Volume 9, Issue 6, 2019, 4522 - 4533 ISSN 2069-5837
Biointerface Research in Applied Chemistry
www.BiointerfaceResearch.com
https://doi.org/10.33263/BRIAC96.522533
Original Research Article Open Access Journal
Received: 16.08.2019 / Revised: 18.10.2019 / Accepted: 19.10.2019 / Published on-line: 25.10.2019

# Separation of D,L-Ampicillin by ligand exchange-micellar electrokinetic chromatography

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## ABSTRACT

In this study, D,L-ampicillin separation was carried out by ligand exchange-micellar electrokinetic chromatography method using L-Lysine monohydrochloride as a ligand and copper (II) sulfate pentahydrate is a central ion supplier. Isomeric separations were performed using capillary electrophoresis (CE) instrument, in which SDS-L-Lys-$Cu^{+2}$ micelle complex was used as a pseudostationary phase. The effect of pH, SDS amount, applied electrical field, pressure, organic solvent ratio and ampicillin D,L-ratios were investigated. Fast, inexpensive and sensitive approach for the simultaneous separation of D,L-ampicillin in both aqueous and real antibiotic sample was performed using CE coupled with UV detector. The separation was achieved in a short period of 7 minutes with high-sensitivity and low-detection limit of 1.25 µM by the developed SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes without using any extra process such as imprinting or spacer arms for the immobilization of the ligands.

**Keywords:** *Ampicillin; Capillary Electrophoresis; Micelle;*

## 1. INTRODUCTION

Chiral compounds contain at least one carbon atom called stereogenic center to which four different atoms or groups are attached. More than half of the pharmaceutically active components, such as antibiotics, are chiral compounds [1-11]. Antibiotics are widely used in the treatment of human infections. They are not only used in human infections but also in the veterinary field in support of the animals growth and in the treatment of animal diseases. In the food industry for preservation of foods, for the growth of aquatic creatures such as fish and also for research activities of the pharmaceutical industry they are used [12,13]. Ampicillin having a beta lactam ring is synthesized semi-synthetically in the laboratory environment and prevents bacterial cell wall synthesis. Doyle and colleagues obtained ampicillin (alpha amino benzyl penicillin) in 1961 by hydrogenation with sodium in the presence of palladium catalysts [14]. The ampicillin side chain has an asymmetric carbon atom. For this reason, optically active D- and L-isomers are present. The D-isomer is used in the treatment since it is more active than the L-isomer [15].

Current trends in the pharmaceutical industry include an increasing need for optically pure dosage forms. Drugs produced synthetically occur as of two or more spatial isomers. Racemic drugs' pharmacological activity is usually associated with the effect of only one isomer. The second one either has a less specific activity or is not active at all and also exhibits other pharmacological effects [16]. Since one isomeric form of a compound having side effects when metabolized, it is necessary to separate these two isomers. One or two isomers may be separated from one another by chiral separation techniques. Methods for separating the isomers of a racemic mixture include capillary electrophoresis, chromatography, crystallization, extraction, methods using membranes, enzymatic kinetic resolution methods. The most common methods used to separate stereoisomers are chromatographic methods. CE which is one of the most common methods of chromatography as a micro-scale analytical technique with high efficiency is used to separate isomers based on their charge/size ratio [17,18]. The analytical variety, the high efficiency of separation, high mass sensitivity, working with sample volumes in very small quantities, low analysis times and low cost are superior to other chromatographic methods [19].

Although the main use of antibiotics is therapeutic, they are also employed as chiral selectors in different separation techniques such as HPLC and CE. Indeed, their structural attributes which include various stereogenic centers and a variety of functional groups provide them with a great capacity to interact stereospecifically with chiral compounds enabling the separation of a variety stereoisomer analytes. The wide use of cationic, anionic and neutral antibiotics as chiral selectors in CE has been demonstrated over the years, in particular, those belonging to the families of macrolides, glycopeptides, polypeptides, aminoglycosides, ansamicins, lactams and lincosamides [20,21].

Ligand exchange chromatography is one of the methods that separates isomeric molecules by using chirality. Ligand exchange is based on the formation of complexes that can act as ligand and the isomeric separation is performed through these complexes. The ligand exchange separation methods are sensitive to the spatial structure of the analyte molecules, which enables the separation of geometric and optical isomers. The ligand exchange chromatography method was used by Davankov and Rogozhin in 1968 to separate the amino acid isomers [22]. Ligand exchange

capillary electrophoresis (LECE), which is accepted as a promising electrophoretic version, is used to analyze a complex mixture of natural compounds [23-38]. LECE is mainly used to separate optical isomers of amino and hydroxy acids. In LECE and also in ligand exchange chromatography, chiral separation is based on the formation of diastereomeric complexes with a metal cation between the ligand of a chiral selector and the analyte isomers. By using the LECE, the important outcomes including 2 to 3 fold decrease in the detection limits of amines and 3 fold increase of amino acids' resolution factors were obtained.

The hydrophilic parts of the micelles formed from surfactants are contacting with water on the outer surface while hydrophobic parts are placed on the inner surface. Micelles occur after a certain change of surfactant. After adding surfactant molecules to aqueous solutions, the monomer surfactants start to form a layer on the liquid-air surface. Micelles are formed in the bulk solution, when the surface becomes saturated with surfactant monomers [39-42]. In this study D,L-ampicilin separation was performed by using a micellar electrokinetic chromatography method by capillary electrophoresis instrument with optimized electrokinetic conditions. The separation was carried out by using micelles which were used as pseudostationary phase in the capillary column. Forthis aim, firstly L-Lysine-copper (II) (L-Lys-$Cu^{+2}$) amino acid-metal complex was formed by using UV-VIS spectrophotometer to determine the optimal molar ratios of the interacting L-Lys amino acid with the $Cu^{+2}$ ion forming the L-Lys-$Cu^{+2}$ complex. Characterization studies of the complex were carried out using FTIR-ATR spectrophotometer. Critical micelle concentration (CMC) was determined by current density measurement. SDS micelles obtained above the CMC interacted with L-Lys-$Cu^{+2}$ complex. Characterization studies of the micelles and the micelles interacting with the chiral selector were made using high contrast transmission electron microscopy (CTEM), dynamic light scattering (DLS) spectrophotometer and zetasizer analyzer. In this chromatography system, the SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex was used as a pseudostationary phase for the D,L-ampicillin separation.

The effect of pH, SDS amount, applied electrical field, pressure, organic solvent ratio and ampicillin D,L- ratios were investigated on the electrokinetic separation of ampicillin isomers in both aqueous solutions and alfasilin antibiotic as a real sample. As a result, the D,L-ampicillin separation was carried out by combining the ligand exchange mechanism with micellar electrokinetic capillary chromatography, the subcategory of the capillary electrochromatography, and the optimum conditions for the isomeric separation were successfully determined.

## 2. MATERIALS AND METHODS

### 2.1. Instrumentation.

Separations were carried out on a Prince CEC-760 equipped with a photodiode array detector (Prince Technologies B. V., Cornelis Houtmanstraat 26, 7825 VG Emmen, The Netherlands). The methods were applied using the long capillary mode system for analysis.

### 2.2. Reagents and Materials.

D-ampicillin was obtained from Sigma Aldrich, and L-ampicillin was obtained from Molcan. L-Lysine monohydrochloride (L-Lys), sodium dodecyl sulfate (SDS), copper sulfate pentahydrate ($CuSO_4.5H_2O$), acetonitrile (ACN), potassium dihydrogen phosphate ($KH_2PO_4$), sodium hydroxide (NaOH) acetic acid ($CH_3COOH$) and other chemicals were obtained from Merck A.G (Darmstadt, Germany). Fused-silica capillaries (i.d. 100 μm and o.d. 375 μm) were supplied by Polymicro Technologies (Phoenix, AZ, USA). All compounds and water used in the experiments were analytical grade.

### 2.3. Preparation of L-Lys-$Cu^{+2}$ complex.

L-Lys-$Cu^{+2}$ complex was prepared according to the literature at pH 10.0 [43]. 2 mmol of L-Lysine amino acid was dissolved in 5 mL of deionized water. 0.33 mL of a 30% NaOH solution was added to the solution for amino acid deprotonation. 2 mL of a solution containing 1 mmol of $Cu(SO_4).5H_2O$ metal salt was added to the deprotonated amino acid solution. The complex was then filtered to remove excess NaOH, and the solvent was removed by Lyophilizer (Christ Alpha 1-2 LD plus, Germany). After that, it was washed with water and dried in a vacuum oven for 24 hours.

### 2.4. Determination of critical micelle concentration (CMC).

CMC was determined by the capillary electrochromatography system (CEC prince 760) with current density method. For that, different concentrations of SDS solutions (2.0 , 3.0, 5.0, 8.0, 10.0, 20.0, 25.0, 30.0, 35.0, 40.0, 45.0 ve 50.0 mM) were prepared with 10.0 mM ammonium acetate buffer. The preparation of the solutions was carried out after the addition of SDS with different concentrations and stirring 10 minutes by means of a magnetic stirrer. Then, L-Lys-$Cu^{+2}$ complex (10.0 mM) was added to the SDS-based solutions to determine the CMC of SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex. All the solutions were homogenized at 6000 rpm for 10 min, and sonicated before using. These solutions were put in inlet and outlet buffers and 10 kV electric field was applied. The CMC of the solutions containing SDS and SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex was found from the curve plotted against concentration (μM).

### 2.5. Preparation of SDS-based electrokinetic columns.

After the appropriate amino acid-to-metal ratio estimation for the preparation of the L-Lys-$Cu^{+2}$ complex, the CMC was found by the measurement of current density. And, then, SDS and SDS-L-Lys-$Cu^{+2}$ micelle complexes at different concentrations were prepared. The activation of the column was achieved by forming silanol groups with 1.0 M NaOH solution on the surface of fused-silica capillaries (i.d. 100 μm and o.d. 375 μm). Before the injections, the column washed with deionized water, 0.1 M $HNO_3$, deionized $H_2O$, 0.1 M NaOH, deionized $H_2O$, respectively and then it was equilibrated with buffer solution [44]. The injection of the samples was carried out hydrodynamically at 34 mbar pressure for 5 sec. For isomeric separations, optimum current and pressure values were determined by using different currents and pressures at 254 nm.

### 2.6. Characterization studies.

UV-VIS spectra of complexes f ormed by L-Lysine ligand and $Cu^{+2}$ metal ion with different molar ratios (mmol:mmol) were obtained by spectrophotometer (SHIMADZU

UV-1601, Japon). The structure analysis of L-Lys and L-Lys-$Cu^{+2}$ complex was obtained by Fourier transform infrared spectrophotometer-attenuated total reflection (FTIR-ATR) (Thermo Fisher Scientific, Nicolet iS10, Waltham, Mass., USA). 2.0 mg of the sample was mixed with 98.0 mg of KBr, and then the pressure applied at 600 kg/$cm^2$ for about 10 minutes to form a thin pellet. FTIR-ATR spectra were taken from the prepared pellets in the range of 4000-400 $cm^{-1}$ wavenumber.

The morphological characterization of SDS micelles (10.0, 25.0 and 50.0 mM) and SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes (10.0 mM) was performed with high contrast transmission electron microscopy (CTEM, FEI Tecnai G2 Spirit Biotwin model). Nano Zetasizer (NanoS, Malvern Instruments, London, UK) was used to determine the size analysis and zeta potential measurements of SDS and SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex solutions. Also, DLS spectrometry (Malvern CGS-3) was used to check the size analysis results of SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes. All experiments were repeated for three times (n=3).

### 2.7. Analysis conditions for D,L-ampicillin separation.

The effect of buffer solution pHs, SDS concentration, applied voltage, pressure, organic solvent ratios and different molar ratios of D- and L-ampicillin isomers were examined on separations. Accordingly, the pH effect on D,L-ampicillin separation efficiency was investigated using SDS and amino acid-metal complex (L-Lys-$Cu^{+2}$) prepared with different pHs at a given concentrations as shown in Table 1a. SDS and L-Lys-$Cu^{+2}$ complex were mixed to obtain SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex solutions and the effect of different SDS concentrations on separation was investigated. The composition of the buffer solution with different SDS concentrations was also given in Table 1b. The effect of the applied voltage on the separation efficiency of D,L-ampicillin isomers has been investigated using buffer solution containing 25.0 mM SDS, 10.0 mM L-Lys-$Cu^{+2}$ complex and 10.0 mM $NH_4OAc$, and electric field was applied as 5, 10 and 15 kV, respectively (Pressure 100 mbar) as shown in Table 1c. In order to investigate the pressure effect on the separation efficiency of D,L-ampicillin, same buffer solution was used at 10 kV, and the pressure was applied as 0, 50 and 100 mbar, respectively as shown in Table 1d.

**Table 1a.** Composition of the SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex solution prepared at different pH values used in the separation of D,L-ampicillin.

| SDS (mM) | L-Lys-$Cu^{+2}$ (mM) | $NH_4OAc$ (mM) | pH | Electrical field (kV) | Pressure (mbar) |
|---|---|---|---|---|---|
| **25.0** | 10.0 | 10.0 | 7.0 | 10 | 100 |
| **25.0** | 10.0 | 10.0 | 8.0 | 10 | 100 |
| **25.0** | 10.0 | 10.0 | 9.0 | 10 | 100 |

**Table 1b.** Composition of the SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex solution containing different SDS concentrations used in the separation of D,L-ampicillin.

| SDS (mM) | L-Lys-$Cu^{+2}$ (mM) | NH4OAc (mM) | pH | Electrical field (kV) | Pressure (mbar) |
|---|---|---|---|---|---|
| **5.0** | 10.0 | 10.0 | 9.0 | 10 | 100 |
| **10.0** | 10.0 | 10.0 | 9.0 | 10 | 100 |
| **25.0** | 10.0 | 10.0 | 9.0 | 10 | 100 |
| **50.0** | 10.0 | 10.0 | 9.0 | 10 | 100 |

To observe organic solvent ratio effect on separation efficiency of D,L-ampicillin, different amounts of ACN were added to the solutions containing SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes. D,L-ampicillin separation was also investigated by varying the ratios for the samples consisting of D- and L-ampicillin. Estimated ratios used for D-ampicillin:L-ampicillin were 1:1, 2:1 and 3:1 (mmol: mmol). The same buffer solution was used for the separation of isomeric compounds, and the analysis was carried out with D-ampicillin sample with a concentration of 1 mg/mL. Alfasilin antibiotic bought from the pharmacy was analyzed as a real sample by SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes. Alfasilin capsule was crushed with muller and mixed by adding 10 mL $NH_4OAc$. Subsequently, the solution was diluted 100 times for analysis.

**Table 1c.** Composition of the SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex solution used on the separation of D,L-ampicillin at different applied electric fields.

| SDS (mM) | L-Lys-$Cu^{+2}$ (mM) | $NH_4OAc$ (mM) | pH | Electrical field (kV) | Pressure (mbar) |
|---|---|---|---|---|---|
| **25.0** | 10.0 | 10.0 | 9.0 | 5 | 100 |
| **25.0** | 10.0 | 10.0 | 9.0 | 10 | 100 |
| **25.0** | 10.0 | 10.0 | 9.0 | 15 | 100 |

**Table 1d.** Composition of the SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex solution used on the separation of D,L-ampicillin at different applied pressure values on the separation of D,L-ampicillin.

| SDS (mM) | L-Lys-$Cu^{+2}$ (mM) | $NH_4OAc$ (mM) | pH | Electrical field (kV) | Pressure (mbar) |
|---|---|---|---|---|---|
| **25.0** | 10.0 | 10.0 | 9.0 | 10 | 0.0 |
| **25.0** | 10.0 | 10.0 | 9.0 | 10 | 50.0 |
| **25.0** | 10.0 | 10.0 | 9.0 | 10 | 100.0 |

### 2.8. Chromatographic parameter.

To understand the physical significants of chromatographic parameter including retention factor (k), resolution value ($R_s$) and separation factor (α) were calculated by the following equations. Retention factor (k) is defined by the Equation (1).

$$k = (n_{mc}/n_{aq}) \quad (1)$$

Here, $n_{mc}$ is the amount of analyte incorporated into the micelle, and $n_{aq}$ is the amount of analyte that surrounds the aqueous solution. In this case, the relationship between retention factor and migration time can be expressed by the Equation (2).

$$k = (t_R - t_0)/[t_0(1 - t_R/t_{mc})] \quad (2)$$

The resolution value $R_s$ can be calculated by the Equation (3).

$$R_s = \left(\frac{N^{\frac{1}{2}}}{4}\right) \times \left[\left(\alpha - \frac{1}{\alpha}\right)\right] \times \left[\frac{k_2}{1+K_2}\right] \times \frac{[1-(t_0-t_{mc})]}{[1+(t_0/t_{mc})k_1]} \quad (3)$$

Where $N$ is the theoretical plate number, $k_1$ and $k_2$ are the retention factors of analytes 1 and 2, respectively. The separation factor, α, is equal to $k_2/k_1$. $R_s$ and α values were calculated using the Equations 4 and 5 from the electropherograms performed for the separation of the D,L-ampicillin by SDS-based micellar electrokinetic columns[45].

$$R_s = (t_D - t_L)/(w_{1,2D} - w_{1,2L}) \quad (4)$$

$$\alpha = (t_D - t_L) \quad (5)$$

The $t_D$ and $t_L$ show retention times for the D- and L-isomers, while $w_{1,2D}$ and $w_{1,2L}$ represent peak widths at the midpoint of the maximum peak height of the D- and L-ampicillin.

### 2.9. Reusability.

The separation of D,L-ampicillin isomers was repeated four times for the reusability experiments. The buffer solution containing 25.0 mM SDS, 10.0 mM L-Lys-$Cu^{+2}$ complex and 10.0

mM $NH_4OAc$ was used for the separation of the D,L-ampicillin with a current at 10 kV and the pressure at 100 mbar.

## 3. RESULTS

### 3.1. Preparation of SDS-based electrokinetic columns.

Separation of D,L-ampicillin isomers was carried out by ligand exchange-micellar electrokinetic chromatography method using L-Lysine monohydrochloride as a ligand and copper (II) sulfate pentahydrate is a central ion supplier. Ligand exchange chromatography is one of the methods that separate isomeric molecules by using chirality and based on the formation of complexes that can act as ligand. D,L-ampicillin separation is performed through these complexes.

In this study, D,L-ampicillin was separated by L-Lys-$Cu^{+2}$ amino acid-metal complex and optimal molar ratios of the interacting L-Lys amino acid with the $Cu^{+2}$ ion forming the L-Lys-$Cu^{+2}$ complex was determined by UV-VIS spectrophotometer. Since there is no significant signal change after the ratio of 2:1 (L-Lys: $Cu^{+2}$) as shown in Figure 1.a and Figure 1.b, this ratio was estimated as the maximum L-Lys:$Cu^{+2}$ ratio and was used for subsequent studies.

The characterization of the L-Lys-$Cu^{+2}$ amino acid-metal complex resulting from the coordination of L-Lys with the $Cu^{+2}$ ion was performed with FTIR-ATR spectrophotometer (Thermo Fisher Scientific, Nicolet iS10, Waltham, MA, USA).

The FTIR-ATR spectrum of the L-Lys amino acid and L-Lys-$Cu^{+2}$ complex were given in Figure 1.c and Figure 1.d respectively. Since the amino acids act as zwitterions in aqueous solutions and solid states, the FTIR spectrum of the amino acids gives significant signals for the $\upsilon NH_3$ and $\upsilon C = 0$ groups. As shown in Figure 1.c, the band at 2947 $cm^{-1}$ belongs to the N-H stretching band while the band at 1625 $cm^{-1}$ stands for the carbonyl $\upsilon$(C=O) stretching band for the –COOH functional group of L-Lysine amino acid. The band of carbonyl group $\nu$(C=O) at 1625 $cm^{-1}$ has shifted to 1633 $cm^{-1}$ when $Cu^{+2}$ ion is coordinated to L-Lysine amino acid when the L-Lys-$Cu^{+2}$ complex is formed. Furthermore, the shift from 2947 $cm^{-1}$ to 3295 $cm^{-1}$ indicates the formation of L-Lys-$Cu^{+2}$ metal-ligand coordination as shown in Figure 1.d. To determine the CMC, 10 kV electric fields were applied to the solutions prepared at different concentrations of SDS in 10.0 mM $NH_4OAc$ and the resulting current was taken as µA and the CMC was determined. Figure 1.e shows the determination of the CMC value obtained from the graph which is plotted of different concentration of the SDS solutions (mM) against the current (µA). The effect of the 10 mM L-Lys-$Cu^{+2}$ amino acid-metal complex which is added to the SDS solutions prepared at different concentrations on the determination of CMC was evaluated. To determine the CMC, 10 kV electric field was applied to the solutions containing SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes prepared at different SDS concentrations in 10.0 mM $NH_4OAc$, and as shown in Figure 1.f the current was found to be 9.85 µA.

Morphological microstructure characterization of SDS micelle and SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes which were prepared in 10.0, 25.0 and 50.0 mM concentrations was performed with CTEM. The mean size distribution of particles and the potentials of these solutions were measured with a zeta size and potential analyzer. In addition, size analysis was performed using a dynamic light scattering spectrometer capable of more accurate measurements in order to verify zeta size measurements of SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes. Microscopic morphological characterization of SDS micelle and SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes were visualized by FEI brand Tecnai G2 Spirit Biotwin model CTEM.

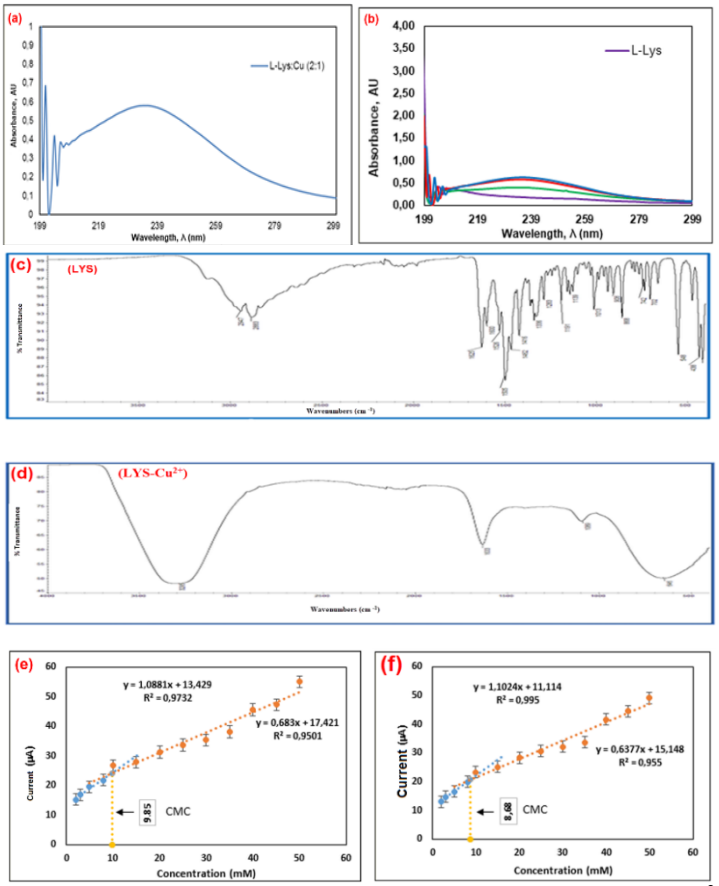


**Figure 1.** Characterization studies of L-Lys amino acid, L-Lys:$Cu^{+2}$ amino acid-metal complex and SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex.

a) Maximum L-Lys:$Cu^{+2}$ ratio (2:1) determined by UV-VIS spectrophotometer.
b) Maximum L-Lys:$Cu^{+2}$ ratio determination by UV-VIS spectrophotometer.
c) The FTIR-ATR spectra of the L-Lys amino acid.
d) The FTIR-ATR spectra of the L-Lys-$Cu^{+2}$ complex .
e) CMC determination of SDS micelles by using different concentration of the SDS solutions (mM).
f) CMC determination of SDS micelles containing SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes prepared at different SDS concentrations (mM).

Figure 2 shows the size of the SDS micelles in the solutions prepared at three different concentrations (Figure 2.a and 2.b for 10.0 mM, Figure 2.c and 2.d for 25.0 mM, and Figure 2.e and 2.f for 50.0 mM) with two different magnifications for each concentration while the size of the SDS-L-Lys-$Cu^{+2}$ micelle complexes which were prepared in the same concentrations was visualized in Figure 3 (Figure 3.a for 10.0 mM, Figure 3.b for 25.0 mM, and Figure 3.c for 50.0 mM). So, the effect of SDS concentration on the size of SDS micelles and SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes were investigated. The sizes of the micelles formed by SDS solutions having 10.0, 25.0 and 50.0

mM concentrations which were prepared over the CMC, were in the range of 60-70, 40-50 and 80-90 nm, respectively.

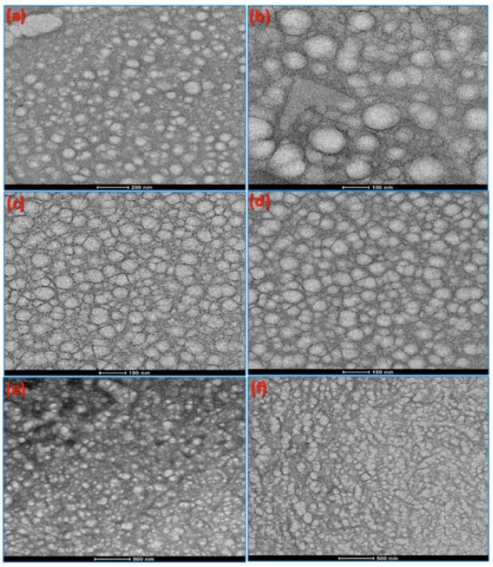

**Figure 2.** CTEM images of the SDS micelles.
a), b) SDS concentration: 10.0 mM; buffer solution: 10.0 mM $NH_4OAc$, pH 9.0.
c), d) SDS concentration: 25.0 mM; buffer solution: 10.0 mM $NH_4OAc$, pH 9.0.
e), f) SDS concentarion: 50.0 mM, buffer solution: 10.0 mM $NH_4OAc$, pH 9.0.

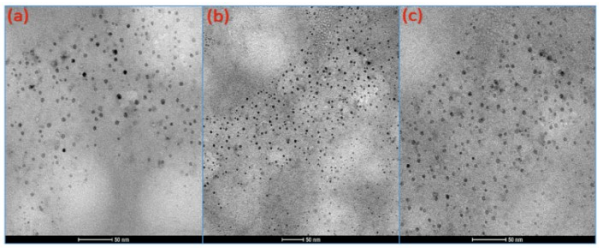

**Figure 3.** CTEM images of the SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes.
a) SDS concentration: 10.0 mM; L-Lys-$Cu^{+2}$ amino acid-metal complex concentration: 10.0 mM; buffer solution: 10.0 mM $NH_4OAc$, pH 9.0.
b) SDS concentration: 25.0 mM; L-Lys-$Cu^{+2}$ amino acid-metal complex concentration: 10.0 mM; buffer solution: 10.0 mM $NH_4OAc$, pH 9.0.
c) SDS concentration: 50.0 mM; L-Lys-$Cu^{+2}$ amino acid-metal complex concentration: 10.0 mM; buffer solution: 10.0 mM $NH_4OAc$, pH 9.0.

The solution with a concentration of 10.0 mM was just above the CMC. For that reason, lesser aggregate formation was observed compared to micelle formations in other prepared solutions. When the concentration was increased to 25.0 mM, micelles shrank due to their negative charges, and more frequent micelle formation was observed. However, by increasing the concentration to 50.0 mM, the micelles began to form large global aggregates. For this reason, the micelles formed in 50.0 mM solution have the largest volumetric sizes. CTEM images show that L-Lys-$Cu^{+2}$ amino acid-metal complexes entered the micelles structure having of 10.0, 25.0, and 50.0 mM SDS concentrations. It has been found that the size of the micelles has started getting smaller considerably by entering the L-Lys-$Cu^{+2}$ complex into micelles structures. The size of micelles has shrunk with increasing concentration but has begun to increase after a certain concentration level.

The zeta size and potential analyzer calculates the zeta potential by determining the velocity of the particles that are actuated under a certain potential. Zeta potential and sizes of 10.0, 25.0 and 50.0 mM SDS micelles prepared in 10.0 mM $NH_4OAc$ medium and Lys-$Cu^{+2}$ micelle complexes consisting of the L-Lys-$Cu^{+2}$ amino acid-metal complex added to the SDS solutions at a concentration of 10.0 mM were calculated by Nano Zetasizer. In Table 2, the mean sizes by number and polydispersity index of the micelle and micelle complexes are tabulated. Due to the interaction of the amino acid-metal complexes with the SDS micelles, size of the micelles starts to shrink and polydispersity indexes are reduced with increased homogenity relative to the micelles formed solely from the SDS. Similar behaviour is observed in the SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes when the SDS concentration is increased above the CMC, the size of micelle starts to shrink with increasing concentration, and then start to grow again after large aggregate formation. Figure 4 shows image for the size distrubution by a number of SDS micelles and SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes determined by Nano Zetasizer instrument.

**Table 2.** Estimated mean sizes by number and polydispersity constants of the SDS micelle and SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes determined by Nano Zetasizer.

| Solution | Mean sizes by number (nm) | Polydispersity constant |
|---|---|---|
| **10.0 mM SDS** | 63.45 | 0.587 |
| **25.0 mM SDS** | 51.84 | 0.481 |
| **50.0 mM SDS** | 90.90 | 0.483 |
| **10.0 mM SDS-L-Lys-$Cu^{+2}$** | 9.394 | 0.309 |
| **25.0 mM SDS-L-Lys-$Cu^{+2}$** | 8.818 | 0.227 |
| **50.0 mM SDS-L-Lys-$Cu^{+2}$** | 9.988 | 0.235 |

When the zeta potential measurements of SDS solutions with different concentrations were evaluated, a sudden increase in the zeta potential was determined by increasing SDS concentration in the solution from 8.0 to 10.0 mM as shown in Table 3 and Figure 5.a. This was due to the starting of micelles formation at a concentration of 8.68 mM SDS.

Zeta potential measurements of SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes prepared with solutions containing 10.0, 25.0 and 50.0 mM SDS were also performed. The zeta potentials of SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes with the increasing SDS concentration were found to be -13.4, -20 and -12.9 mV, respectively. The zeta potentials of SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes are shown in Figure 5.b, Figure 5.c and Figure 5.d. Zeta potentials of micelle solutions prepared with only SDS are quite negative. However, when the L-Lys-$Cu^{+2}$ complex, which has a relatively positive charge, enters the medium, the negativity of the zeta potentials present in the medium is reduced. The solution of SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex with a concentration of 10.0 mM SDS, prepared just above the CMC, has a zeta potential of -13.4 mV. However, when the SDS concentration in the solution increased to 50.0 mM, a large number of micelles formed in the solution, resulting in the formation of large aggregates. For this reason, zeta potential did not increase negatively as expected.

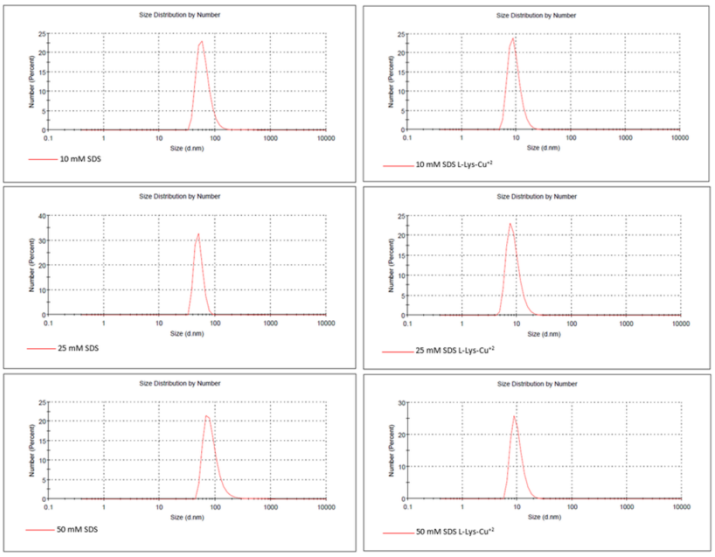


**Figure 4.** Size images as size distrubution by number of SDS micelles and SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes determined with Nano Zetasizer instrument.
a) SDS concentration: 10.0 mM; buffer solution: 10.0 mM $NH_4OAc$, pH 9.0.
b) SDS concentration: 25.0 mM; buffer solution: 10.0 mM $NH_4OAc$, pH 9.0.
c) SDS concentration: 50.0 mM; buffer solution: 10.0 mM $NH_4OAc$, pH 9.0.
d) SDS concentration in SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: 10.0 mM; L-Lys-$Cu^{+2}$ amino acid-metal complex concentration: 10.0 mM; buffer solution: 10.0 mM $NH_4OAc$, pH 9.0.
e) SDS concentration in SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: 10.0 mM; L-Lys-$Cu^{+2}$ amino acid-metal complex concentration: 10.0 mM; buffer solution: 10.0 mM $NH_4OAc$, pH 9.0.
f) SDS concentration in SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: 10.0 mM; L-Lys-$Cu^{+2}$ amino acid-metal complex concentration: 10.0 mM; buffer solution: 10.0 mM $NH_4OAc$, pH 9.0.

Zetasizer results were verified by the particle size obtained as a result of measurements made with DLS. The change in size due to the increase in the size distribution and density obtained by Zetasizer was very similar. In Figure 6, particle size distributions of three different SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex solutions having 10.0, 25.0 and 50.0 mM SDS concentrations were shown by measuring the intensity and variation of the scattered light by particles. The mean particle sizes as shown in the graph for the micelle-chiral selector complexes containing 10.0, 25.0 and 50.0 mM SDS solutions were; 7.125, 6.707 and 7.400 nm, respectively.

### 3.2. Electrokinetic studies.

Separations were carried out using a micellar electrokinetic chromatography using a capillary electrophoresis instrument by combining the ligand exchange mechanism with micellar electrokinetic capillary chromatography. In this system, the SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex was used as a pseudostationary phase for the D-, L- separation of ampicillin.

**Table 3.** Zeta potentials for the SDS solutions prepared with different SDS concentration; SDS concentrations: 2-20 mM.

| SDS (mM) | 2.0 | 3.0 | 5.0 | 8.0 | 10.0 | 15.0 | 20.0 | 25.0 | 30.0 |
|---|---|---|---|---|---|---|---|---|---|
| Zeta Potential (mV) | -7.4 | -11.6 | -15.9 | -29.7 | -70.1 | -78.4 | -84.8 | -88.3 | -83.5 |

| SDS (mM) | 35.0 | 40.0 | 45.0 | 50.0 | 55.0 | 60.0 | 65.0 | 70.0 |
|---|---|---|---|---|---|---|---|---|
| Zeta Potential (mV) | -75.3 | -70.6 | -69.5 | -67.3 | -66.9 | -67.1 | -66.8 | -67.1 |

After the optimization of electrokinetic conditions, D,L-ampicillin was separated. The effect of pH, SDS amount, applied electrical field, pressure, organic solvent ratio and ampicillin D,L-ratios were investigated on the electrokinetic separation of ampicillin isomers in both aqueous solutions and alfasilin antibiotic as a real sample.

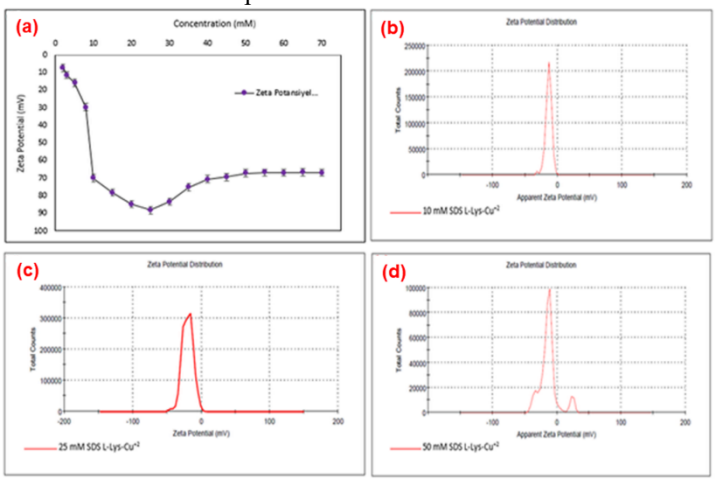


**Figure 5.** Zeta potential measurement of SDS micelles and SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes determined with Nano Zetasizer instrument.

a) Graph of the zeta potentials for the SDS solutions prepared with different SDS concentration; SDS concentration: 2-20 mM.
b) Image of the zeta potential distrubutions for the SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: SDS concentration in SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: 10.0 mM; L-Lys-$Cu^{+2}$ amino acid-metal complex concentration: 10.0 mM; buffer solution: 10.0 mM NH4OAc, pH 9.0.
c) Image of the zeta potential distrubutions for the SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: SDS concentration in SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: 25.0 mM; L-Lys-$Cu^{+2}$ amino acid-metal complex concentration: 10.0 mM; buffer solution: 10.0 mM NH4OAc, pH 9.0.
d) Image of the zeta potential distrubutions for the SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: SDS concentration in SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: 50.0 mM; L-Lys-$Cu^{+2}$ amino acid-metal complex concentration: 10.0 mM; buffer solution: 10.0 mM NH4OAc, pH 9.0.

pH is one of the parameters which affects the solubility control of the ionisable substances along with surface tension reducing substances [46]. Kinetic studies were carried out at pH 7.0, 8.0 and 9.0 to examine the effect of pH. It was observed that the best separation was obtained at pH 9.0 as shown in Figure 7.a. Because of the charge balance between the structures of Cu (L-Ligand-D-ampicillin) and Cu (L-Ligand-L-ampicillin) which are formed by the competitive binding of the D- and L- forms of ampicillin to Cu $(L\text{-}Ligand)_2$ complex at pH 9.0, ligand exchange has been successfully accomplished.

The effect of SDS concentrations on the separation of D,L-ampicillin isomers was also investigated. For this, buffer solutions (pH 9.0) containing 5.0, 10.0, 25.0 and 50.0 mM SDS, 10.0 mM amino acid-metal complex and 10.0 mM $NH_4Oac$ were tested separately. No separation was observed with the buffer solution containing 5.0 mM SDS concentration which was below the CMC. The formation of large aggregates in the solution medium with a concentration of 50.0 mM SDS also inhibited the efficient separation of the isomers. As a result, it was found that the active electrolyte buffer which contains 25.0 mM SDS concentration. In Figure 7.b. overlapped electropherograms show using of the micelles with different SDS concentrations on the separation of D, L ampicillin. With increasing amounts of the anionic surfactant SDS, the retention time of the ampicillin isomers was also

increased. This is because of the micelle interactions caused by increasing SDS concentration.

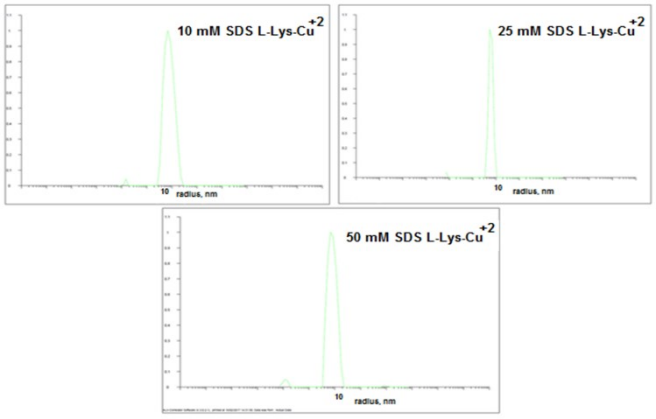


**Figure 6.** DLS spectrometry measurements for the mean particle size distribution estimation of SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex.

a) Image of the mean particle size distrubutions for the SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: SDS concentration in SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: 10.0 mM; L-Lys-$Cu^{+2}$ amino acid-metal complex concentration: 10.0 mM; buffer solution: 10.0 mM $NH_4OAc$, pH 9.0.

b) Image of the mean particle size distrubutions for the SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: SDS concentration in SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: 25.0 mM; L-Lys-$Cu^{+2}$ amino acid-metal complex concentration: 10.0 mM; buffer solution: 10.0 mM $NH_4OAc$, pH 9.0.

c) Image of the mean particle size distrubutions for the SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: SDS concentration in SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: 50.0 mM; L-Lys-$Cu^{+2}$ amino acid-metal complex concentration: 10.0 mM; buffer solution: 10.0 mM $NH_4OAc$, pH 9.0.

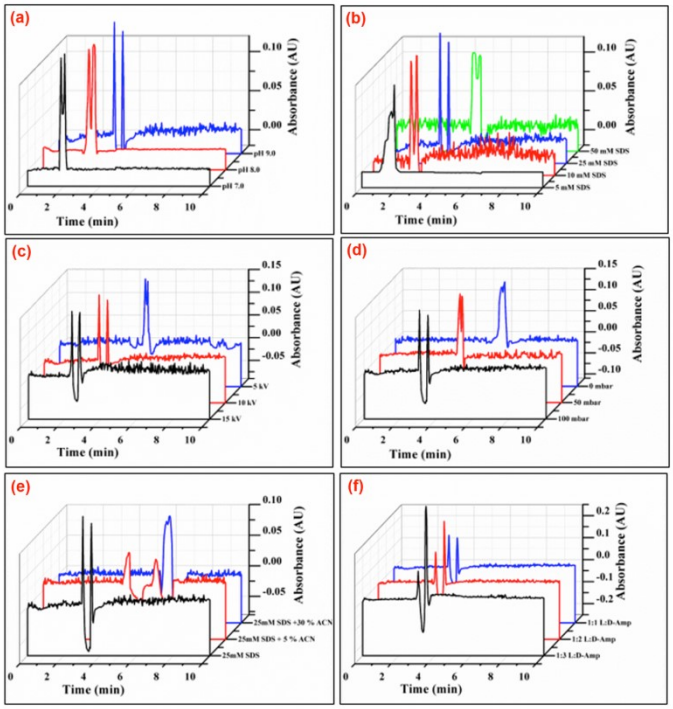


**Figure 7.** Electroknetic studies performed with SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes with CEC instrument.

a) pH effect on the separation of D,L-ampicillin by the SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex; SDS concentration in SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: 25.0 mM; L-Lys-$Cu^{+2}$ amino acid-metal complex concentration: 10.0 mM; buffer solution: 10.0 mM $NH_4OAc$; applied electrical field: 10 kV; applied pressure: 100 mbar; pHs: 7.0, 8.0, 9.0.

b) SDS concentration effect on the separation of D,L-ampicillin by the SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex; L-Lys-$Cu^{+2}$ amino acid-metal complex concentration: 10.0 mM; buffer solution: 10.0 mM $NH_4OAc$, pH: 9.0; applied electrical field: 10 kV; applied pressure: 100 mbar: SDS concentrations in SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: 5.0, 10, 25, 50 mM.

c) Electrical field effect on the separation of D,L-ampicillin by the SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex; SDS concentrations in SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: 25 mM; L-Lys-$Cu^{+2}$ amino acid-metal complex concentration: 10.0 mM; buffer solution: 10.0 mM $NH_4OAc$, pH: 9.0; applied pressure: 100 mbar; applied electrical field: 5.0, 10, 15 kV.

d) Pressure effect on the separation of D,L-ampicillin by the SDS-L-Lys- $Cu^{+2}$ micelle-chiral selector complex; SDS concentrations in SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: 25 mM; L-Lys-$Cu^{+2}$ amino acid-metal complex concentration: 10.0 mM; buffer solution: 10.0 mM $NH_4OAc$, pH: 9.0; applied electrical field: 10 kV; applied pressure: 0, 50, 100 mbar.

e) Organic solvent ratio effect on the separation of D,L-ampicillin by the SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex; SDS concentrations in SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: 25 mM; L-Lys-$Cu^{+2}$ amino acid-metal complex concentration: 10.0 mM; buffer solution: 10.0 mM $NH_4OAc$, pH: 9.0; applied electrical field: 10 kV; applied pressure: 100 mbar; ACN%: 0.0, 15, 30.

f) Isomeric ratio effect of D-and L-ampicillin molecules separately on the separation of D,L-ampicillin by the SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex; SDS concentrations in SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: 25 mM; L-Lys-$Cu^{+2}$ amino acid- metal complex concentration: 10.0 mM; buffer solution: 10.0 mM $NH_4OAc$, pH: 9.0; applied electrical field: 10 kV; applied pressure: 100 mbar; D,L-Ampicillin ratio: 1:1; 2:1; 3:1 (mmol:mmol).

The effect of the electric field on the separation of the ampicillin isomers by the prepared solution containing 25.0 mM SDS, 10.0 mM L-Lys-$Cu^{+2}$ amino acid-metal complex and 10.0 mM $NH_4OAc$ was investigated. For separation, different voltages (5, 10, 15 kV) of the electric field were applied. It has been determined that best separation performance was obtained by the electric field applied at 10 kV. Since at 5 kV, the applied electric field did not produce sufficient EOF, separation of the D- and L-ampicillin from each other could not be performed efficiently. By increasing the electric field to 10 kV, separation of the D,L-ampicillin isomers was successfully carried out. When the applied electric field is increased to 15 kV, the D- and L- isomers are separated from each other efficiently. Biggest problem with CE systems is the joule heating, so, the use of a pseudostationary phase loaded with charged particles, such as SDS, results in an excess current by the applied electric field. For this reason, the work was carried out at 10 kV and no higher electrical field values were used. In Figure 7.c., overlapped electropherograms show the effect of the applied electric field on the separation of D,L-ampicilline for different voltage values.

The effect of pressure on the chiral separation of ampicillin isomers has also been investigated. Experiments were done by applying electrolyte buffer solution containing 25.0 mM SDS, 10.0 mM amino acid-metal complex and 10.0 mM $NH_4OAc$ at 0, 50 and 100 mbar pressure, respectively. The best separations were obtained when the 100 mbar of pressure was applied. Figure 7.d shows the overlapping of the electropherograms obtained at different pressure applications. No isomeric separation was observed for the D,L-ampicillin by applying a solely electric field to the system. When 50 mbar of pressure was applied, the EOF flow was increased and the retention times of the D,L-ampicillin isomers in the column were reduced, but D,L-ampicillin separation was not achieved yet. With the application of 100 mbar of pressure to the column, the retention time of the D,L-ampicillin is further reduced and effective separation was observed. Since the

structures of micelles are thought to be momentarily distorted with the pressure applications, the workings were limited to a maximum pressure of 100 mbar.

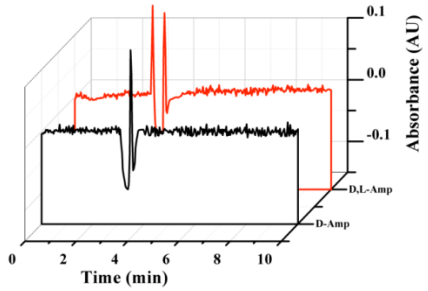


**Figure 8.** Isomeric species detection of D,L-ampicillin molecules separately by the electroknetic separation performed with SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes with CEC instrument; SDS concentrations in SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: 25 mM; L-Lys-$Cu^{+2}$ amino acid-metal complex concentration: 10.0 mM; buffer solution: 10.0 mM $NH_4OAc$, pH: 9.0; applied electrical field: 10 kV; applied pressure: 100 mbar; samples; D- and D,L-ampicillin.

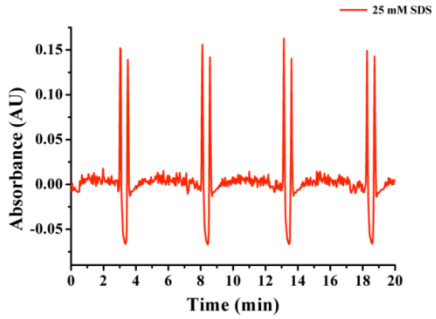


**Figure 9.** Reusability studies of the isomeric D,L-ampicillin separation by SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex; SDS concentrations in SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: 25 mM; L-Lys-$Cu^{+2}$ amino acid-metal complex concentration: 10.0 mM; buffer solution: 10.0 mM $NH_4OAc$, pH: 9.0; applied electrical field: 10 kV; applied pressure: 100 mbar; repeat number: 4.

Water-miscible organic solvents (such as short chain alcohols, acetonitrile) increase the CMC of surfactants and inhibit micelle formation at sufficiently high concentrations [47-49]. The instability of the micelles limits the normal composition variation range to <25% (v/v) organic solvent. The selectivity changes can result from the organic solvents' capability of changing the solvophobic properties of the electrolyte solution. The EOF is reduced by increasing the amount of ACN. For this reason, separated species are subjected to further retention. By increasing the ACN amount to 30% by volume, the EOF is considerably reduced and the retention of the species is increased. In addition, by increasing the amount of ACN to 30%, the micelle formation is also deteriorated. In this case, the isomers were not separated from each other since there is no stationary phase in the column buffer. Figure 7.e shows the overlapped electropherograms which were obtained for the different ACN volume values in the separation of D,L-ampicillin.

### 3.3. Investigation of the D- and L-ampicillin ratio change on the separation.

D-, and L-ampicillin ratios have been varied to separate the isomers in optimized conditions. By keeping the L-ampicillin ratio constant, at first the amount of D-ampicillin was taken same with L-ampicillin, and then the D-ampicillin concentration was doubled and tripled, respectively. There are hydrophobic interactions between the hydrophobic portion of the micelles and long chain -$(CH_2)$ groups of L-Lys amino acids. There is interaction also between amino acid amino groups and $Cu^{+2}$ ions. The increase in the intensity of the lately eluted signal with increasing concentrations of the D-isomers results from the formation of Cu(L-Ligand-D-ampicillin) complex, which instantaneously forms during the ligand exchange by the Cu(L-Ligand)$_2$ complex with the D-isomer during the chiral separation. Figure 7.f shows overlapped electropherograms which indicate that D,L-ampicillin ratio effects the isomeric separation.

### 3.4. Kinetic analysis with D-isomer.

There is a growing interest in drugs consisting of isomerically pure compounds. For this reason, the pharmaceutical industry has given a great deal of effort to develop single isomers. For this purpose, the detection process of a single isomeric species in the optimized conditions of the D,L-ampicillin isomeric mixture was performed. Figure 8 shows the overlapped figures of two analyses including a single isomeric signal and two isomeric signals.

### 3.5. Reusability studies.

Reusability studies were performed for the separation of D,L-ampicillin in conditions optimized with the solution containing SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes. D,L-ampicillin samples were injected repeatedly (4 times) into the system. As shown in Figure 9, After four separation cycles, there was no remarkable change or decrease in the separation capacity of the SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex.

The retention times, peak widths and selectivity factors of each D,L-ampicillin signal repeatedly injected into the system were compatible with each other. These quantities were obtained by calculation of the chromatographic separation parameters.

### 3.6. Real sample analysis.

The acquisition of isomerically pure compounds is of great importance for the pharmaceutical industry. However, many of the drugs sold in the pharmacies are marketed as racemates or diastereomers. For this purpose, the Alfasilin antibiotic sample containing the ampicillin active substance sold on the market was injected into the CEC system by SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes solution in an optimized condition. Electropherogram shown in Figure 10 belongs to Alfasilin antibiotic, while in Figure 11 overlapped electropherograms of samples containing 1:1 mmol D,L-ampicillin and alfasilin used in this experiment were observed together. When the electropherograms of two different samples are examined, the signal of the D-isomer in the alfasilin antibiotic resembled the signal of the D-isomer in the D,L-ampicillin sample. At the same time, a very small amount of the L-isomer was found in the electropherogram of this sample.

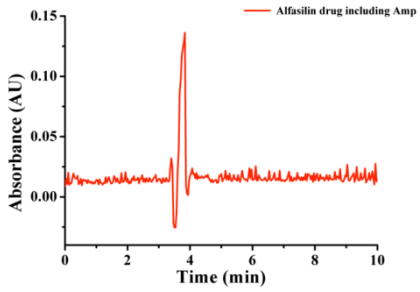


**Figure 10.** Purity estimation studies of alfasilin antibiotic by SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes; SDS concentrations in SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: 25 mM; L-Lys-$Cu^{+2}$ amino acid-metal complex concentration: 10.0 mM; buffer solution: 10.0 mM

$NH_4OAc$, pH: 9.0; applied electrical field: 10 kV; applied pressure: 100 mbar; sample: alfasilin drug including ampicillin.

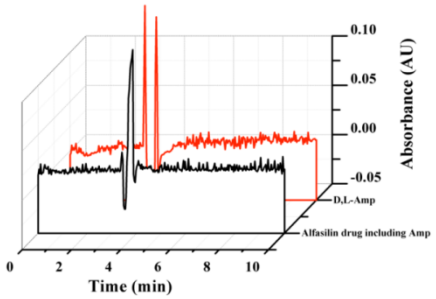


**Figure 11.** Purity estimation studies of alfasilin antibiotic compared with the enatiomeric D,L-ampicillin separation by SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes; SDS concentrations in SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex: 25 mM; L-Lys-$Cu^{+2}$ amino acid-metal complex concentration: 10.0 mM; buffer solution: 10.0 mM $NH_4OAc$, pH: 9.0; applied electrical field: 10 kV; applied pressure: 100 mbar; sample: alfasilin drug including ampicillin.

### 3.7. Chromatographic separation efficiency parameters.

Resolution ($R_s$), selectivity factors (α) and retention times of the D,L-ampicillin isomers were calculated by means of electropherograms obtained from the isomeric separation of D,L-ampicillin by SDS-based micelle solutions. For the isomeric separation, $R_s$ values were not calculated since no effective separation was observed at pH 7.0 and 8.0 as different pH conditions. At pH 9.0, the isomeric separation was achieved successfully. The calculated $R_s$ value (3.47) confirmed this fact. When the effect of the different electric field on the separation of the isomers was examined, no separation was observed when 5 kV electric field was applied to the system.

**Table 4.** Chromatographic separation parameters ($t_D$, $t_L$, $R_s$ and k values) for the separations carried out under different conditions.

| | $t_D$ | $t_L$ | $R_s$ | $A$ |
|---|---|---|---|---|
| **pH** | | | | |
| **7.0** | 2.103 | 1.903 | - | - |
| **8.0** | 2.770 | 2.503 | - | - |
| **9.0** | 3.503 | 3.037 | 3.47 | 1.153 |
| **Current (kV)** | | | | |
| **5** | 4.870 | 4.737 | - | - |
| **10** | 3.503 | 3.037 | 3.47 | 1.153 |
| **15** | 2.837 | 2.403 | 2.59 | 1.181 |
| **Pressure (mbar)** | | | | |
| **0** | 6.033 | 5.967 | - | - |
| **50** | 4.503 | 4.403 | - | - |
| **100** | 3.503 | 3.037 | 3.47 | 1.153 |
| **SDS (mM)** | | | | |
| **5.0** | - | - | - | - |
| **10.0** | 2.370 | 2.103 | 1.36 | 1.126 |
| **25.0** | 3.503 | 3.037 | 3.47 | 1.153 |
| **50.0** | 4.633 | 3.433 | - | - |
| **Organic solvent content (% ACN)** | | | | |
| **0** | 3.503 | 3.037 | 3.47 | 1.153 |
| **15** | 6.170 | 4.703 | 2.31 | 1.312 |
| **30** | - | - | - | - |
| **D-L ratio (D,L)** | | | | |
| **1:1** | 3.503 | 3.037 | 3.47 | 1.153 |
| **2:1** | 3.637 | 3.170 | 3.51 | 1.147 |
| **3:1** | 3.503 | 3.070 | 3.25 | 1.141 |
| **Real sample (Alfasilin antibiotic containing Ampicillin)** | | | | |
| **Alfasilin** | 3.837 | 3.403 | 1.09 | 1.127 |
| **Reusability** | | | | |
| **1. Analysis** | 3.503 | 3.037 | 3.47 | 1.153 |
| **2. Analysis** | 8.570 | 8.103 | 3.51 | 1.058 |
| **3. Analysis** | 13.60 | 13.13 | 3.47 | 1.035 |
| **4. Analysis** | 18.73 | 18.27 | 3.51 | 1.026 |

When 10 kV electric field was applied, a good separation was achieved and the calculated $R_s$ value confirmed this. The retention times of the isomers were reduced because the EOF velocity was accelerated by increasing the electric field intensity to 15 kV and the $R_s$ value was obtained as a lower value (2.59) than the applied 10 kV electric field. To evaluate pressure effect on the separation of D,L-ampicillin isomers, 0, 50 and 100 mbar pressure values were applied to the system separately at 10 kV electric field.

The D,L-ampicillin isomers at 0 and 50 mbar were not separated from each other, so the $R_s$ values were not calculated. An effective separation was observed at 100 mbar. Isomeric separations obtained by different SDS concentration based micelle solutions showed that the separation couldn't be achieved at 5.0 mM SDS concentration. The reason is that this concentration is under CMC. The calculated $R_s$ value for the 10.0 mM SDS containing solution is 1.36, while the calculated value for the solution containing 25.0 mM SDS is 3.47. When these values are compared, it is seen that the separation obtained by the solution containing 25.0 mM SDS is more acceptable. Due to the formation of large aggregates in the solution containing 50.0 mM SDS concentration, the retention times of the species at the pseudostationary phase increased and the $R_s$ value was not calculated because they could not be separated from each other. Experimental results have shown that the organic solvent added to the buffer solution in different volumes adversely affected the isomeric separation. The value of $R_s$ decreased due to the deterioration of the micelle structure with increasing ACN volumes. The retention times of the D- and L-ampicillin in experiments conducted with different D,L-ampicillin concentrations prepared in the same buffer were almost identical to each other. The $R_s$ values calculated by the retention times were found to be 3.47, 3.51 and 3.25, respectively for the ratios of 1:1, 2:1 and 3:1 D,L-ampicillin. As a real sample, alfasilin antibiotic containing ampicillin was injected into the column system and the calculated $R_s$ value for separation of the isomers was found to be 1.09. Looking at the $R_s$ value, it appeared that the separation was not very effective.

For reusability studies, a solution having 25.0 mM SDS concentration was used as the electrolyte solution. As the calculated $R_s$ values for four repeated measurements were close to each other, it appeared that the electrolyte solution containing 25.0 mM SDS which was determined as the optimized separation conditions for the D,L-ampicillin sample can be used as a pseudostationary phase. Table 4 summarized chromatographic separation efficiency parameters.

## 4. CONCLUSIONS

A majority of the pharmaceuticals are chiral, i.e. having one or more chiral centers in their structures. Usually, these molecules are synthesized through achiral synthesis in the form of racemates. Hence, special measures are undertaken to isolate

individual isomers from these isomeric mixtures. Frequently, only one isomer is pharmaceutically active while the other may be toxic, ballast, or inactive [50]. Therefore, to avoid the toxicities, side effects, and other problems, these agents should be administered in the form of pharmaceutically active pure isomers.

The scientists, clinicians, industrialists, and government authorities are asking data on the chiral resolution of biologically important molecules including antibiotics [51].

Due to growing importance of homochiral drugs and interest to metabolic transformations of racemic drugs [52], there is a great demand for analytical chiral separation methods to control isomeric purity of pharmaceutical formulations and determination of chiral drugs in biological samples. In spite of the research and development during the last three decades, only moderate progress has been made in the separation of the chiral antibiotics. Although there are some methods for chiral separation of antibiotics, most of the them are time consuming and they result in large peaks which negatively affects the sensitivity and resolution. Furthermore, a large majority of the proposed HPLC methods is not compatible with mass spectrometric detection. For this reason, the need for more efficient separation methods is obvious.

Ampicillin is a β-lactam antibiotic and is widely used in clinical practice because of its wide pharmacological action, low toxicity and low price if medication is not used in a reasonable way, there will be a risk of ampicillin residues with L-isomer in foods or drugs, which present a potential threat to human health [53].

In this study, SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex was used as a pseudostationary phase for the separation of D,L-ampicillin in CEC system. Different type of parameters influencing separation of D,L-ampicillin in CE such as concentration of SDS, applied pressure, pH, and applied voltage were evaluated from both aqueous solution and real antibiotic sample, alfasilin. Optimization of the parameters improved the isomeric separation remarkably, and the results proved that the combination of micelles with chiral selector producing SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex is suitable as pseudostationary phase and can be used efficiently in isomeric resolution of D,L-ampicillin when looked at $R_s$ values. Factors such as the content of buffer reagents and the preparation of SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex may essentially affect the reproducibility of migration times. Also, high voltage and short analysis time can affect the resolution. Very high voltage causes a decrease in S/N and overheating.

As a result, according to the $R_s$ values calculated for all kinetic analyses performed by SDS-based micelles used for the separation of the D,L-ampicillin showed that the most efficient separation was achieved with a 10.0 mM $NH_4OAc$ buffer solution containing 25.0 mM SDS, 10.0 mM L-Lys-$Cu^{+2}$ amino acid-metal complex at pH 9.0 medium, 10 kV electric field and 100 mbar pressure. Hereby, the separation of the D,L-ampicillin isomers was performed by combining the mechanism of ligand exchange method with micellar electrokinetic chromatography. So, superior properties of the CE such as high efficiency, short analysis time, low amount of solution were successfully used for the separation of the D,L-ampicillin isomers with the predetermined optimum conditions.

In this study, D,L-ampicillin molecule was selected as the isomeric molecule to be separated by using pseudostationary phase prepared with SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complex as a sorbent. So, the separation was achieved in a short period of 7 minutes with high-sensitivity and low-detection limit of 1.25 μM by the developed SDS-L-Lys-$Cu^{+2}$ micelle-chiral selector complexes without using any extra process such as imprinting or spacer arms for the immobilization of the ligands.

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