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Application of the Azo Coupling Reaction for Ceftriaxone Assay in Drugs using Spectrophotometric Method

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A spectrophotometric method for determining ceftriaxone based on the formation of a red-colored azo compound from the diazotized antibiotic and the phenolic reagent o-cresol has been developed. The optimal conditions for ceftriaxone diazotization were determined to be a 12.0M hydrochloric acid medium with sodium nitrite in 10-fold excess relative to ceftriaxone. Conditions for the azo coupling reaction between ceftriaxone diazonium salt and o-cresol were optimized, with maximum yield achieved in an alkaline medium (final NaOH concentration of 0.16 M) and a 10-fold reagent excess relative to ceftriaxone. The azo compound formed remains stable for 1 hour. Effective molar absorptivity at the absorption maximum (516 nm) for the ceftriaxone–o-cresol azo compound was found to be (6.90 ± 0.16)·10³ L·mol⁻¹·cm⁻¹. The developed spectrophotometric method is sensitive and rapid, allowing ceftriaxone determination within the concentration range of 0.79–51.30 μg·mL⁻¹, with a detection limit of 4.3·10⁻⁷ M. The method was successfully validated for ceftriaxone analysis in pharmaceutical preparations, including mono-component drugs and injection powders.

Keywords: ceftriaxone, *o*-cresol, diazotization, azo coupling, spectrophotometry, drug

Застосування реакції азосполучення для визначення цефтріаксону в лікарських засобах із використанням спектрофотометричного методу

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Розроблено методику спектрофотометричного визначення антибіотика цефтріаксону, яка базується на отриманні забарвленої в червоний колір азосполуки діазотованого антибіотика з фенольним реагентом о-крезолом. Вивчено оптимальні умови діазотування цефалоспоринового антибіотика цефтріаксону: середовище 12,0 М хлоридної кислоти; концентрація натрій нітриту в 10-кратному надлишку по відношенню до концентрації цефтріаксону. Досліджено умови азосполучення солі діазонію цефтріаксону з о-крезолом для максимального виходу забарвленої сполуки: лужне середовище з кінцевою концентрацією NaOH 0,16 М, надлишок реагенту по відношенню до цефтріаксону – 10-кратний. Стабільність утвореної азосполуки становить 1 годину. Ефективний молярний коефіцієнт світлопоглинання з максимумом світлопоглинання за 516 нм для азосполуки ЦЕФТР-КРЕЗ становить (6,90±0,16)·10³ л·моль⁻¹·см⁻¹. На основі встановлених оптимальних умов реакції розроблено нову чутливу, досить швидку методику спектрофотометричного визначення цефтріаксону з о-крезолом. Дана методика дозволяє визначати ЦЕФТР в діапазоні концентрацій 0,79-51,30 мкг·мл⁻¹, межа виявлення цефтріаксону становить 4,3·10⁻⁷ М. Запропонована методика була успішно апробована при визначенні ЦЕФТР в монокомпонентному препараті, порошках для приготування розчину для ін'єкцій.

Ключові слова: цефтріаксон, *о*-крезол, діазотування, азосполучення, спектрофотометрія, лікарський засіб

Introduction

Antibiotics are substances of natural, semi-synthetic and synthetic origin that have a selective bacteriostatic or bactericidal effect [1]. Cephalosporin antibiotics are bicyclic compounds consisting of β-lactam and dihydrothiazine rings, which form 7-aminocephalosporinic acid the same for all cephalosporins molecules. Ceftriaxone (Table S1, [2-5]) is one of the most common β-lactam antibiotics on the pharmaceutical market. It is a reason, that drugs based on ceftriaxone are most often falsified, so it is necessary to control their quality at various stages of production and distribution.

The ceftriaxone molecule has several functional groups in its structure (Scheme S1), which allows the use of various approaches and methods for the development of CEFTR quantification techniques. To identify the CEFTR in the substance are suggests pharmacopoeia methods such as infrared absorption spectrophotometry (IR) and the thin-layer chromatography (TLC) as well as liquid chromatography with UV detection is used to determine the quantitative content of CEFTR in drugs [1].

According to the literature data [2], in addition to chromatography, spectrophotometry is most often used for the quantitative determination of CEFTR, and electrochemical methods, in particular, voltammetry, are used less. A new trend in the analysis of ceftriaxone is the use of fluorescent [6-8], optical [9], and electrochemical [10, 11] sensors. The most important factor when choosing an analytical method is the composition of the analyte samples. Thus, spectrophotometry is used for the analysis of active pharmaceutical ingredient in bulk and simple drugs. When monitoring the content of AN in complex objects such as biological fluids, food products, combined medicinal and veterinary drugs or environmental objects, it is advisable to use chromatography [2,12, 13].

The active use of spectrophotometry in the analysis of pharmaceutical products can be explained by the availability, cheapness, and ease of implementation in combination with the good accuracy of this method. It is known that most pharmaceutical substances can be determined due to their own absorbance in the UV spectrum range. In addition, some of them enter into chemical reactions that lead to the formation of colored compounds. In general, all spectrophotometric methods for CEFTR determining can be divided into several groups:

• Spectrophotometry in the UV range based on own absorbance. It is known to use UV spectrophotometry in the range of 230–265 nm for the analysis of ceftriaxone due to the formation of its alkaline hydrolysis products [2] or in the availability of a phosphate buffer solution at pH 7.4 with maximum absorbance λ_{max} = 340 and 360 nm [14]. The described methods are sufficiently sensitive, but not selective, since many other organic substances of different classes absorb the light in this spectrum range.

• Formation of colored derivatives in diazotization-azo coupling reactions. The reaction underlying this method is two-stage, and relatively long in time, but it does not require complex reagents. Cephalosporin antibiotics undergo an azo coupling reaction after diazotization of the primary amino group in the thiazole ring. In the literature, there are several methods for cephalosporins determination, which are based on its diazotization and subsequent azo coupling with *p*-dimethylamino benzaldehyde [15, 16], *N*-(1-naphthyl)-ethylenediamine dihydrochloride, resorcinol and chloroglucin [17], 2-naphthol in an aqueous solution [18] and methanol solution [19], β-naphthol with subseqwent cloud point extraction [20], 3-amino phenol [21], 2,5-dimethylphenol (2,5-DMP) and 4-tret-butylphenol (4-TBP) [22]. However, the conditions of CEFTR diazotization are very controversial. The main problem is the formation of the by-product of self-azo coupling of the diazotized cephalosporin molecules already in an acidic medium [23], which many researchers do not consider in their studies. To overcome this problem, when obtaining colored azo compounds of ceftriaxone, like ceftazidime, concentrated (12.0 M) hydrochloric acid should be used for diazotization [24,25]. The high acidity of the medium makes it impossible the side reactions undergone during diazotization, in particular, the CEFTR's self-azo coupling reaction [26].

• Different methods based on the formation of colored compounds of the ceftriaxone interaction with metal ions [2], dyes [27], and colored products obtained as a result of the oxidation reaction [2].

Despite the large number of known spectrophotometric methods for determining ceftriaxone, many of them have some disadvantages: the use of hard-to-reach and rare reagents, the use of organic solvents, are multistage (previous hydrolysis), time-consuming and require heating.

To obtain colored azo compounds, phenolic compounds are widely used as an azo component. We chose cresol (CRES) as a reagent (Table S2) for our research, which easily undergoes an electrophilic substitution as well as condensation reactions with aldehydes, when mixed with chlorine water in the presence of HCl it forms yellow-brown colored compounds. In the literature, there is no data on the use of *o*-cresol as an analytical reagent. Instead, *o*-cresol is widely used in the synthesis of azo dyes and for the production of resins. The forms of existence and some characteristics of *o*-cresol are presented at Scheme S2 [28].

Experimental part

The solutions of ceftriaxone (USP Reference Standards with the main substance content not less than 99%, Switzerland) were prepared by dissolving the appropriate amounts of the reagent in distilled

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water. The working solutions were stored at room temperature in a dark place for no longer than two days.

The solutions of *o*-cresol (Sigma-Aldrich, Germany) were prepared by dissolving appropriate amounts of the reagents of pharmacopoeial grade (≥99%) in a 4.0 M sodium hydroxide solution immediately before the experiment.

The solutions of hydrochloric acid, sulfamic acid, sodium hydroxide, and sodium nitrite were prepared by dissolving the reagent from chemicals of the analytical grade in distilled water.

UV-VIS measurements were performed with UV-VIS scanning spectrophotometer SPECORD M-40 (Carl Zeiss Jena, Germany) using l cm cuvettes. All absorbance measurements were performed at 20–25°C.

Voltammetric measurements were performed by computer controlled digital device MTech OVA-410 [29] with temperature-controlled three-electrode cell (10 mL). An indicator dropping mercury electrode, a saturated calomel reference electrode and platinum wire auxiliary electrode were used. The utilized DME had the following characteristics: m = 1.46 mg·s^{-1}; τ = 6.0 s at μ = 0.3 (blank solution). Analytical studies were performed at the linear potential range from -0.2 to -1.1 V, potential sweep rate was 2.5 mV·s^{-1}, the delay imposition voltage was 3.6 s. The accuracy of potential measurements is 1 mV. The uncertainty of current measurement is 0.1%. Voltammograms were recorded at a room temperature (*ca.* 20°C). Dissolved oxygen was removed from the test solutions by means of purified argon bubbling for 15 ± 1 min.

The procedure of sample preparation for CEFTR determination in the powders for the preparing a solution for injections "Ceftriaxone" and "EMSEF"

The accurate amount of powder, containing ~ 1000 mg of CEFTR, was placed into a 100.0 mL volumetric flask and dissolved in 50.0 mL of distilled water. Then the solution was interfusing for 10 minutes and diluted with the same solvent to the full volume of 100.0 mL (stock solution). The working solution of CEFTR is prepared by diluting the stock solution 10 times. For this purpose, 5.0 mL of the stock solution is placed into a 50.0 mL volumetric flask diluted with distilled water to the total volume of 50.0 mL, and mixed thoroughly. For the assay solution, an aliquot of 1.0 mL was picked out and then treated as described below in the recommended procedure for ceftriaxone determination.

General procedure of ceftriaxone determination with o-cresol

An aliquot of the tested ceftriaxone solution containing 0.21–51.30 μg·mL^{-1} of CEFTR (final concentration) was placed into a 25.0 ml volumetric flask, then 0.5 mL of 12.0 M hydrochloric acid solution and 2.5 mL of 9.3·10^{-3} M sodium nitrite solution were added. Next 2.5 mL of 4.6·10^{-3} M *o*-cresol solution prepared by dissolving appropriate amounts of the reagents in 4.0 M sodium hydroxide solution was added into the flask and distilled water was added to the full volume of 25.0 mL. Then the solution was mixed thoroughly and the absorbance measurements (at the room temperature ~ 20 °C) were carried out against all corresponding reagents blank solution at 516 nm in 1 cm cuvettes. Ceftriaxone concentration was calculated using the methods of the calibration curve and single-point calibration.

Results and discussion

It is established that ceftriaxone does not directly interact with *o*-cresol, but CEFTR diazonium salts azocouple with CRES in an alkaline solution and form the red-colored azo compound, which is characterized by a new absorbance maximum at 516 nm (Fig. 1). As can be seen from Fig. 1, the use of sulfamic acid to eliminate unreacted excess of sodium nitrite leads to a slight decrease in the analytical signal, therefore it is inappropriate to eliminate unreacted sodium nitrite in further studies. This effect can be explained by nitrosation of the reagent under the action of nitrite ions, which contributes to the electron density conjugation in the formed azo product and, accordingly, increases solution absorbance.

The molecule of CEFTR includes a primary aromatic amino group. It is known from the literature that the diazotization process is influenced by some factors such as the nature and concentration of the acid for the reaction medium, temperature, the concentration of the diazotizing agent sodium nitrite, and the diazotation duration.

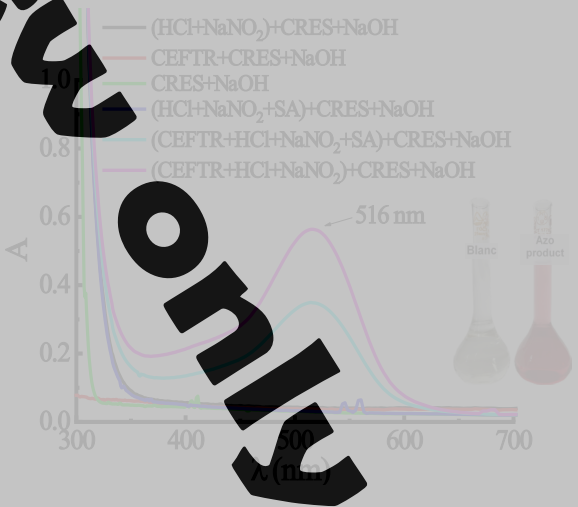

Fig. 1. Absorption spectra of solutions of ceftriaxone, CEFTR diazonium salt, *o*-cresol and azo products of CEFTR diazonium salt interaction with CRES. Diazotization conditions: C_{CEFTR} = 7.4·10^{-5} M; C_{HCl} = 12.0 M (initial conc., V = 0.5 mL); C_{NaNO2} = 7.4·10^{-4} M, $t_{diazot.}$ = 5 min, C_{SA} = 1.0·10^{-3} M. Conditions for azo coupling: C_{CRES} = 7.0·10^{-4} M, C_{NaOH} = 0.26 M; l = 1 cm.

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For analytical purposes, the diazotization reaction is carried out when the reaction medium is cooled (0-5 ºC) or at room temperature, but there are known cases of diazotization with heating. The temperature regime of the reaction largely depends on the nature of the amine involved in diazotization [30]. In our previous studies, it was shown that the reaction temperature affects the duration of the diazotization reaction of sulphanilamides. At room temperature (20 ºC), the maximum amount of the reaction product is formed a little slower, as when an ice bath was used [31]. However, in the case of cephalosporins, the diazotization reaction undergoes very quickly even at room temperature (less than 5 min), so there is no reason to use an ice bath for cooling.

According to the reaction mechanism, diazotization theoretically requires a two-fold excess of the acid relative to the aromatic amine. In fact, strong mineral acid is taken in more than a threefold excess. The high acidity of the medium is necessary to stop two side reactions involving the analyte, namely the formation of a triazene (diazo imino compound), or an amino azo compound. In a solution with a low pH value, the concentration of free amine is sharply reduced, thereby suppressing both undesirable processes [27, 30].

As shown in the paper [25] the undesirable co-product of the diazotized CEFTR's azo coupling when using 1.0 M HCl for diazotization is not formed within 5 min. However, during the diazotization of more than 5 min, these own azo coupling product of ceftriaxone diazo salt with maximum absorbance at λ_{max} = 460 nm is formed even when diazotization is carried out in a medium of concentrated hydrochloric acid. Since the acidity of the medium in which diazotization of ceftriaxone is carried out affects the self-azo compound formation, it was important to determine the lowest concentration of hydrochloric acid at which this product is not formed, still, the yield of the azo compound of diazotized CEFTR with the *o*-cresol is maximal. The results of studies on the influence of the concentration of hydrochloric acid on a medium for CEFTR diazotization and the subsequent azo coupling of obtained diazonium salt with CRES (Fig. 2a) are showed that the formation of the maximum amount of the azo coupling product of diazotized CEFTR with reagent occurs when diazotization is carried out in a medium of 12.0 M hydrochloric acid.

The next stage was the study of the duration of ceftriaxone diazotization for further formation of the azo compound CEFTR with CRES. The results of the investigation (Fig. 2b) show that the highest absorbance of the obtained azo compound is reached when the time for the diazotization reaction is 4–6 minutes. With a further increase in the diazotization time, the yield of the target products much decreases, as evidenced by a decrease in product solutions absorbance. It is probably concerned with the simultaneous formation of the product of CEFTR's azo compound and, as a consequence, a decrease of the available diazonium salt for azo coupling with the investigated reagent. However, 5 min is sufficient for diazotization and to addition of an alkaline solution of the reagent to obtain the expected product, which is formed almost instantly. Therefore, we chose 5 min for CEFTR diazotization in the next studies.

The influence of the concentration of diazotizing reagent sodium nitrite on the formation of the CEFTR diazonium salt and its subsequent azo coupling with CRES (Fig. 2c) was investigated. According to the mechanism of the diazotization reaction, two molecules of nitric acid are required to obtain a nitrosonium cation, besides, excess of one of the reagents shifts the reaction equilibrium towards forming more reaction products. However, at the large excess of sodium nitrite, the unstable forms of diazo compounds can be obtained, and oxidation and asphaltization can take place.

As shown in Fig. 2c to use sodium nitrite with a concentration of $1.0 \cdot 10^{-3}$ M, that is, its 10-fold excess toward the ceftriaxone concentration is optimal.

According to the literature data, the azo coupling reaction between diazonium salt and phenolic compounds occurs in an alkaline medium. Since we used concentrated hydrochloric acid for CEFTR diazotization, it was necessary to use a concentrated alkali solution to neutralize a significant amount of hydroxonium ions. The effect of NaOH concentration on the interaction of diazotized CEFTR with CRES was studied (Fig. 3a). The maximum yield of the azo coupling product is observed at an alkali residual concentration in the reaction medium of 0.16 M.

To establish the maximal azo coupling product yield, the search for the optimal reagent excess of the *o*-cresol was carried out. We found (Fig. 3b) that CRES in excess towards diazotized CEFTR to obtain the maximum yield of colored azo product 10-fold reagent is required. The azo product absorbance does not change when the CRES concentration is further increased.

Since the consecutive stages of an azo compound formation take place in a different media, which has a significant effect on the reagents and the yield of colored products it was important to investigate the order of adding components to the mixture. The results of these studies are shown in the Table S3.

The maximum absorbance of the obtained azo compound is observed if diazotization and subsequent azo coupling of reagents are carried out in the following sequence (Table S3). An aliquot of the CEFTR solution, the 12.0 M hydrochloric acid, and sodium nitrite solution are added first for CEFTR diazonium salt formation. Finally, the appropriate alkaline solution of the reagent *o*-cresol is added for azo coupling.

The absorbance stability of the obtained azo product of diazotized CEFTR with CRES was investigated. As follows from experimental data, absorbance value of

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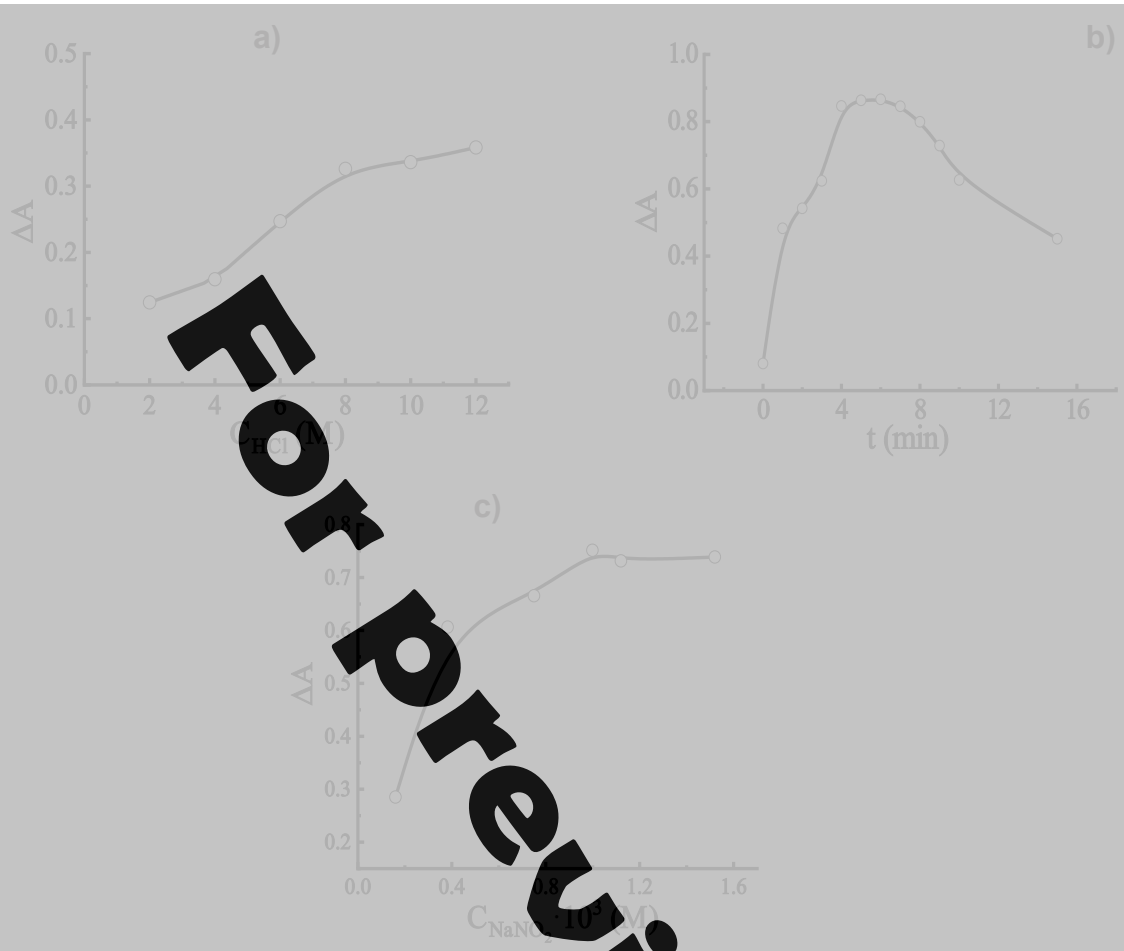

Fig. 2. Effect of a) hydrochloric acid concentration, b) time of CEFTR diazotization, c) sodium nitrite concentration on the ceftriaxone diazotization and the subsequent azo coupling with *o*-cresol. λ_{max} = 516 nm, *l* = 1 cm. Diazotization conditions a): C_{CEFTR} = 7.4·10^{-5} M, C_{NaNO2} = 7.4·10^{-4} M, $t_{diazot.}$ = 5 min. Conditions for azo coupling a): C_{CRES} = 7.0·10^{-4} M, C_{NaOH} = 0.26 M; Diazotization conditions b): C_{HCl} = 12.0 M (initial conc., V = 0.5 mL), C_{CEFTR}=7.4·10^{-5} M, C_{NaNO2}=7.0·10^{-4} M. Conditions for azo coupling b): C_{CRES} = 7.0·10^{-4} M, C_{NaOH} = 0.26 M; Diazotization conditions c): C_{HCl} = 12.0 M (initial conc., V = 0.5 mL), C_{CEFTR} = 1.0·10^{-4} M, $t_{diazot.}$ = 5 min. Conditions for azo coupling c): C_{CRES} = 7.0·10^{-4} M, C_{NaOH} = 0.16 M.

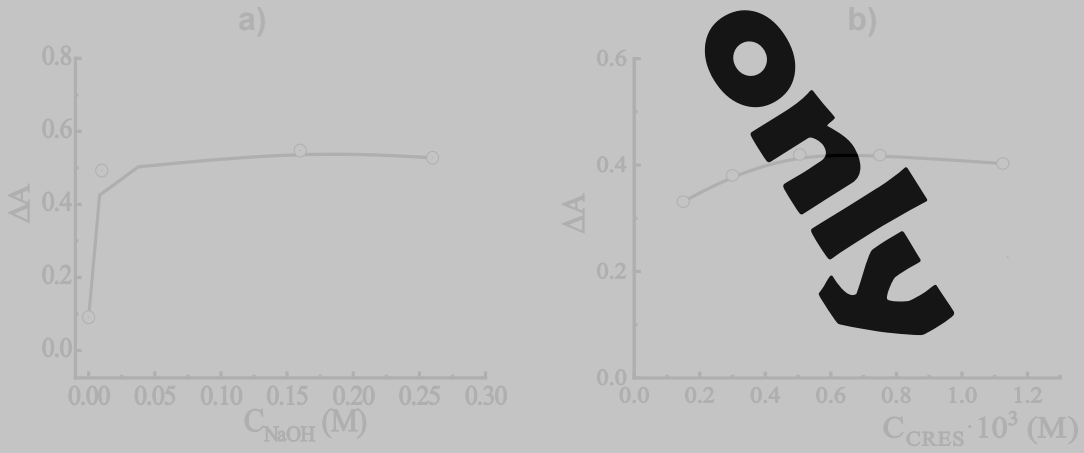

Fig. 3. Effect of a) sodium hydroxide concentration and b) reagent (CRES) concentration on the absorbance of CEFTR diazonium salt azo coupling products with CRES. λ_{max} = 516 nm, *l* = 1 cm. Diazotization conditions a): C_{HCl} = 12.0 M (initial conc., V = 0.5 mL), C_{CEFTR} = 1.0·10^{-4} M, C_{NaNO2} = 7.4·10^{-4} M, $t_{diazot.}$ = 5 min. Conditions for azo coupling a): C_{CRES} = 7.0·10^{-4} M; Diazotization conditions b): C_{HCl} = 12.0 M (initial conc., V = 0.5 mL), C_{CEFTR} = 5.0·10^{-5} M, C_{NaNO2} = 5.0·10^{-4} M, $t_{diazot.}$ = 5 min. Conditions for azo coupling b): C_{NaOH} = 0.16 M.

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examined azo compound did not change for a long time (more than 50 minutes), what is sufficient for assay.

The method of continuous variations was used to establish the mole ratio of the components in the compounds of diazotized CEFTR with *o*-cresol (Fig. 4). As can be seen from Fig. 4, when diazotized CEFTR interacts with CRES, the azo coupling product is formed with the stoichiometric ratio of components equal to 1:1.

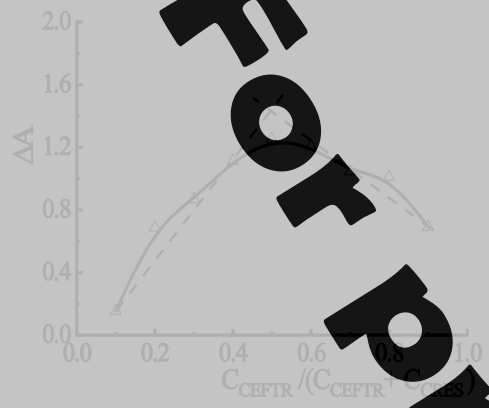

Fig. 4. The continuous variation curve for the azo compound of diazotized ceftriaxone and CRES. $C_{CEFTR+CCRES}$ = 3.7·10⁻⁴ M. Diazotization conditions: C_{HCl} = 12.0 M (initial conc., V = 0.5 mL), C_{NaNO2} = 1.0·10⁻³ M, $t_{diazot.}$ = 5 min. Conditions for azo coupling: C_{NaOH} = 0.16 M; λ_{max} = 516 nm, *l* = 1 cm.

To propose a possible scheme for the formation of azo products based on CEFTR, additional voltammetric studies of the obtained azo compound were conducted using cyclic voltammetry. The cyclic voltammograms of the azo coupling products solution of the diazotized CEFTR with CRES in the presence of unreacted sodium nitrite in the reaction medium and when it was removed using sulfamic acid are shown in Fig. 5.

As can be seen from Fig. 5a on the voltammograms of the solutions of the diazotized CEFTR azo coupling products with CRES two new reversible peaks at the potentials $E_c^{p3'}$ = -0.76 V, $E_c^{p4'}$ = -0.84 V are observed, which are conformed to the two-stage reduction of the newly formed azo group. Besides, two other peaks at $E_c^{p1'}$ = -0.32 V, and $E_c^{p2'}$ = -0.68 V, which correspond with the reduction of the cephalosporins methoxy imino group [32-34] on the voltammogram of the azo product solution are the same as on the voltammogram of the CEFTR solution. Removing the unreacted sodium nitrite from the solution of the obtained CEFTR azo compound with CRES did not affect the polarogram shape. Instead, as can be seen in Fig. 5b sodium nitrite significantly affects the CRES polarogram shape in an alkaline medium. At the nitrite ions availability at the *o*-cresol solution, the peak at $E_c^{p1''}$ = -0.67 V appears on the voltammogram, which is presumably conformed to the reduction of the nitroso group introduced into the aromatic ring under the experimental conditions. CRES is polarographically inactive. The use of sulfamic acid (SA) to remove sodium nitrite from the reaction medium leads to a significant decrease in peak 1" (for the complete disappearance of this peak, a higher concentration of SA must be used or the reaction time should be increased). This data confirms the nitrosation of *o*-cresol under the action of unreacted sodium nitrite in the process of azo coupling with diazotized CEFTR. On the polarogram of azo product solutions, it is difficult to record the effect of unreacted sodium nitrite, since the peak 1" of the nitroso group reduction is superimposed on the own peak of CEFTR reduction (peak 2').

Based on the spectrophotometric and voltammetric studies, we propose a probable scheme for the formation of the azo compound of the diazotized CEFTR with CRES. As can be seen from Scheme 1, nitrite-ions diazotize the primary aromatic amino group of CEFTR in acid media with the formation of

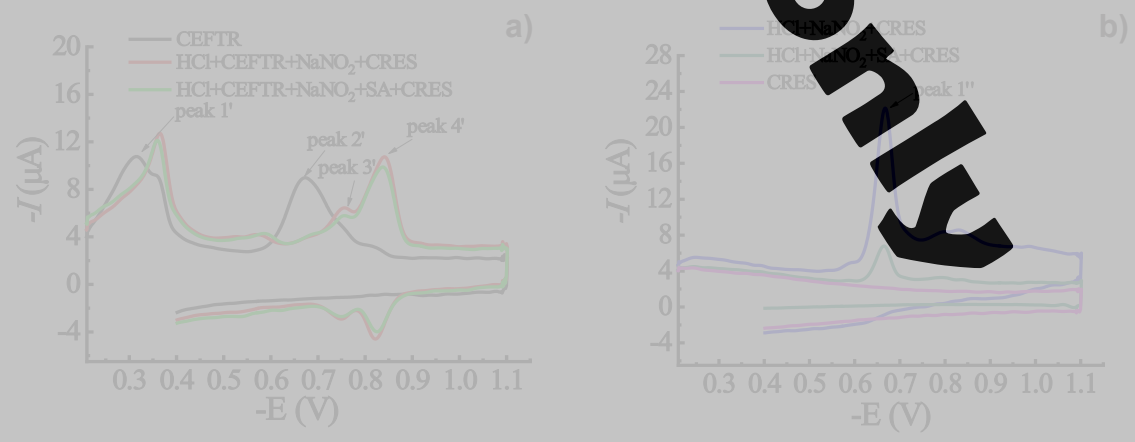

Fig. 5. Cyclic voltammograms of solutions of CEFTR azo coupling products with CRES. *υ* = 2.5 V·s⁻¹. Diazotization conditions: C_{HCl} = 12.0 M (initial conc., V = 0.5 mL), C_{CEFTR} = 7.5·10⁻⁵ M, C_{NaNO2} = 7.5·10⁻⁴ M, C_{SA} = 1.1·10⁻⁴ M. Conditions for azo coupling: C_{CRES} = 7.5·10⁻⁴ M, C_{NaOH} = 0.16 M.

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the diazonium salt and the following azo coupling with the reagent in an alkaline medium, forming a red-colored azo product. The obtained azo compound CEFTR-CRES undergoes nitrosation in the reagent fragment, as evidenced by higher absorbance for CEFTR-CRES azo compound solution that contains unreacted sodium nitrite residue.

The analytical parameters of the proposed spectrophotometric method of CEFTR determination using its azo compound with *o*-cresol are summarized in Table 1. The LOD and LOQ for the determination are based on three and ten times the blank standard deviation ($3.3S_a/b$, $10S_a/b$, respectively (S_a – residual standard deviation or standard deviation of the y-intercept) [35].

As can be seen from Table 1, the elaborated methods for the spectrophotometric determination of ceftriaxone with *o*-cresol possess wide linear ranges (two orders of concentration); it is simple, repeatable, sensitive, and rapid (in general, the antibiotic determination technique proceeds for 10 min) and cheap. The sensitivity of CEFTR determination with CRES is higher to some known sensitive spectrophotometric methods based on the azo coupling reaction (Table S4). Our proposed methods do not require the use of an ice bath, temperature control, or organic solvent and can be used for ceftriaxone assay in drugs.

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Scheme 1. Scheme of ceftriaxone interaction with *o*-cresol.

Table 1. Analytical parameters for the spectrophotometric ceftriaxone determination using *o*-cresol, C_{CEFTR} = 15.0 μg·mL^{-1}, n = 5, P = 0.95. Diazotization conditions: C_{HCl} = 1.2.0 M (initial conc., V = 0.5 mL), C_{NaNO2} = 2.1·10^{-3} M, $t_{diazot.}$ = 5 min. Conditions for azo coupling: C_{CRES} = 5.3·10^{-4} M, C_{NaOH} = 0.16 M; *l* = 1 cm.

λ_{max}, nm	516
Stability, h	1
Molar absorptivity, $\varepsilon_{\lambda max}\cdot10^{-3}$, L·mol^{-1}·cm^{-1}	3.30 ± 0.16
Optimum photometric linear range, μg·mL^{-1}	0.79–31.30
Regression equation (A)[a] Slope, $b \pm \Delta b$	0.019±2.67·10^{-4}
Intercept, $a \pm \Delta a$	0.009±0.005
Correlation coefficient, R	0.9995
Limit of Quantification, μg·mL^{-1}	0.79
Limit of Quantification, M·10^{6}	1.42
Limit of Detection, μg·mL^{-1}	0.22
Limit of Detection, M·10^{7}	4.30
Repeatability (RSD, n = 5) %	1.4
Recovery (n = 5) %	100.9

[a]A = bC+a, where C is the concentration of CEFTR in μg·mL^{-1}.

Formulated drug products of ceftriaxone are not released in combined oral forms, only in the form of a powder for preparing injection solutions, because CEFTR is quickly destroyed under the influence of gastric juices. The developed technique of CEFTR spectrophotometric determination with CRES was successfully tested during ceftriaxone assay in powders for preparing a solution for injections. The results of CEFTR determination in these medicines are given in Table 2.

As follows from data of Table 2 the CEFTR content in both powders for preparing a solution for injections obtained due to the developed technique using *o*-cresol correlates rather well with the content of the CEFTR specified by the manufacturer (it is acceptes 5% deviation from regulated content) and the method of the high performance liquid chromatography, which is recommended as an official method for determination of ceftriaxone in these drugs according to The State Pharmacopoeia of Ukraine [1] and the Pharmacopoeia of the United States of America [36]. The S_r value does not exceed the common values of spectrophotometry errors. However, the developed method is much simpler and cheaper. Moreover, it is characterized by high reproducibility and rapidity.

We used the AGREEprep [37], Blue Applicability Grade Index (BAGI) [38] and AGREEmetric [38] for assessing the developed analytical procedure due to the principles of green chemistry. As follows from the assessment results using AGREEprep and AGREEmetric (Fig. 6a,c), a score of 0.58-0.62 on the AGREEnness scale indicates that the process already has a significant level of eco-friendliness and aligns with some principles of green chemistry. This means that important aspects of safety, not using organic solvents and energy efficiency were considered in its development.

The BAGI method is considered an additional concept to existing green metrics tools that combine the characteristics of sample preparation and analytical determination [38]. On the BAGI icon, ten sectors correspond to the ten evaluation criteria, with a more intense shade of blue corresponding to a higher score. According to the BAGI criteria, the developed procedure for the CEFTR determination received a score of 70.0 (Fig. 6b). The final BAGI score is considered acceptable if it exceeds 60 points.

Conclusions

The manuscript deals with novel experimental aspects of spectrophotometric studies of ceftriaxone with phenolic compound *o*-cresol used as an analytical reagent for the first time, particularly CEFTR determination. It was shown that diazotization and azo coupling reactions can proceed well at room temperature with a short time for diazotation and without establishing azo product solution pH, because of using an alkali reagent solution. This greatly simplifies and speeds up the determination.

For the first time, the formation of azo compounds by the interaction of diazotized ceftriaxone with *o*-cresol has been established. Optimum conditions for examined CEFTR diazotization in the medium of 12.0 M HCl, as well as the following azo coupling with CRES in the alkali medium (C(NaOH)=0.16 M) have been investigated. The components ratio in the obtained azo compounds is CEFTR : CRES = 1:1. It has been demonstrated that azo coupling occurs due to the cresol group of the second component (CRES), which has concordantly oriented substitutes.

Table 2. Spectrophotometric determination of CEFTR with CRES in pharmaceuticals (powders for preparing a solution for injections). Diazotization conditions: C_{HCl} = 12.0 M (initial conc., V = 0.5 mL), C_{NaNO2} = $9.3 \cdot 10^{-4}$ M, $t_{diazot.}$ = 5 min. Conditions for azo coupling: C_{CRES} = $9.3 \cdot 10^{-4}$ M, C_{NaOH} = 0.16 M; λ_{max}=516 nm, l = 1 cm; P =0.95, n=5.

Ceftriaxone (regulated amount in medecines – 1 g in vial)	Determined amount of Ceftriaxone			
	HPLC official method according to analytical specification		Spectrophotometric method with SA CRES	
	$\bar{x} \pm \frac{S \cdot t_{\alpha}}{\sqrt{n}}$	RSD	$\bar{x} \pm \frac{S \cdot t_{\alpha}}{\sqrt{n}}$	RSD
“Ceftriaxone”, NSPS Hebei Huamin Pharmaceutical Company Limited, China				
Ceftriaxone 90 – 120% (0.90 – 1.20 g in vial)	100.11±0.11 (1.001±0.001)	0.1	98.4±0.4 (0.984±0.04)	1.7
“EMSEF 1000”, Nektar Lafsciences Limited, Unit-VI, India				
Ceftriaxone 90 – 120% (0.90 – 1.20 g in vial)	100.09 ± 0.15 (1.001±0.002)	0.1	99.1± 1.4 (0.991±0.145)	3.0

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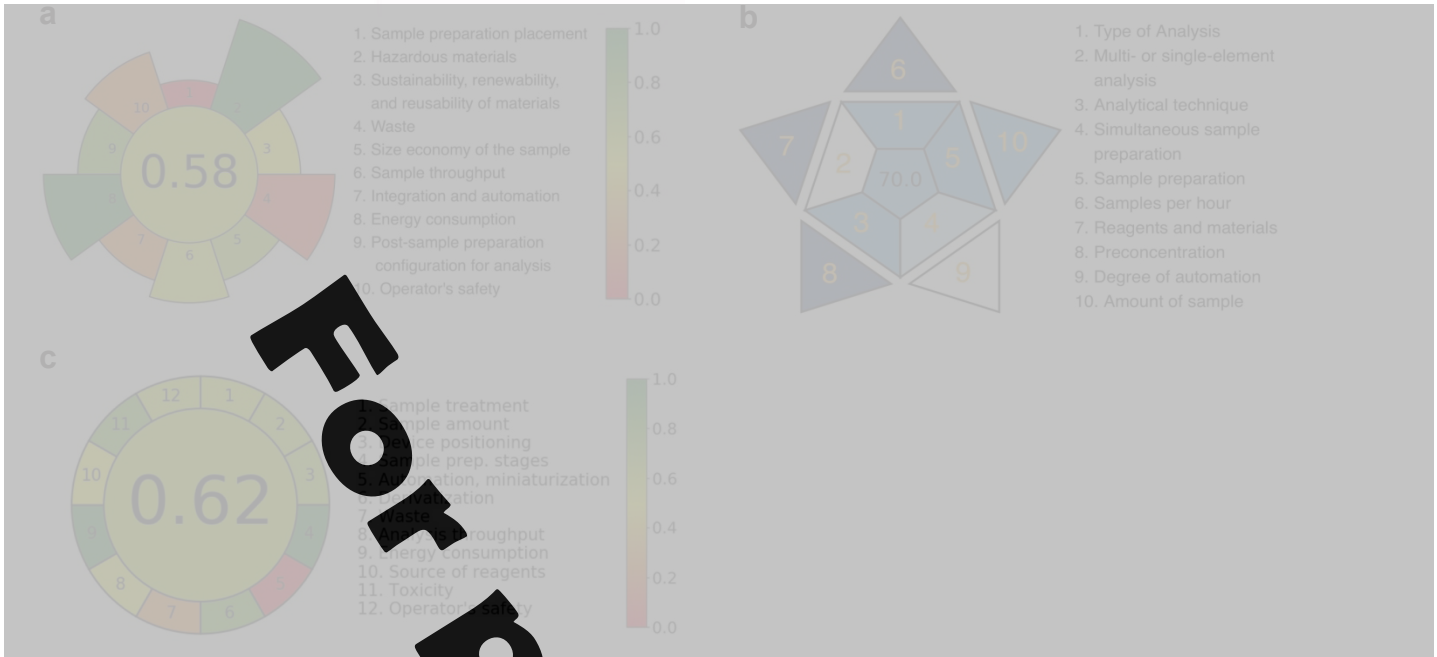

Fig. 6. Generic result of assessment of the developed spectrophotometric procedure for the determination of ceftriaxone using *o*-cresol: a) AGREEprep; b) BAGI; c) AGREE metric.

Spectroscopic and validation characteristics of ceftriaxone determination with CRES have been established. The effective molar absorptivity of obtained azo compounds of CEFTR with CRES $6.9 \cdot 10^3$ $L \cdot mol^{-1} \cdot cm^{-1}$. The method allows us to determine the wide range of CEFTR concentrations ($0.79 – 51.30$ $\mu g \cdot mL^{-1}$) and competes with most of the spectrophotometric methods available in the literature. The limit of detection for CEFTR determination using CRES is $4.30 \cdot 10^{-7}$ M. The proposed method is simple, repeatable, sensitive, rapid, and cost-efficient and competes with most of the spectrophotometric methods for CEFTR determination available in the literature. The developed technique is advantageous over many spectrophotometric methods because it does not require the extraction of organic solvents, using an ice bath and corresponds to the principles of green chemistry.

The elaborated methods of CEFTR spectrophotometric determination with *o*-cresol have been approved during the analysis of single-component commercial pharmaceuticals. Obtained data correlated rather well with the content of the CEFTR specified by the manufacturer. The validation parameters of CEFTR determination in powder for preparing a solution for injections using CRES indicate the reproducibility (RSD ≤ 3.0), the robustness, the precision, and the accuracy of tested methods corresponding to modern requirements of the specification. Therefore, the recommended procedure is well-suited for the assay of drugs to assure a high standard of quality control. Since ceftriaxone is excreted renally as an unchanged drug, this method is suitable for the assay of CEFTR for metabolite control in biological fluids.

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