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Levofloxacin as potent chiral selector for enantioseparation of DL-amino acids using ligand exchange chromatographic approach on TLC

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ABSTRACT

A simple, efficient, cost-effective and less time consuming analytical method was developed to separate the enantiomers of certain DL-amino acids using Levofloxacin as chiral selector. Levofloxacin was used as chiral ligand exchange reagent on TLC plates. Chiral separation was achieved under different conditions like pH of reaction mixture, mobile phase, polarities of solution or reaction mixture and temperature. Separation mechanism of the enantioseparation was also discussed.

Keywords: Levofloxacin, DL-Amino acids, Enantioseparation and Ligand exchange chromatography

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INTRODUCTION

The impetus for advances in separation and detection of various chiral analytes have been highest in the past several years and this still continues to be an area of high focus particularly in food industries, pharmaceutical, archeological, clinical and forensic field. The separation of chiral compounds has been of great interest because the majority of bioorganic molecules are chiral. Living organisms are composed of chiral biomolecules such as amino acids, sugars, proteins and nucleic acids. In nature these biomolecules exist in only one of the two possible enantiomeric forms, e.g., amino acids in the L-form and sugars in the D-form. L-Amino acids are found exclusively in the peptides and proteins of living beings. However, D-amino acids were recently detected as residues in peptides and proteins of various living systems [1]. Chirality is a major concern in the modern pharmaceutical industry. This interest can be attributed largely to a heightened awareness that enantiomers of a racemic drug may have different pharmacological activities, as well as different pharmacokinetic and pharmacodynamic effects. The body being amazingly chiral selective, will interact with each racemic drug differently and metabolize each enantiomer by a separate pathway to produce different pharmacological activity [2]. There are various techniques that have been used for enantioseparation of several drugs. For this purpose, enantioselective chromatographic techniques, namely, high-performance liquid chromatography (HPLC), thin layer chromatography (TLC), and gas chromatography (GC) have extensively been used for decades, because chromatographic procedures are rapid, efficient, reliable, sensitive and easy to perform. Among various chromatographic approaches chiral ligand exchange chromatography (CLEC), which was introduced by Davankov and Rogozhin was seen to be a powerful liquid chromatography (LC) technique with high enantioselectivity for the chiral resolution of chelate or complex-forming compounds, such as amino acids and hydroxyl acids [3, 4].

The ligand-exchange chromatographic HPLC separation of certain natural and unnatural underivatized amino acids was achieved using three cysteine-based coated chiral selectors viz., (*S*)-diphenylmethyl-(*R*)-cysteine, (*S*)-trityl-(*R*)-

cysteine and (*S*)-benzyl-(*R*)-cysteine [5]. The ligand exchange chromatographic resolutions of selected DL-amino acids and their stability studies were carried out by applying the potential of the click chemistry for preparation chiral stationary phase [6]. Three new hybrid organic and inorganic polymeric based ligand-exchange chiral stationary phases (CSP) were developed on silica gel, and successfully applied for the enantioresolutions of some DL-amino acids and DL-hydroxyl acids [7]. The mono-sodium salt of (*R*)-*N*,*N*-carboxymethyl undecyl phenylglycinol was applied as chiral selector onto silica gel to develop a new ligand exchange CSP which was further employed for the resolution of some α- and β-amino acids [8]. Chiral ionic liquid method (having *L*-Proline anions and imidazolium cation as a chiral selector) was used to separate the enantiomers of tryptophan (Trp) on reversed phase C18 column using ligand exchange chromatography [9]. Bhushan et al., developed four different approaches of plate impregnation in the use of Cu divalent complexes of L-amino acids (L-Pro, L-Phe,L-Trp, L-His, and N,N-Me_2-L-Phe) for direct resolution of the enantiomers of racemates of the certain β-blockers like propranolol, atenolol, and salbutamol by ligand-exchange chromatography on silica gel coated TLC plates [10, 11]. Bhushan *et al*. [12] had resolved some racemic α-amino acids (Phe, Tyr, Ile and Trp) using a Cu(II)-L-proline complex by TLC. A variety of compounds have also been enantioseparated using different chiral selectors on ligand exchange TLC and well described in earlier literature which includes [13-15]. Literature shows certain monographs and handbooks [16, 17] on enantioresolution of amino acids. Recently, a review article has also been appeared on enantioresolution of amino acids in which variety of chiral selectors have been discussed [18].

There is a clear evidence of TLC which can be used as complimentary technique to HPLC as it is inexpensive, simple, less time consuming, simultaneous analysis of both standard and sample, impregnation of desirable chiral selector, no need of sophisticated and expensive instrument, analysis of number of samples in one single run and detection is easy, static and off-line. Other advantages of TLC includes: (a) variety of visualizing agents (b) it consumes little amount of mobile phase (c) many samples can be analyzed in one single run (d) there is flexibility in choice of stationary and mobile phases; and (e) it can be coupled to more selective detection techniques such as infrared spectroscopy (IR), mass spectrometry, or gas chromatography [19-21].

During literate survey, a variety of separation methods had been found but still a need to develop a new, simple and cost-effective method for enantioseparation. In view of the cost-effectiveness of TLC, success of the ligand exchange chromatography (LEC) and in search of certain new diverse chiral selectors, Levofloxacin (LEVO) is used as chiral selector. For the enantioseparation of DL-amino acids, diverse chiral selectors had already been applied but LEVO has not been employed as chiral selector to separate enantiomers. The main objective of our study was to apply LEVO as chiral selector or chiral ligand exchange reagent to separate the DL-amino acids on TLC. Levofloxacin (LEVO), is the laevorotatory isomer of ofloxacin, and having (*S*) absolute configuration. Biologically, it is a fluoroquinolone broad-spectrum antibacterial agent and chemically, chiral fluorinated carboxyquinolone (IUPAC: (*S*)-9-fluoro-2, 3-dihydro -3-methyl-10-(4-methyl-1- piperazinyl)-7-oxo- 7H-pyrido [1, 2, 3-de]-1, 4-benzoxazine-6-carboxylic acid) [22-25]. It is used in the treatment of a range of illnesses including respiratory tract, urinary tract, and tissue-based infections. In present study, LEVO was tested as chiral ligand exchange reagent due to presence of carboxylic and adjacent C=O group. The structure of LEVO or (*S*)-Ofloxacin is given below in **Fig. 1**.

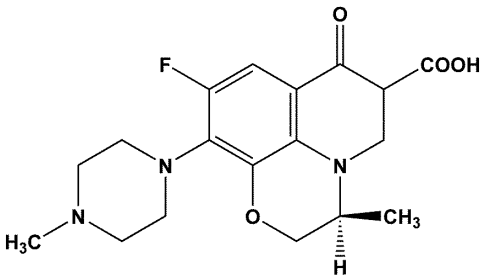


Fig. 1: Structure of Levofloxacin (chiral selector)

Literature review proved that demand for enantiomers of DL-amino acids has continued to grow in various discipline of sciences such as clinical, forensic, pharmaceuticals, biochemistry, archaeology, chiral synthesis etc. Thus there is greater need to develop rapid, robust, highly sensitive methods for enantioseparation of DL-amino acids. For this purpose we developed a simple, novel, cost-effective and versatile enantioseparation method in which LEVO was applied as chiral selector in ligand exchange approach on TLC for the first time.

EXPERIMENTAL SECTION

2.1 Chemicals, Reagents and Apparatus:

The kit of amino acids was from Lobachemie Pvt Ltd. (Mumbai, India), Cupper (II) sulphate pentahydate was purchased from CDH (P) Ltd. (New Delhi, India) and solvents like methanol, ethanol, dichloromethane (pure), acetone etc. used to prepare stock solution were purchased from Merck (AR grade) and silica gel-G was from (Molychem). UV-visible absorption spectrophotometer (Shimadzu, Tokyo, Japan) was used to perform UV-spectrum. FT-IR spectrometer (Nicolet Shimadzu, Tokyo, Japan), polarimeter (model Krüss P3001RS, Germany), Incubator, digital pH meter, TLC plates, developing chambers, glassware and other required chemicals were provided by Lovely Professional University (LPU) laboratory. Most of solutions and solvents were prepared in methanol. Double distilled water was used throughout the study. Tablets of Levofloxacin were purchased from local market.

2.2. Extraction and purification of Pharmaceuticals:

The extraction and purification of Levofloxacine (LEVO) drug was performed from their tablets as per procedure given below.

Six LEVO tablets, each containing 750 mg, were ground to a fine powder in a pestle motor which was shaken with methanol (70 mL) in a small conical flask. The solution was filtered and the residue was further treated with methanol and filtered. The combined filtrate was concentrated in vacuo and left to cool until crystals appeared. The mother liquor was decanted and the crystals were dried and recrystallized from methanol–water. The purity of LEVO was ascertained by determination of melting point, recording UV absorption λmax and IR spectrum followed by comparison with standard values. Above said values were in agreement with reported values (Sigma Aldrich catalogue, 2007). The pure LEVO so obtained were examined by polarimeter for their optical purity and then applied for further studies.

2.3. Preparation of TLC Plates and Development of Chromatograms:

- ***Preparation of plane plates***

An unmodified plane TLC plates were prepared by spreading slurry of silica gel (10 g) in distilled water (20 mL), followed by drying or activation of TLC plates into hot air oven at 70 ± 2˚C temperature for 2 hours.

- ***Preparation of chiral impregnated plates by different approaches***:

A. Mixing of solution of Cu (II)-LEVO complex with silica gel slurry and spreading over the TLC plate followed by activation of plates into hot air oven at 70 ± 2˚C temperatures for 2 hours **(Approach A)**.

B. Ascending development (10-15 min) of unmodified plates in a solution of Cu (II)-LEVO. Unmodified plates were prepared as described above. The plates were dried in hot air oven at 70 ± 2˚C temperature for 2 hours **(Approach B)**.

C. Mixing of the chiral selector (LEVO) with slurry of silica gel. Plates were prepared from same slurry followed by drying in hot air oven at 70 ± 2˚C temperature for 2 hours. Cu (II) (aq) solution was used as mobile phase additive **(Approach C)**.

Cleaned, dried and paper lined rectangular glass chamber was used for developing the chromatograms which was pre equilibrated with the mobile phase at 18 ± 2°C for 12–15 min inside an incubator. TLC plates (10 cm × 5 cm × 0.5 mm) were prepared by spreading a slurry of silica gel G (25 g) prepared in these solutions. The plates were activated for 2 hours at 70 ± 2˚C. Solutions (10^{-2} M) of the DL-amino acids were prepared in methanol (few amino acids in aq. HCl) and applied to the plates with the help of glass capillary. After development of TLC, chromatograms were dried at 50°C in an hot air oven and cooled to room temperature; finally spots were located in an iodine chamber. The chromatograms were bluish or brown.

2.4. Mobile phases for TLC development:

Different mobile phases with different ratio were tried; these were:
MeCN-CH_3COOH-MeOH (4:3:3), MeCN-CH_3COOH-MeOH (6:2:2), MeCN-MeOH (4:6), MeOH-CH_2Cl_2 (4:6), MeCN–MeOH-CH_2Cl_2, C_4H_9OH-H_2O-CH_3COOH (4:1:1), Ethyl acetate-Methanol-Water (1:1:1), Cu (II) aq.-MeCN-MeOH (5:3:4); among these MeCN-CH_3COOH-MeOH (4:3:3), and C_4H_9OH-H_2O-CH_3COOH (4:1:1) were found to give successful results for the separation of DL-amino acid using LEC approach. Details of all mobile phases used and their respective results are given in Table 1.

2.5. Formation of Cu (II)-LER Complex and in-situ diastereomers of DL-Leucine:
Solutions of Levofloxacine (4 mM) and Cu (II) sulphate (2 mM) were prepared in purified methanol-water (90:10). The solutions of chiral selector and Cu (II) ion were mixed in a ratio of 1:2, and the pH of the reactive medium was adjusted (pH ~ 8) by addition of a few drops of aqueous solution of ammonia. The Cu (II)-LER (ligand exchange reagent) chiral complexes so obtained was further treated with DL-Leucine (2 mM) in presence of basic medium. The completion of the reaction was checked on unmodified TLC development. The formation of Cu-chiral complex and their respective diastereomers was confirmed by comparative analysis of UV spectra of the Cu(II) solution, Cu(II)-LER complex and diastereomeric solution. The temperature of the reaction mixture was maintained around 18 ± 2°C.

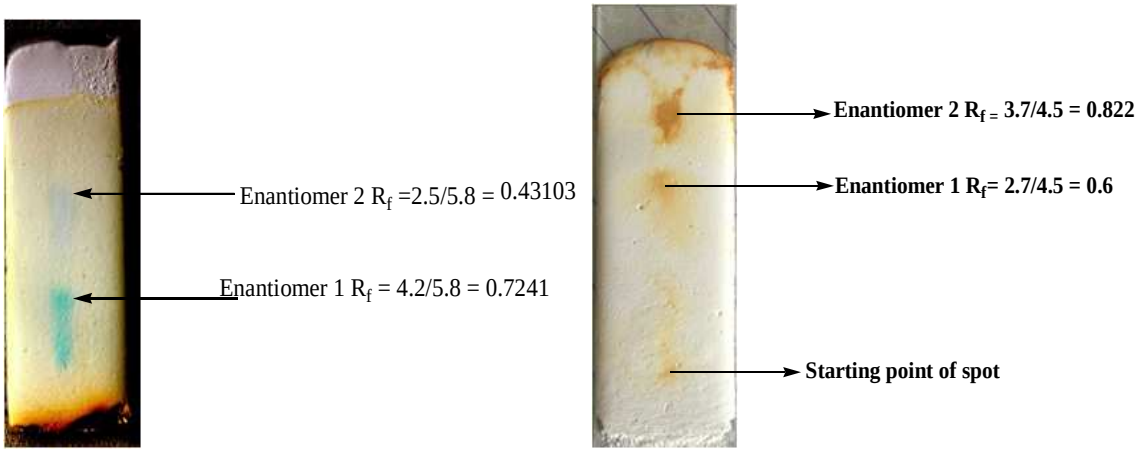
2.6. Effect of Temperature & pH on enantioseparation:
Earlier studies on enantioseparation of different chiral compounds by ligand exchange chromatographic TLC had shown to affect the enantioseparation on variation in temperature and pH. The temperature increase or decrease was done in systematic manner to obtain the variable results in terms of tailing or clearly resolved spots of enantiomers. Each temperature was maintained inside an incubator and the chromatographic chambers were placed inside and allowed to attain the specific temperature before development (pre-equilibration time: 15 min). The optimum temperature of enantioseparation was found at 18 ± 2°C. To find out the optimum condition for good enantioseparation, pH of the chiral selector/ reaction medium was adjusted in the range of pH 6-9 using ammonium hydroxide solution. Most of the successful separations were achieved in basic pH (pH around 8).

RESULTS AND DISCUSSION

TLC separation of selected amino acid enantiomers was successful using LEVO as ligand exchange reagent (chiral selector). Three different approaches (A-C) were employed to achieve the direct enantioseparation on TLC. The basic requirement of direct enantioseparation is chiral medium. In present study, TLC plates were made chiral using approaches A, B and C. In approach A, Cu (II)-LEVO complex was prepared and mixed inside the slurry to prepare chiral TLC plates. Approach B represents ascending development of unmodified plates in a solution of Cu (II)-LEVO solution (chiral mobile phase additive; CMPA). In third approach C, chiral selector (LEVO) was mixed with slurry of silica gel-G and plates were prepared from same slurry followed by development of unspotted plates in Cu (II) solution (aq).

3.1. Ligand Exchange Chromatography on TLC:
The separation on TLC using ligand-exchange approach is a typical case of complexation chromatography. LEC has been shown to be a great approach for chiral separation of chelate complex forming compounds like amino acids, amino alcohols etc. Selected DL-amino acids were enantioseparated using LEC in which copper sulphate (source of Cu (II) ions) was used as divalent central metal ion and LEVO as chiral ligand exchange reagent (LER). Three different approaches (A-C) were employed on TLC. The complex of Cu (II)-LER was used as a chiral impregnating agent as well as chiral mobile phase additive (CMPA) for the resolution of DL-amino acids by TLC.

The best results were obtained using approach "A" (Table 1). A range of solvent systems were tried as mobile phase. The successful solvent combinations for enantioseparation along with hR_F (R_F ×100) values are highlighted in Table 1. Same mobile phase systems were applied on the approaches B & C. However, approach B was comparatively less effective than approach A. Eight shaped spots were obtained using approach B which shown very less resolution. DL-2-amino butyric acid and DL-Isoleucine were merely enantioseparated using approach B. However, very poor or no clear enantioseparation was obtained using approach C. The best results among all amino acids were obtained for DL-Isoleucine using solvent system MeCN-CH_3COOH-MeOH (4:3:3 v/v/v). Two blue colored spots representing the two enantiomers of amino acids were located by exposure to iodine vapors in a glass chamber. The polarimeter studies of each spot confirmed the separation of two enantiomers. The relative distances travelled by two enantiomers were measure by retention factor (R_f) and $hR_f = 100 \times R_f$. Actual photographs of TLC for the enantioseparation of Tryptophan **(a)** and DL-2-amino butyric acid **(b)** are shown in **Fig. 2** as representative chromatograms.



(a) **(b)**

Fig. 2. Actual photographs of TLC for the enantioseparation of (a) Tryptophan and (b) DL-2-amino butyric acid

Table 1: hR_F (R_F × 100) values of amino acid enantiomer using LEVO as chiral ligand exchange agent and approach "A"

S. NO.	*DL*-Amino acids	Solvent Systems		Run time (min)	hR_F	
		Components	Composition (*v/v*)		Ist enanti-omer	IInd enanti-omer
1	*DL*-2-amino butyric acid	MeCN-CH_3COOH-MeOH	4:3:3	10	41	70
		C_4H_9OH-H_2O-CH_3COOH	**4:1:1**	**10**	**50**	**79**
		MeCN–MeOH-CH_2Cl_2	6:2:2	12	NR	NR
2	DL-Threonine	MeCN-CH_3COOH-MeOH	6:2:2	10	58	66
		C_4H_9OH-H_2O-CH_3COOH	4:1:1	10	NR	NR
		MeCN–MeOH-CH_2Cl_2	6:2:2	12	NR	NR
3	DL-Methionine	**MeCN-CH_3COOH-MeOH**	**4:3:3**	**10**	**30**	**41**
		C_4H_9OH-H_2O-CH_3COOH	4:1:1	10	NR	NR
		MeCN–MeOH-CH_2Cl_2	6:2:2	12	NR	NR
4	DL-Tryptophan	MeCN-CH_3COOH-MeOH	4:3:3	10	NR	NR
		C_4H_9OH-H_2O-CH_3COOH	**4:1:1**	**10**	**43**	**72**
		MeCN–MeOH-CH_2Cl_2	6:2:2	12	NR	NR
5	DL- Isoleucine	**MeCN-CH_3COOH-MeOH**	**4:3:3**	**12**	**68**	**93**
		C_4H_9OH-H_2O-CH_3COOH	4:1:1	12	40	58
		MeCN–MeOH-CH_2Cl_2	6:2:2	12	NR	NR

Separation Conditions: NR= No Resolution; pH of mobile phase ~ 8, development distance 8 cm, temperature 18±2°C, and spots were located with iodine vapor in a glass chamber

3.2. Influence of temperature and pH on enantioseparation:

A change in temperature might influence the formation and mobility of diastereomers, resulting in poor or failure of resolution. On account of this, the studies were also carried out with respect to effect of pH and temperature using successful solvent systems only. Table 2, clearly shows that at higher temperature (>30 °C) the separation was very poor or no separation because run time of mobile phase was very fast which leads to lesser or no interaction of enantiomer with chiral selector resulting into poor separation [15, 26]. Since enantiomeric resolutions were observed at 18 ± 2°C and at pH> 8, it can be suggested that only an optimum temperature and pH provide the desired mobility to the transient *in-situ* diastereomeric ion pair complex and therefore any change in these parameters adversely affects the separation of enantiomers.

Table 2: Effect of temperature and pH on enantioseparation of selected DL-Amino acids using successful mobile phase (given in table 1)

DL-Amino acids	Temperature °C±2					pH of Cu-LER complex solution			
	16	**18**	20	25	30	3	5	7	**>8**
DL-2-amino butyric acid	***R***	***R***	***ES***	***NR***	***NR***	***NR***	***R***	***NR***	***R***
DL-Threonine	***R***	***R***	***ES***	***NR***	***NR***	***NR***	***R***	***NR***	***R***
DL-Methionine	***R***	***R***	***ES***	***NR***	***NR***	***NR***	***NR***	***NR***	***R***
DL-Tryptophan	***R***	***R***	***ES***	***NR***	***NR***	***NR***	***NR***	***NR***	***R***
DL- Isoleucine	***R***	***R***	***ES***	***NR***	***NR***	***R***	***R***	***NR***	***R***

Separation Conditions: Successful mobile phases were used during all these studies as listed in Table 1; R: Resolved into enantiomers; NR: not resolved; ES: eight shaped spot; TS: spot with tailing; temperature 18±2 °C, and spots were located with iodine vapor

3.3. Separation Mechanism:

Basic separation mechanism of ligand exchange chromatography is considered to be the same in the present case as is reported earlier [26, 27]. The separation mechanism of chiral ligand exchange chromatography (CLEC) separation is based on the formation of ternary diastereomeric complexes between the chiral ligand exchange reagent (*i.e.* LEVO), enantiomeric analytes, and the metal ion. Since the chiral selector may be exist as stationary phase or mobile phase; thus chiral ligand exchange chromatography has been performed by two modes: the chiral stationary phases (CSPs) and the chiral mobile phases (CMPs). General mechanism for the synthesis of transient ternary diastereomeric complex has been shown in **Fig. 3**.

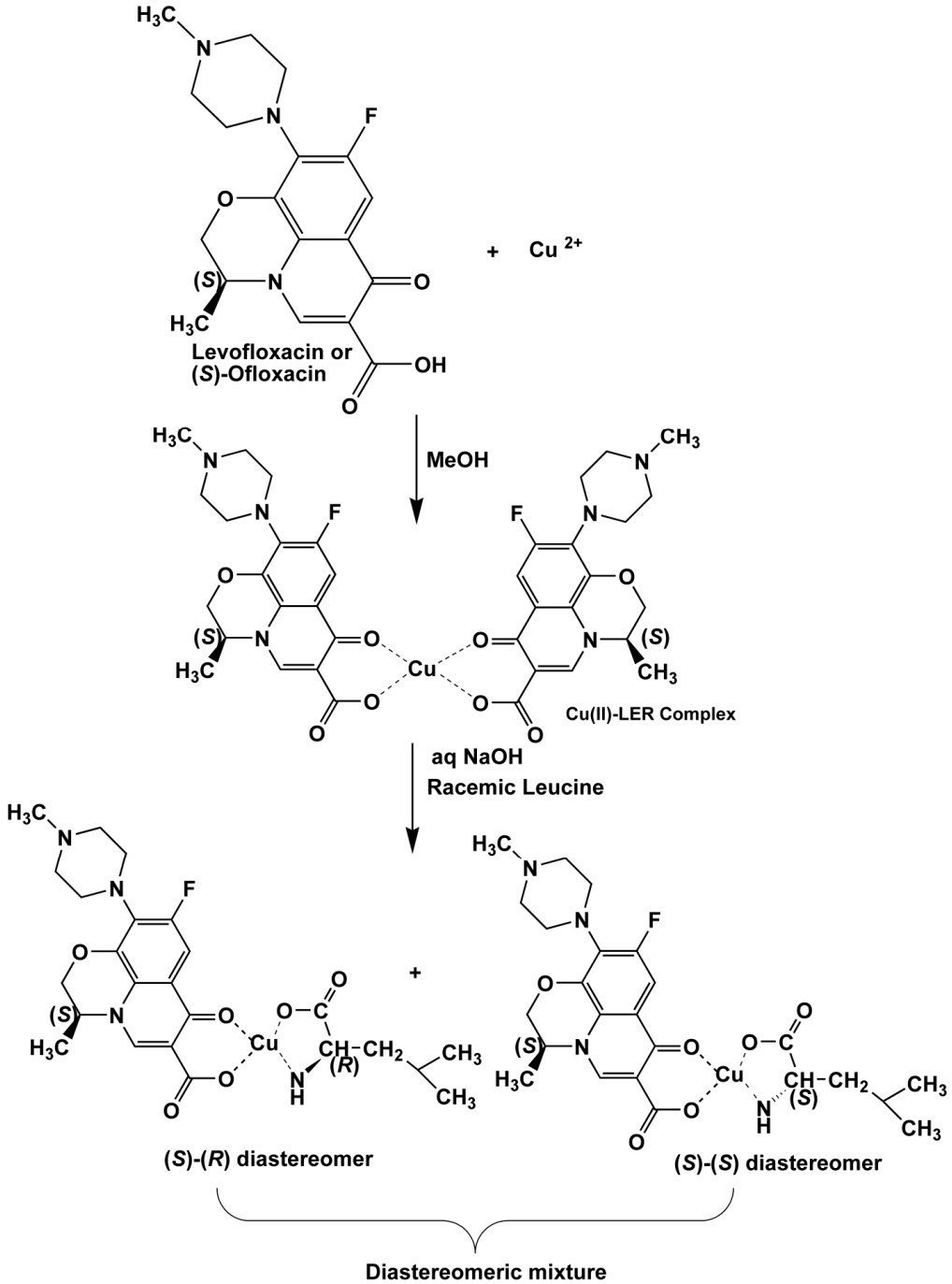


Fig. 3: Separation mechanism for amino acids using Levofloxacin as ligand exchange reagent

3.4. Scope of the Study:
The scope of the current study has been concluded as:
In current study, TLC was made chiral by impregnation with LEVO as chiral selector using different approaches. These approaches are very simple and cost-effective. The direct method was adopted for the enantioseparation of DL-amino acids using ligand exchange approach. This approach can be applied for the separation of structurally similar chiral pharmaceuticals into their individual enantiomers.

CONCLUSION

The present work describes improved analytical method to separate mixture of some DL-amino acids which demonstrated the simplicity, flexibility, versatility and sensitivity of TLC techniques for the enantioseparation of certain racemic analytes via direct method. Direct enantiomeric resolution of selected DL-amino acids was successful *via* ligand exchange on thin silica layers impregnated with Cu (II) complexes of LEVO (chiral selector). Being a direct approach, TLC allowed the recovery of the original enantiomers without chemical modification, simply based on solubility difference in the enantiomer and the chiral selector. There is a clear evidence of TLC being a complimentary technique to HPLC as it is inexpensive, simple, less time consuming, simultaneous analysis of both standard and sample, impregnation of desirable chiral selector, no need of sophisticated and expensive instrument, analysis of number of samples in one single run and detection is easy, static and off-line. The developed method can be applied to recover the pure enantiomer for further use. Thus, the method has potential applications in quality control, forensic, analytical laboratories, and pharmaceutical industries for routine analysis and R&D activities.

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