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PRACTICAL APPLICATION OF CHROMATOGRAPHIC METHODS IN ANALYZING THE IDENTIFICATION OF CEPHALOSPORIN ANTIBIOTICS¹

Abstract. The paper explores the possibility of applying HPLC-MS methods for the identification of cephalosporin antibiotics during routine analyses with the aim of detecting adulterated preparations of this group. To obtain additional information on the chemical structure by selecting the mass detector, the authors investigated the possibility of fragmentation of molecular ions of different generations of cephalosporin antibiotics. Carried out in optimal conditions for HPLC with mass spectrometric detection, the study presents the results of development of a standardized methodology for qualitative and quantitative analysis of cefazolin and cefuroxime using standard samples.

Keywords: cephalosporin antibiotics, pharmaceutical products, ionization mode, neutral molecules, falsification of drugs, fragmentation of molecular ions, validation

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ПРАКТИЧЕСКОЕ ПРИМЕНЕНИЕ ХРОМАТОГРАФИЧЕСКИХ МЕТОДОВ ДЛЯ АНАЛИЗА И ИДЕНТИФИКАЦИИ ЦЕФАЛОСПОРИНОВЫХ АНТИБИОТИКОВ¹

Аннотация. В статье представлены результаты возможности применения методов высокоэффективной жидкостной хроматографии и тандемной масс-спектрометрии (ВЭЖХ-МС) для идентификации цефалоспориновых антибиотиков в ходе рутинных

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анализов для выявления фальсифицированных лекарственных препаратов данной группы. С целью получения дополнительной информации о химической структуре с помощью выбора масс-детектора в работе была изучена возможность фрагментации молекулярных ионов различных поколений цефалоспориновых антибиотиков. С учетом оптимальных условий применения ВЭЖХ с масс-спектрометрическим детектированием, в статье представлены результаты по разработке унифицированной методики качественного и количественного анализа цефазолина, цефуроксима с использованием стандартных образцов.

Ключевые слова: цефалоспориновые антибиотики, лекарственные препараты, режим ионизации, нейтральные молекулы, фальсификация лекарственных препаратов, фрагментация молекулярных ионов, валидация

With regard to the practical aspects of drug identification issues, it can be challenging to achieve rapid and quantitative transfer of the compounds being analyzed from a solution to a high vacuum (10–12 Torr) within the chromatographic time scale. Equally interesting is the necessity of ionizing substances, as liquid chromatography is designed to separate mixtures of neutral molecules of different types, while the mass detector can sort ions only, meaning molecules carrying at least one charge [1, p. 285; 2, p. 48–58; 5, p. 809–812; 8, p. 13–17; 7, p. 501–505]. Ion sources are used in HPLC-MS to separate molecules in the studied sample from a large volume of eluent and ionize them, converting neutral molecules into ions with one or more electrical charges [4, p. 1565–1587; 11, p. 15–28; 6, p. 804–817].

It is important to note that the combination of HPLC and mass spectrometry results in a significant reduction in analysis time, allowing for quantitative analysis and selective detection of chosen ions. The use of software facilitates mathematical processing of the obtained data, library search, and identification of individual chemical substances or components in unknown mixtures. Using optimal conditions for HPLC with mass spectrometric detection, we have developed a standardized methodology for qualitative and quantitative analysis of cefazolin and cefuroxime using standard samples.

Materials and Methods

Materials and Methods: The study utilized an interface based on the atmospheric pressure electrospray ionization method (AP-ESI). Under carefully selected conditions of HPLC-MS, cephalosporin antibiotics, namely cefazolin and cefuroxime, exhibited improved ionization, producing more ions. Additionally, methods of ‘soft’ ionization, without significant fragmentation, were successfully implemented, allowing for the registration of molecular ions of the analyzed samples. The experimental evidence demonstrated the possibility of fragmentation of molecular ions of various generations of cephalosporin antibiotics through their collision

with neutral nitrogen atoms (CID) inside the mass detector under conditions of low vacuum. This allowed for obtaining additional information about the chemical structure by choosing the appropriate mass detector. It should be noted that all ionization modes at atmospheric pressure are sensitive to the eluent composition, meaning the type of mass spectrum depends on the conditions of chromatographic separation.

The presented work employed electrospray ionization (ESI) through the atmospheric pressure electrospray ionization method (AP-ESI). This method ionizes a broad range of organic compounds with different molecular masses and polarities. The absence of eluent heating during ionization provides extensive opportunities for studying thermally sensitive compounds [3, p. 45–54; 9, p. 145–149; 10, p. 147–150; 12, p. 287].

Discussion of Results

The selected interface for the conducted research operated on the principle of electrospray ionization of the eluent. After exiting the liquid chromatograph column, the eluent was charged with several thousand volts. The charged eluent then entered the nebulizer and transformed into mist droplets. A heated nitrogen counterflow was introduced into the mist stream and into the chamber externally. The droplets of eluent mist, which carry uncompensated charges, evaporated quickly. During evaporation, the charge density on the surface of the droplets continuously increased. Once the droplets reached a certain limit, known as the Rayleigh limit, they underwent explosive fragmentation. This process repeated multiple times until individual ions of the analyzed substances appeared in the ionization chamber atmosphere. The remaining eluent was removed from the lower ionization chamber through a drain. The cephalosporin antibiotics ions formed in this manner were directed into the entrance capillary of the mass detector under the influence of the electrostatic field.

The investigation into the qualitative and quantitative determination of cefazolin and cefuroxime was conducted using a single quadrupole mass detector. It was also used to identify unknown compounds on the basis of their molecular mass.

The study examined retention time values, molecular masses of the analyzed substances, and mass spectra of molecular ion fragments. The obtained results suggest that this method can be used to determine the chemical structure of a substance, which is crucial in identifying falsified pharmaceuticals belonging to this group. Chromatograms, the UV spectra, and the mass spectra of cefazolin and cefuroxime are characterized by the presence of stable fragments and characteristic ions. These ions are formed by common pathways of molecular ion fragmentation. Furthermore, all analyzed substances exhibited unique UV spectra with absorption maxima that are specific to each compound.

The analysis of spectral and chromatographic characteristics allowed establishing the presence of molecular and typical fragment ions for cefazolin and cefuroxime,

as presented in Tables 1 and 2. To identify and quantify the sodium salt of cefazolin, a step-by-step analysis was conducted. This included examining chromatograms obtained through HPLC, analyzing UV spectra, and mass spectra parameters.

Figure 1 shows the research results, indicating one peak with a slight shoulder at a retention time of 3.906 minutes in the HPLC chromatogram.

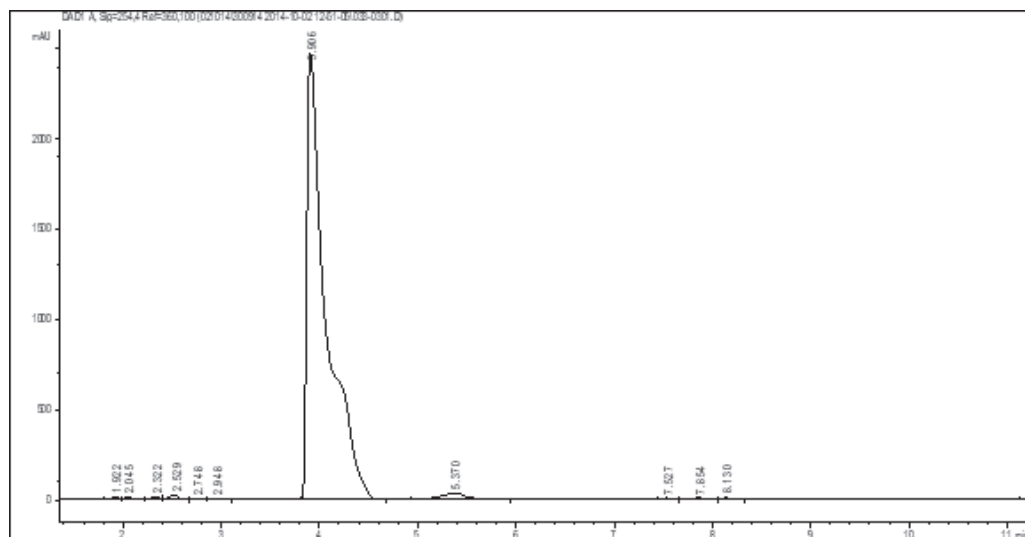


Fig. 1. HPLC chromatogram of cefazolin at a wavelength of 254 nm

Based on this, we have identified the possible presence of a small number of isomers. The examination of fixed absorption parameters in the UV spectrum at a retention time of 3.906 minutes explains the presence of two absorption maxima in the 208 and 274 nm range, confirming the presence of isomeric compounds in the pharmaceutical formulation (see Fig. 2).

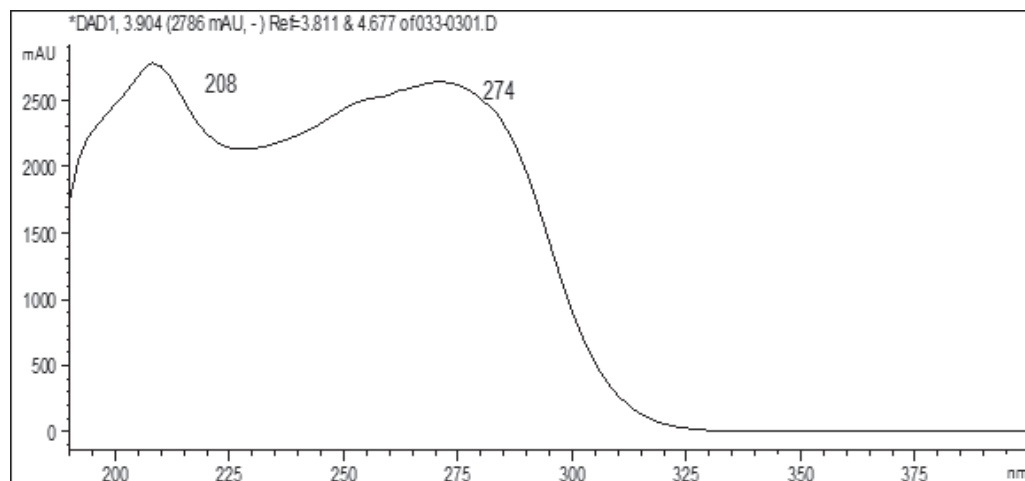


Fig. 2. UV spectrum of the cefazolin peak at a retention time of 3.906 minutes

Figure 3 presents the results of the research on the dependence of molecular mass parameters on ion charge coordinates. It has been established that two characteristic peaks, differing in retention time, corresponding to 4.2 minutes and 5.44 minutes, respectively, were identified during positive ionization in HPLC-MS chromatograms of cefazolin.

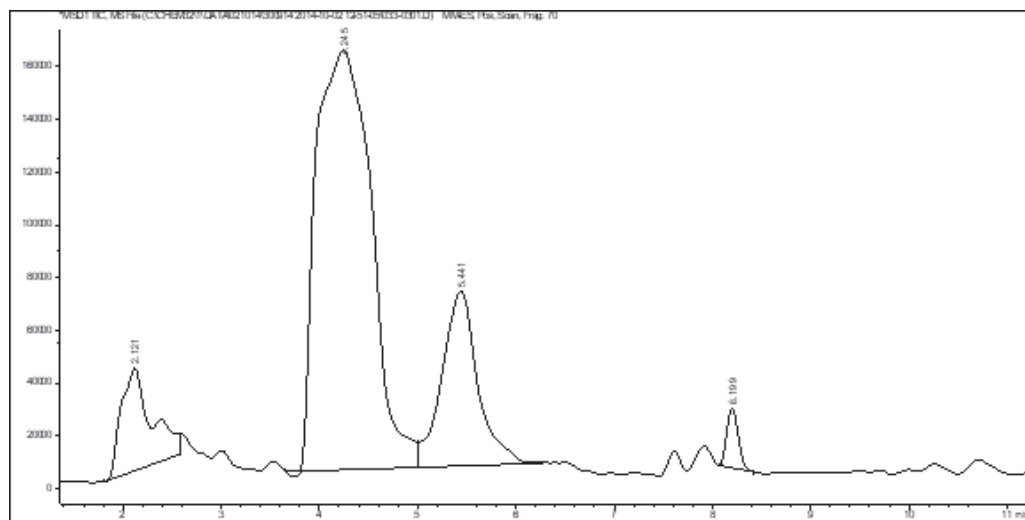


Fig. 3. HPLC-MS mass chromatogram of cefazolin obtained in positive ionization mode

In the experimental conditions, the mass spectra data at retention times of 4.2 and 5.44 minutes showed similarity. According to the experimental studies, a protonated ion with a minor intensity of a fragment ion at m/z 272 was present in the positive ionization mode at a retention time of 4.2 minutes (Fig. 4).

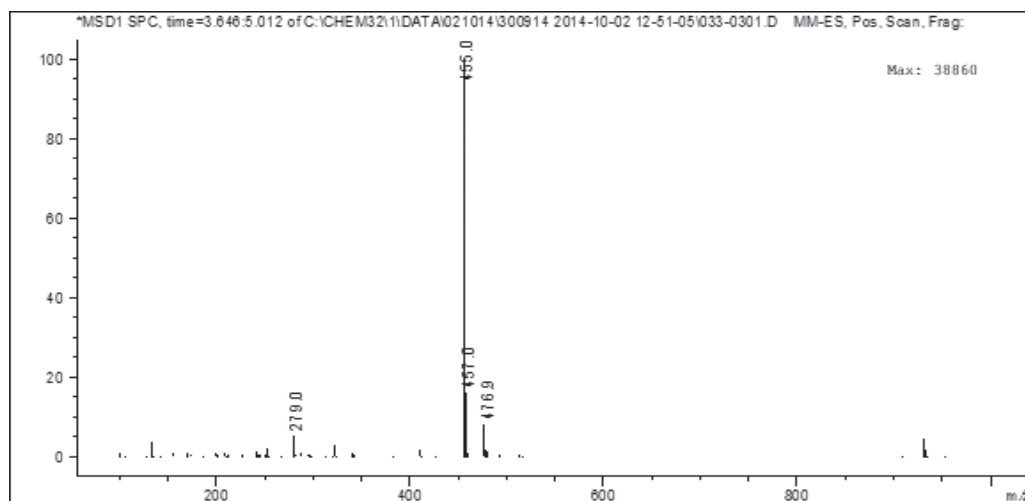


Fig. 4. Mass spectra of the cefazolin peak obtained at a retention time of 4.245 minutes in positive ionization mode

The results obtained during the study of cefazolin mass spectra in positive ionization mode confirm the presence of a protonated molecular ion with fragment ions at m/z 323, 279, 253, 170, 173, 133 (Fig. 5). The obtained mass spectra of cefazolin indicated that the examined samples were separated into two isomeric compounds during chromatography. The identification of this compound was facilitated by the presence of the molecular ion in the mass spectra obtained in positive ionization mode.

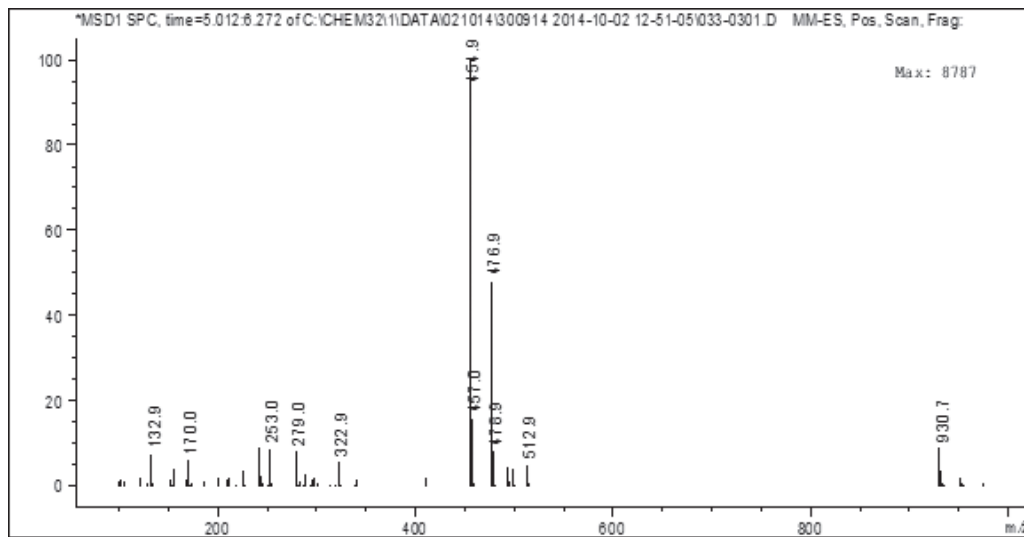


Fig. 5. Mass spectra of the cefazolin peak obtained at a retention time of 5.44 minutes in positive ionization mode

The HPLC chromatogram of cefuroxime was obtained at a wavelength of 254 nm, revealing two peaks with fixed retention times of 3.9 and 4.6 minutes (Fig. 6).

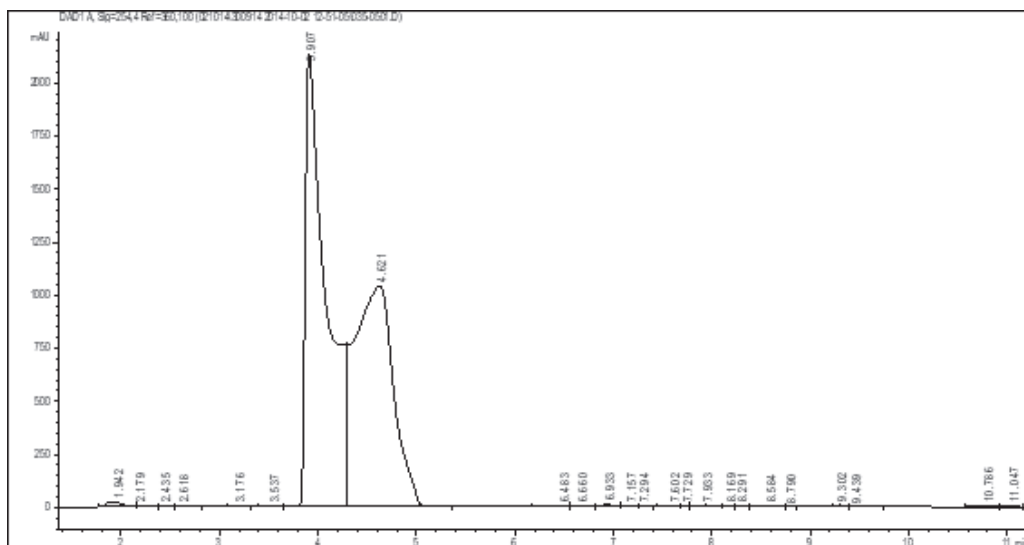


Fig. 6. HPLC chromatogram of cefuroxime at a wavelength of 254 nm

Figure 7 presents the characteristics of the UV spectrum of the peak at a retention time of 3.9 minutes, indicating the presence of two absorption maxima at 200 nm and 272 nm. At a retention time of 4.6 minutes, the absorption maxima corresponded to 194 nm and 274 nm, as shown in Figure 8.

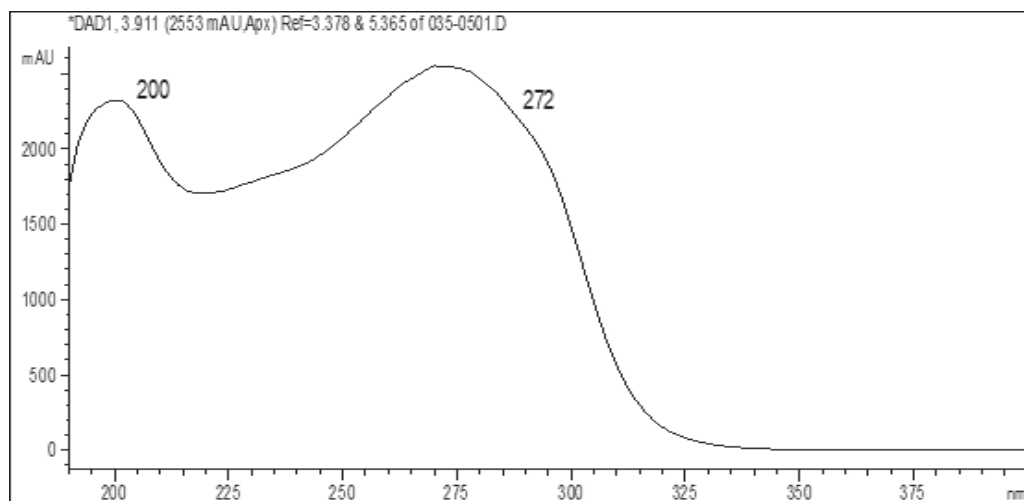


Fig. 7. UV spectrum of the cefuroxime peak obtained at a retention time of 3.9 minutes

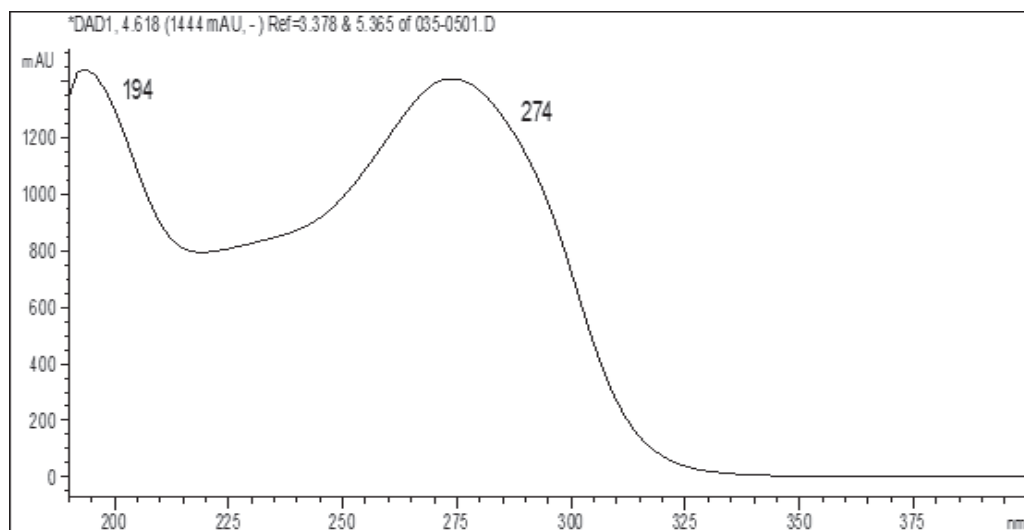


Fig. 8. UV spectrum of the cefuroxime peak obtained at a retention time of 4.6 minutes

The results of the HPLC-MS chromatograms of cefuroxime in positive ionization mode, presented in Fig. 9, indicate the presence of one intense peak with a retention time of 4,5 minutes.

The mass spectra of cefuroxime displayed characteristic peaks of ions with m/z values of 444, 442, 381, 364, 321, 294, and 253 (Fig. 10). As shown by the analysis of literature data [2, p. 1098–1105; 5, p. 283–293; 4, p. 34–36; 7, p. 588–590; 11, p. 763–772],

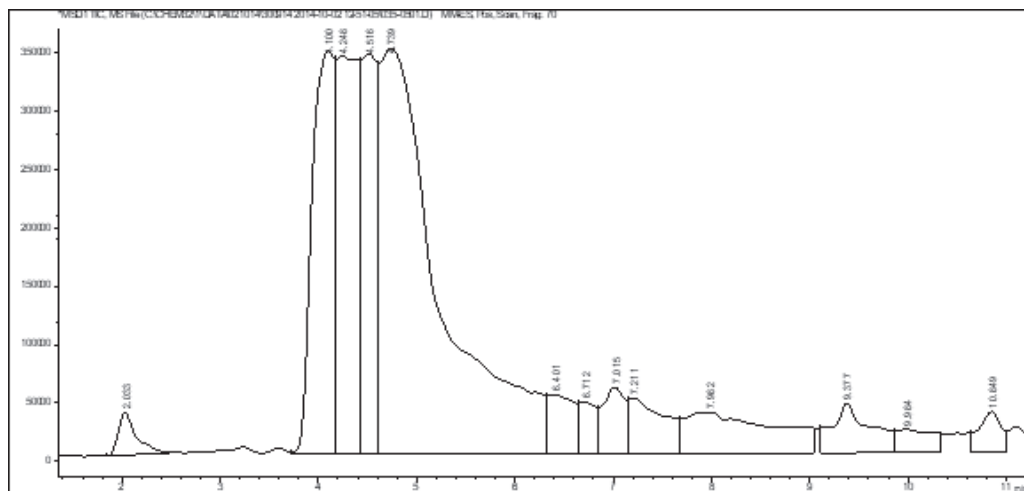


Fig. 9. HPLC-MS chromatogram of cefuroxime obtained in positive ionization mode

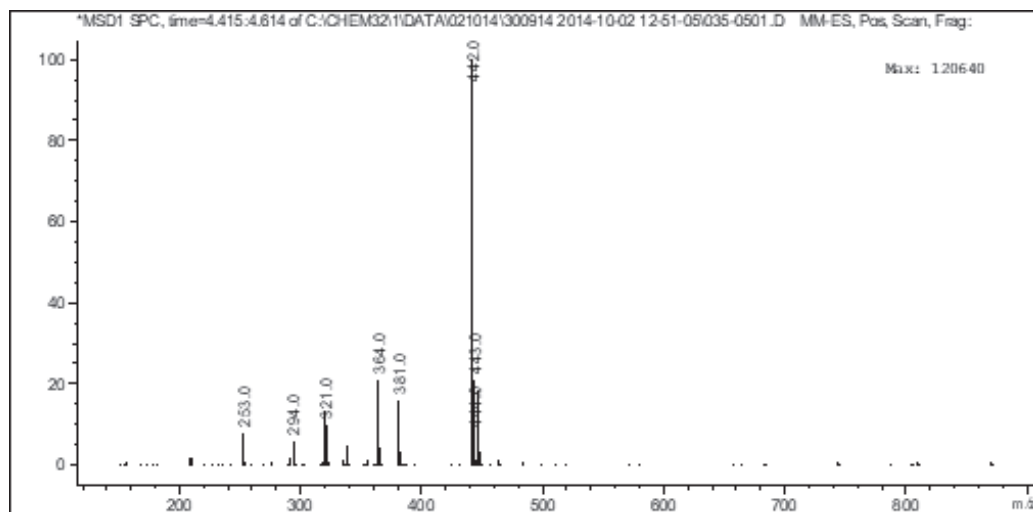


Fig. 10. Mass spectra of cefuroxime at a retention time of 4.5 minutes.

comparing the intensities of fragment ions allows for the determination of the brute formula of the investigated compounds.

Figure 11 shows the mass chromatogram of cefuroxime obtained in negative ionization mode. The results allowed establishing the presence of two intense peaks with retention times of 4.02 minutes and 4.69 minutes.

The mass spectra at a retention time of 4.02 minutes showed the presence of a molecular protonated ion ($M^+ + H^+$ 424) and fragment ions with m/z 423, 362, 318, and 251. Similarly, at a retention time of 4.69 minutes, a mass spectrum resembling the initial peak was recorded (Fig. 12).

During the investigation, the mass spectra of cefuroxime indicated that the compound under study during chromatography was separated into two isomeric compounds.

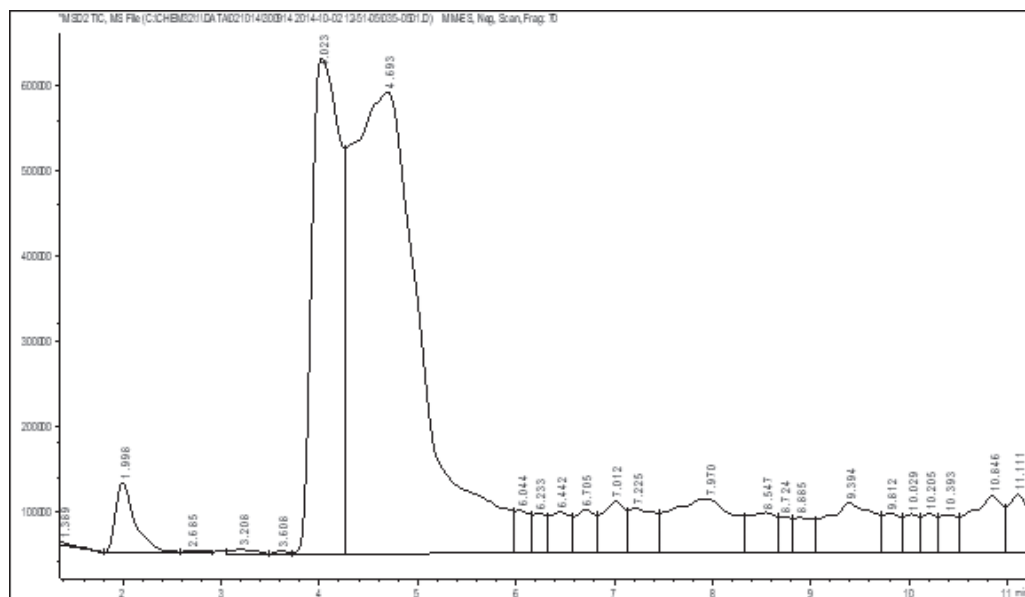


Fig. 11. HPLC-MS chromatogram of cefuroxime obtained in negative ionization mode

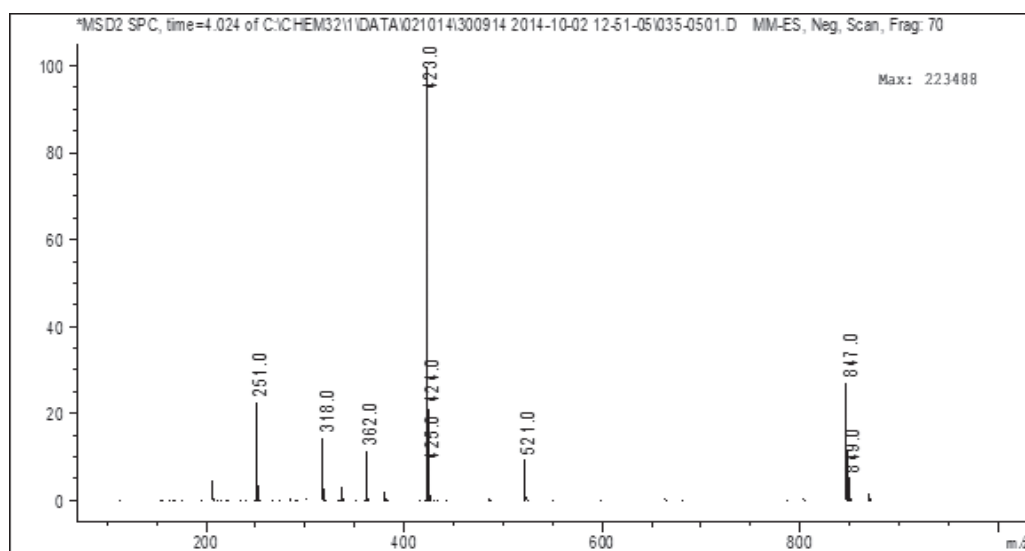


Fig. 12. Mass spectrum of the cefuroxime peak obtained at a retention time of 4.02 minutes in negative ionization mode

It is worth mentioning that the optimal identification of cefuroxime occurred under conditions of negative ionization mode due to the presence of the molecular ion. On the total ion current chromatogram, two peaks with identical mass spectra were observed (Fig. 13).

The data presented clearly indicates that positive ionization is the optimal mode for analyzing cefepime. This mode allows for the identification of a molecular ion, which greatly facilitates the identification of the substance.

Tables 1 and 2 present the typical ions and intensities of the investigated compounds. The absence of molecular ions in the mass spectra suggests that the compounds are unstable under electro spray ionization conditions. Thus, the structure and structural features of the active moiety of the studied substances are well reflected in the mass spectra.

Table 1

Molecular and typical fragment ions of cephalosporin antibiotics

Name of the compound	Retentiontime (R_f) minutes	Molecular ion (M^+)	Typicalions (m/z)
Cefazolin	5.4	($M^+ + H^+$) 455	323; 279; 253; 170; 173; 133
	4.2	($M^+ + H^+$) 455	272
Cefuroxime	3.9 in positive Ionization mode	($M^+ + H^+$) 445	444, 442, 381, 364, 321, 294, 253
	4.02	($M^+ + H^+$) 424	423, 362, 318, 251

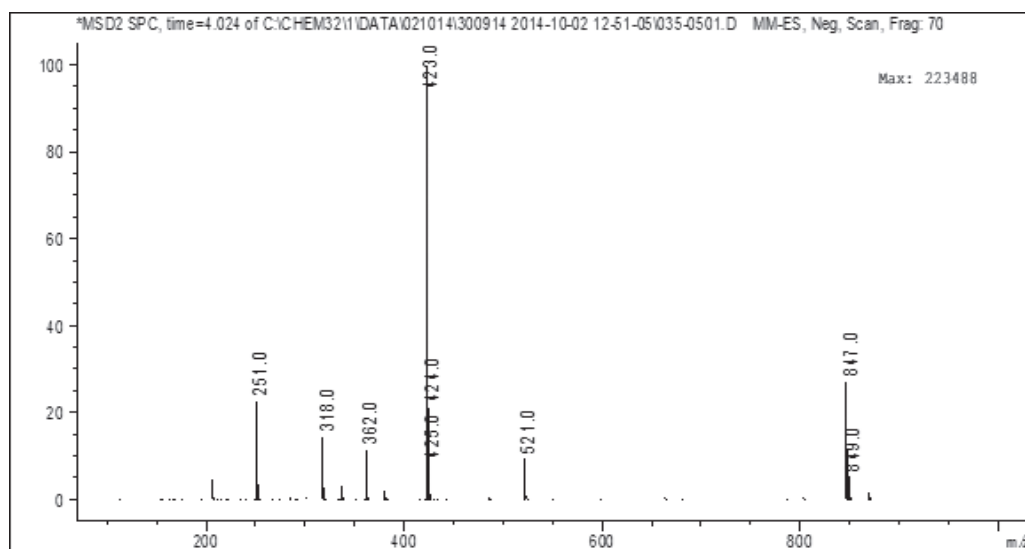


Fig. 13. Mass spectrum of the cefuroxime peak obtained at a retention time of 4.69 minutes in negative ionization mode

Table 2 presents the main results of the spectral-chromatographic characteristics of cefazolin and cefuroxime. These parameters are quantitative and qualitative characteristics used to determine the authenticity of pharmaceuticals.

Table 2

Spectral-chromatographic characteristics of cephalosporin antibiotics

Name of the compound	Retentiontime (R_f) minutes	Characteristic maxima on UV spectra, nm
Cefazolin	3.9	208, 274
Cefuroxime	3.9	200; 272
	4.62	194; 274

Table 3

Metrological characteristics of quantitative determination of cefazolin

Quantitative content (mg)	Quantitative content (mg)
972.18	Number of degrees of freedom $f = 4$
971.19	Standard deviation $S = 1.95$
967.04	Mean value $X_{av} = 970.34$
970.94	Standard deviation from the mean result $S_{av} = 0,87$
979.35	Relative accuracy of test results $\varepsilon = \pm 0.134 \%$
	Confidence probability $P = 95 \%$
	Critical value of the Student's t -criterion at a given confidence probability = 2.78

Table 4

Metrological characteristic of the quantitative determination of cefuroxime

Quantitative content (mg)	Quantitative content (mg)
754.99	Number of degrees of freedom $f = 4$
736.17	Standard deviation $S = 7.069$
746.11	Mean value $X_{av} = 746.60$
744.93	Standard deviation from the mean result $S_{av} = 3.16$
750.80	Relative accuracy of test results $\varepsilon = \pm 1.17 \%$
	Confidence probability $P = 95 \%$
	Critical value of the Student's t -criterion at a given confidence probability = 2.78

The results of the studies on identifying cephalosporin antibiotics using HPLC-mass spectrometry have enabled the establishment of an algorithm for assessing authenticity and detecting falsification of pharmaceuticals using HPLC-MS methods (see Fig. 14).

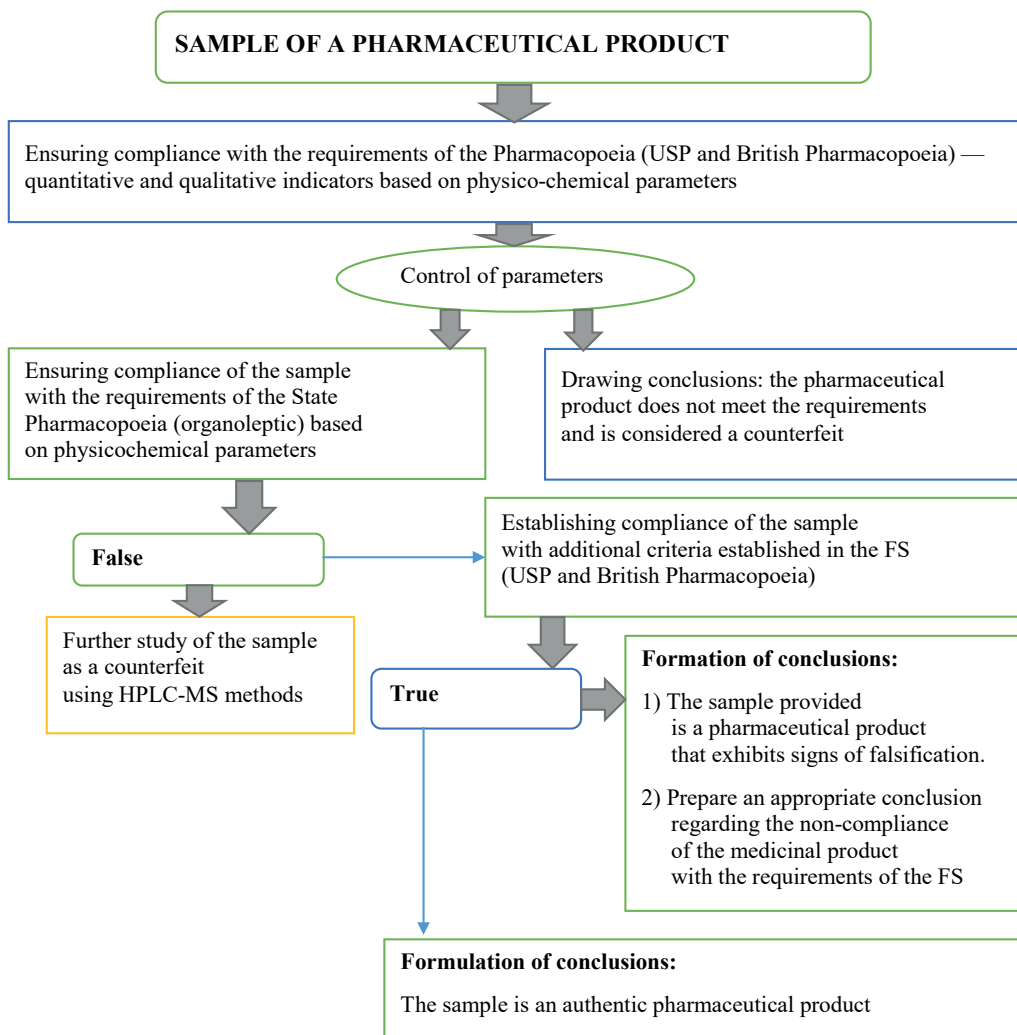


Fig. 14. Algorithm for assessing the authenticity and identifying counterfeit drugs using HPLC-MS analysis methods

Conclusions

The results of the qualitative and quantitative determination of the antibiotics cefazolin and cefuroxime indicate that the use of HPLC with a mass spectrometric detector (HPLC-MS) made it possible to determine the retention time, molecular and fragment ions, their intensity, the individual fragmentation of each substance, as well as absorption maxima in the UV region of the spectrum.

The results of the studies indicate that using molecular ion (M^+) and fragment ions as characteristic ions for each of the studied drugs as diagnostic markers enables the detection of cefazolin and cefuroxime in unknown samples using HPLC-MS in modes of positive and negative electrospray ionization. In addition, this technique, during routine tests, makes it possible to establish the presence of counterfeit drugs. This technique can also detect counterfeit drugs during routine tests. It is evident from the above that the fragmentation rate of the studied cefazolin and cefuroxime is determined by their individual molecular structure, composition, and physicochemical features that affect the appearance of the molecular ion.

The HPLC method with mass spectrometric detection was validated according to generally accepted statistical criteria. From the given parameters, the arithmetic mean, dispersion, standard deviation, confidence probability and relative error of the average result were determined.

The results show that for cefazolin, the standard deviation from the mean was 0.87, with a confidence level of $P = 0.05$ (95 %) and a relative error of the mean of 0.25 %. For cefuroxime, the standard deviation from the mean was 3.16 and the relative error of the mean was 1.17 %. The confidence level of the obtained values is 95 %.

It should be clearly stated that the data obtained on the validation of statistical processing of cefazolin and cefuroxime data prove the compliance of the metrological indicators of the developed method for quantitative analysis of HPLC with mass spectrometric detection with the above requirements.

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