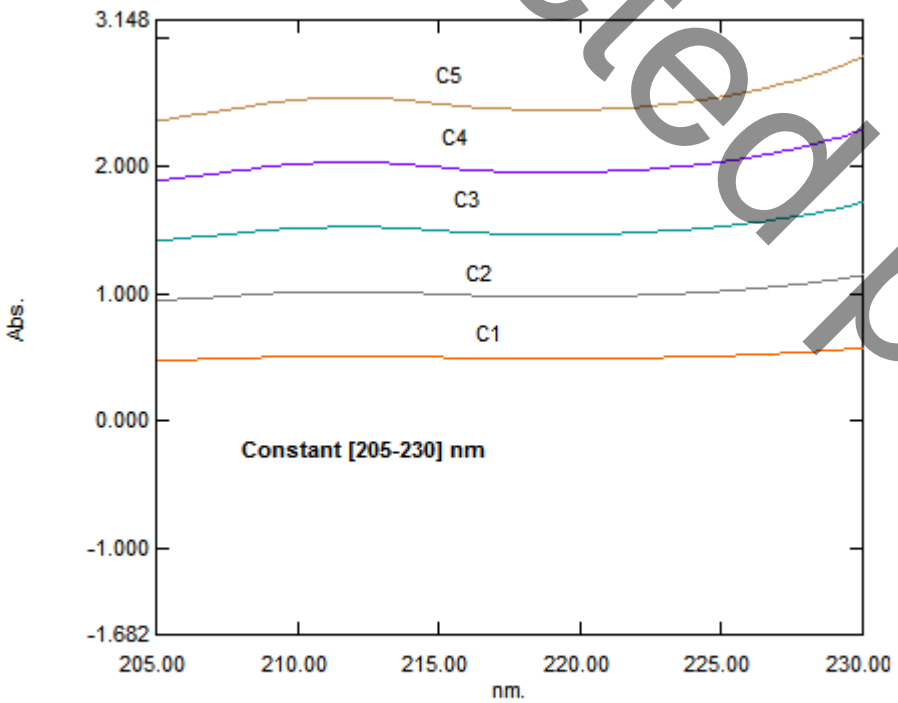
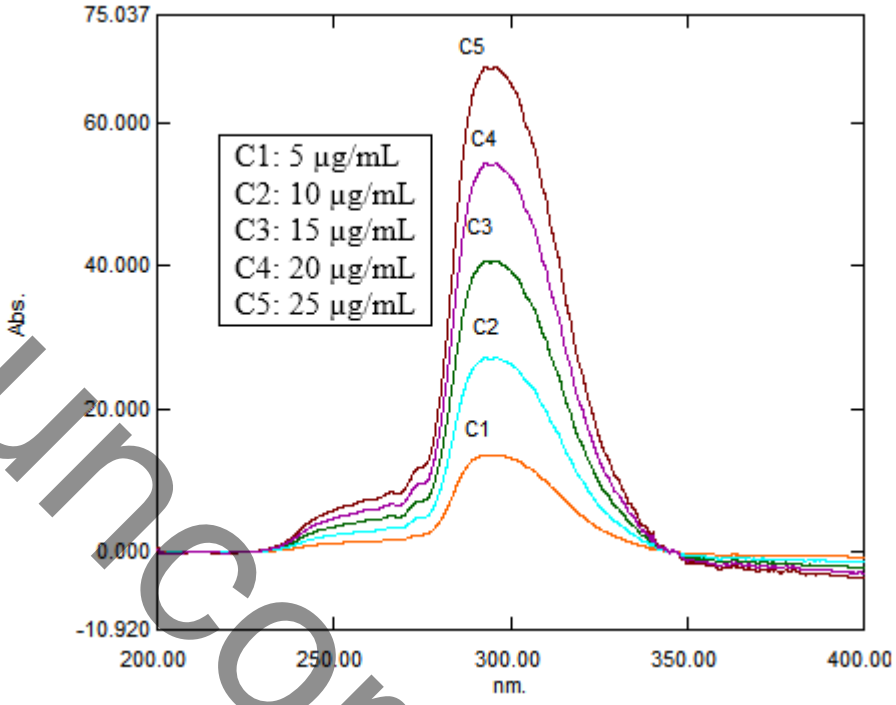


(c)

Figure 6. (a) Ratio spectra of a mixture of CFR and SDB using CFR (10 µg/mL) as a divisor. (b) Subtracting the value of the constant from the ratio spectra (c) The obtained SDB spectrum in zero order.



(a)



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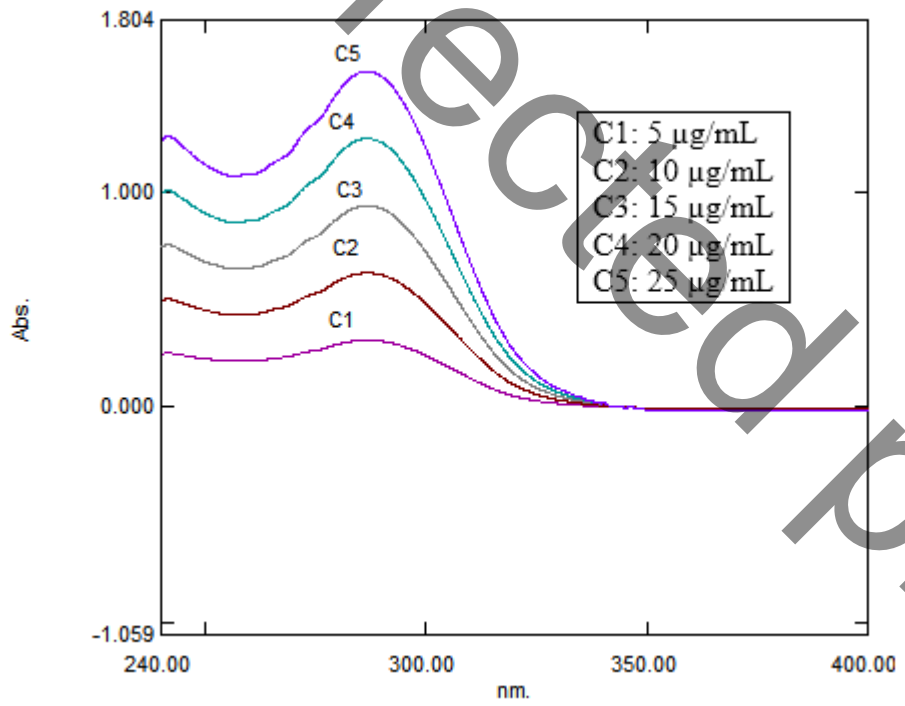
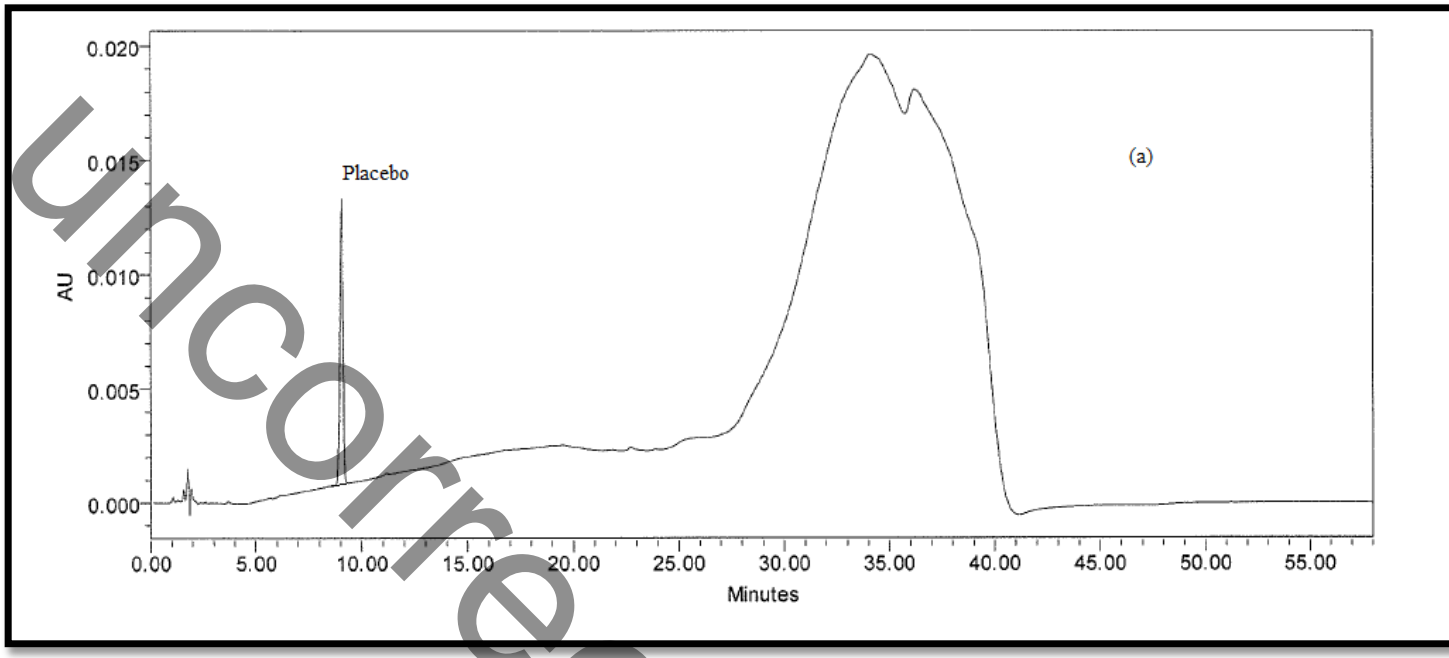
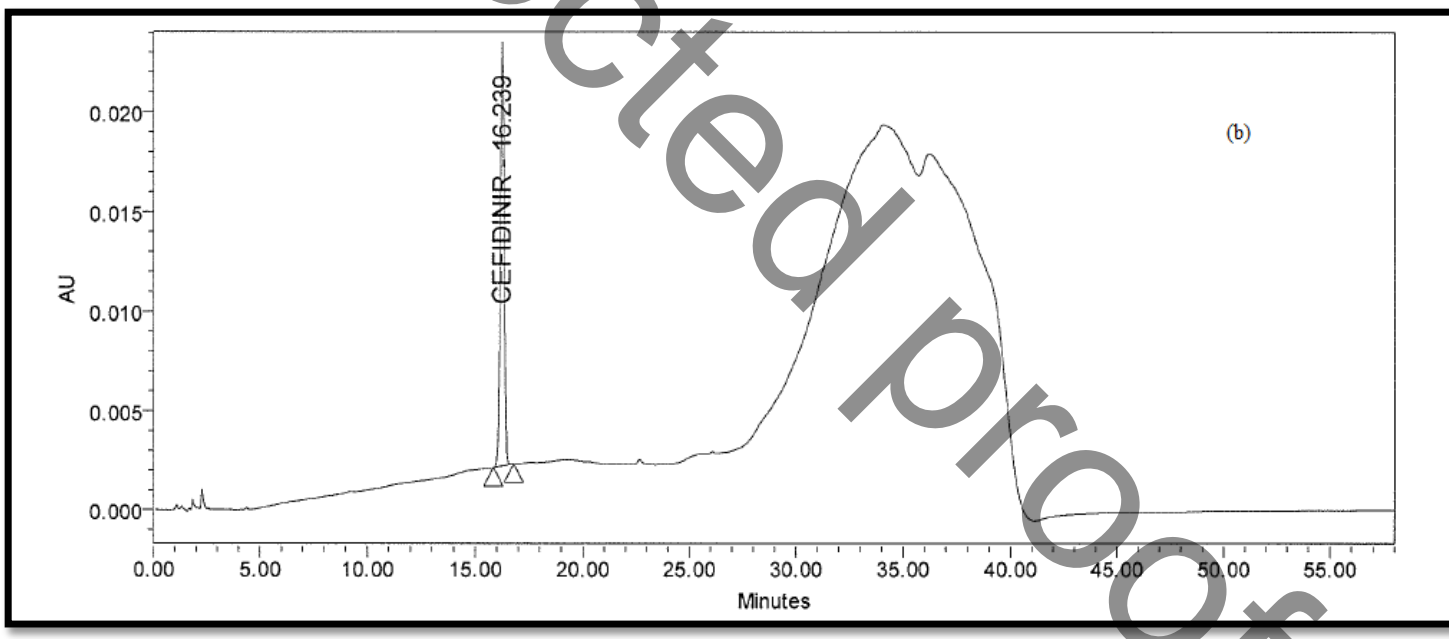
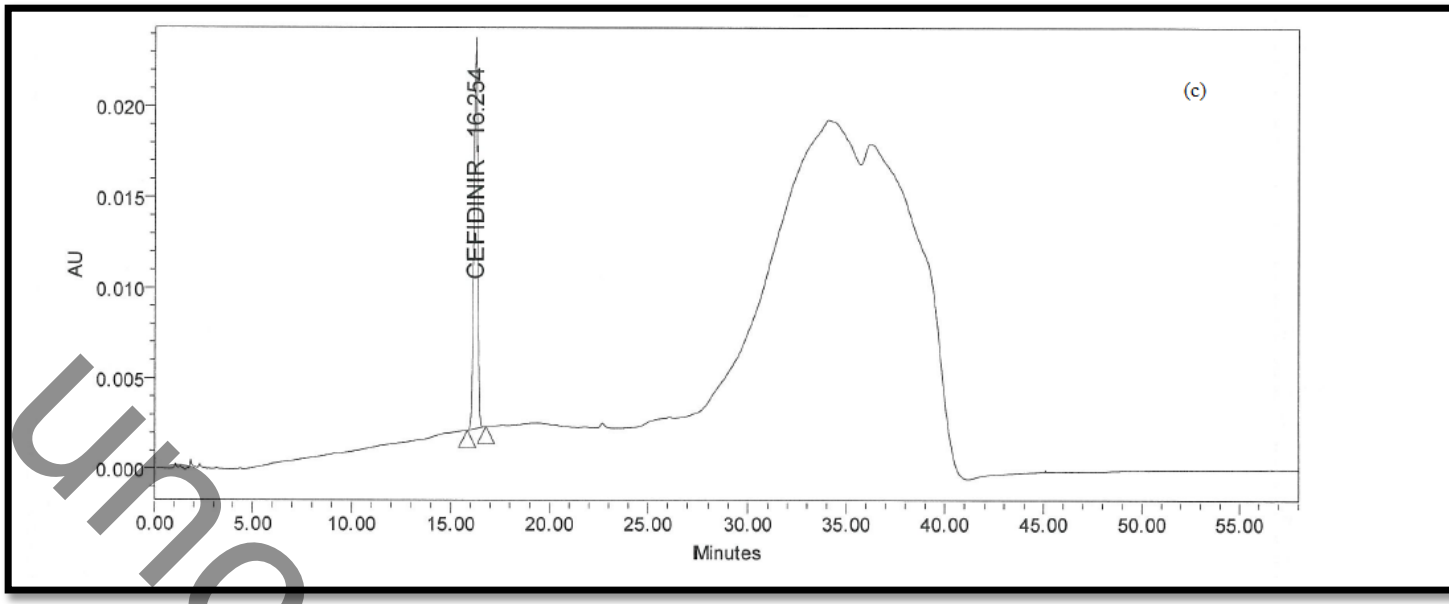


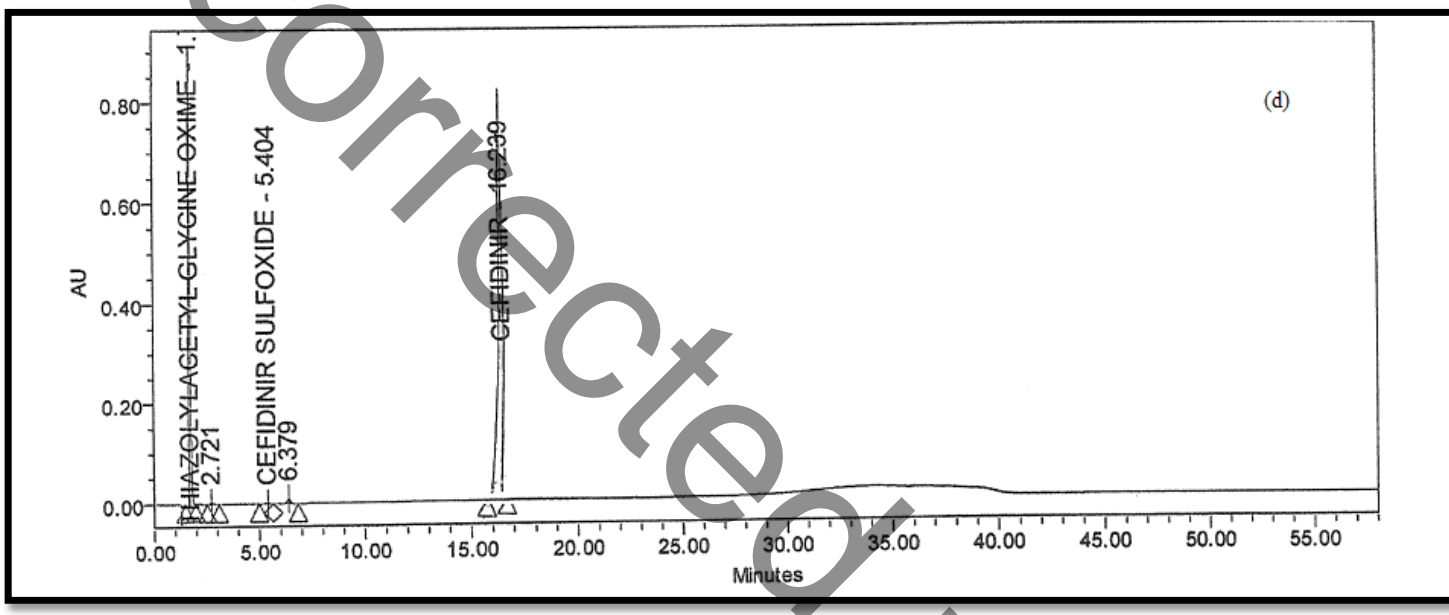
(c)

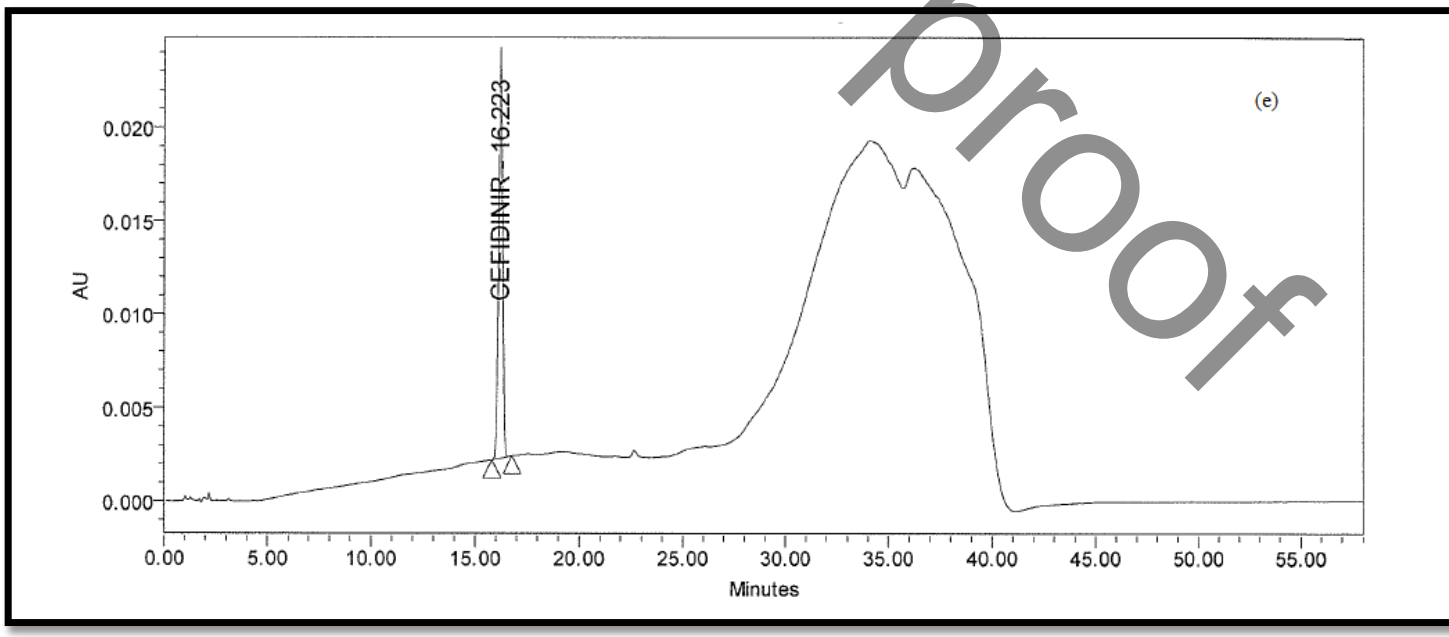
Figure 7. (a) Ratio spectra of a mixture of CFR and SDB using SDB (5 µg/mL) as a divisor. (b) Subtracting the value of the constant from the ratio spectra (c) The obtained CFR spectrum in zero order.











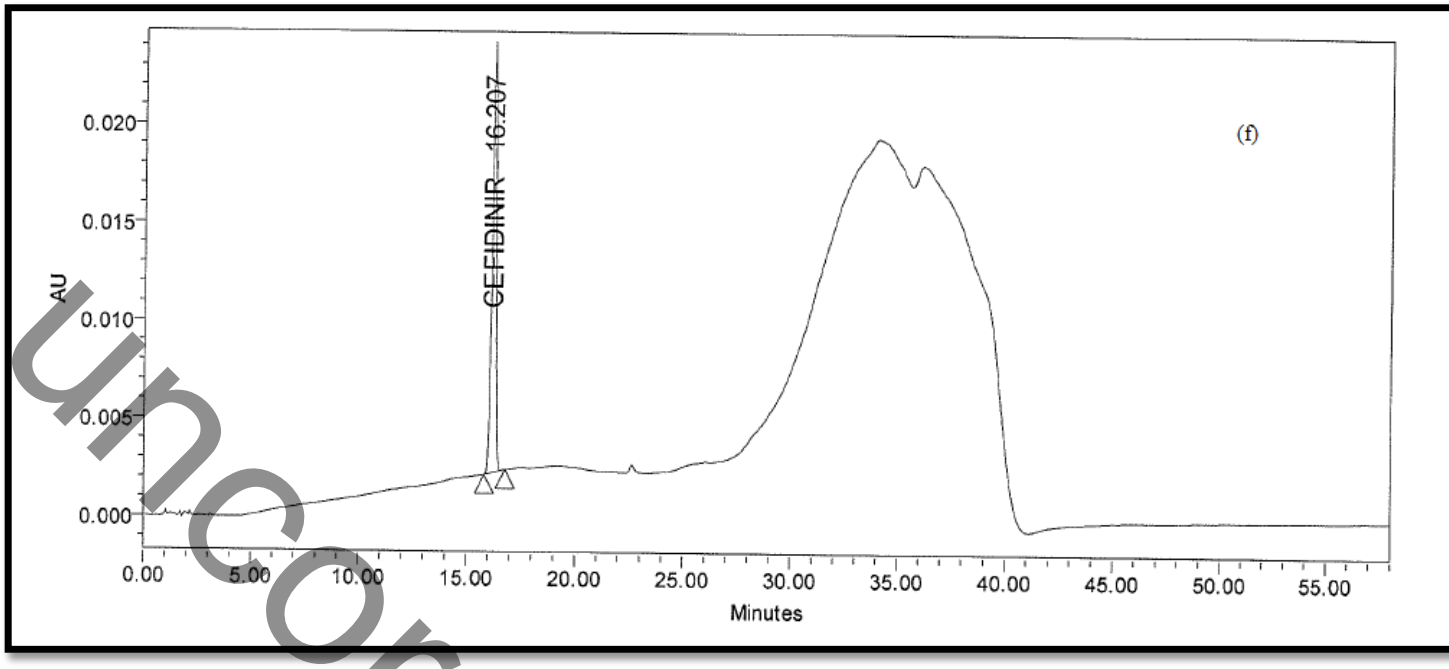


Figure 8. Chromatograms of (a) Placebo, (b) Acid hydrolyzed degraded sample, (c) Base hydrolyzed-degraded sample, (d) Oxidative-degraded sample, (e) Thermal degraded sample, (f) Sun light degraded sample.

Table 1. Regression and validation parameters

Parameter	HPLC		DD[1]		RSM	
	CFR	SDB	CFR	SDB	CFR	SDB
Wavelength	254 nm	254 nm	283.5 nm	216.7 nm	283 nm	225 nm
Range (µg/mL)	3-75	2-50	5-25	5-25	5-25	5-25
Slope	18651283	1850.2	0.0705	0.3150	0.0563	0.3234
Intercept	988.5	1093.6	0.028	0.400	0.0063	0.0250
Correlation coefficient	0.9999	0.9999	0.9996	0.9997	0.9995	0.9996
Repeatability	0.2	0.1	0.3	0.2	0.4	0.2
LOD [a] (µg/mL)	0.41	~~1.42~~ 0.42	0.73	0.36	0.33	0.30
LOQ [a] (µg/mL)	1.22	~~4.29~~ 1.29	2.20	1.11	1.03	0.92

[a] Limit of detection (3.3 × σ /Slope) and limit of quantitation (10 × σ /Slope).

Table 2. **Ruggedness, Robustness and stability of analytical solution of the proposed methods.**

Parameter	**HPLC**		**UV**		**Limit %**
	CFR	**SDB**	**CFR**	**SDB**	
Day to Day	0.80	0.75	0.70	0.64	**RSD ≤ 2.0%**
Analyst to Analyst	1.22	1.13	0.90	0.74	

Column to Column	0.77	0.79	-	-
Flow rate change (±0.1 mL/min)	0.71	0.85	-	-
pH change of mobile phase (±0.2)	0.88	0.79	-	-
Wavelength change (254±2.0 nm)	0.80	0.78	0.76	0.57
Column temperature change (30,25°C)	0.93	0.82	0.89	0.59
Fresh sample	0.12	0.14	0.19	0.22
Stored sample in fridge	0.66	0.47	0.54	0.49
Stored sample at room temperature	0.89	0.94	0.83	0.77

Table 3. Accuracy and recovery of CFR in the proposed method

Conc	Test	Result (%)	Average Result (%)	RSD (%)
50 %	T1 inj-1	99.98 %	99.88 %	0.18 %
	T1 inj-2	99.76 %		
	T2 inj-1	99.72 %		
	T2 inj-2	99.68 %		
	T3 inj-1	100.12 %		
	T3 inj-2	99.98 %		
100%	T1 inj-1	99.66 %	99.49 %	0.10 %
	T1 inj-2	99.53 %		
	T2 inj-1	99.48 %		
	T2 inj-2	99.49 %		

	T3 inj-1	99.40 %		
	T3 inj-2	99.36 %		
150%	T1 inj-1	99.93 %	99.75 %	0.13 %
	T1 inj-2	99.88 %		
	T2 inj-1	99.63 %		
	T2 inj-2	99.73 %		
	T3 inj-1	99.74 %		
	T3 inj-2	99.60 %		

Table 4. Stability indicating capability of the related substances

Cefdinir

Condition	Peak Area	% Degradation	Peak Purity Match
Normal	290534	-	pass
Thermal	283365	2.47 %	pass
Light	283087	2.56 %	pass
Acidic	279986	3.63 %	pass
Basic	283603	2.39 %	pass
Oxidative	19087	93.43 %	pass

Table 5. **System suitability testing parameters of the developed methods.**

Item	**HPLC**	**Reference values**
	CFR	
Tailing factor	0.92	T ≤ 1.5
Injection precision	0.17	RSD ≤1%
Number of theoretical plates (N)	4850	N > 2000
Resolution	3.0	Rs>1.5
Retention time (R_t)(	0.10	RSD ≤1%