A Simple and Cost-effective Synthesis of Sulfated β-cyclodextrin and its Application as Chiral Mobile Phase Additive in the Separation of Cloperastine Enantiomers

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ABSTRACT

A simple and cost-effective method for the synthesis of sulfated beta-cyclodextrin, one of the most widely used chiral mobile phase additives, using sulfamic acid as a sulfonating agent has been described. The method was optimized, and the synthesized product was characterized by spectroscopic, size-exclusion chromatographic, thermal, and microscopic methods and was compared to the marketed Sigma Aldrich sulfated beta-cyclodextrin. Beta cyclodextrin, hydroxypropyl beta-cyclodextrin, sulfated beta-cyclodextrin (marketed and synthesized) were evaluated as chiral mobile phase additives (CMPAs) for the enantiomeric separation of cloperastine, an antitussive agent, using reversed-phase HPLC. Under optimized conditions, a resolution of 3.14 was achieved within 15 minutes on an achiral Kromasil C_8 (150 x 4.6 mm, 5 µm) column, with a mobile phase of 5 mM monopotassium phosphate containing 10 mM synthesized sulfated beta-cyclodextrin pH 3.0 and 45% methanol. The method utilizing synthesized sulfated beta-cyclodextrin as CMPA was validated as per ICH guidelines and applied for the quantitative determination of cloperastine enantiomers in active pharmaceutical ingredients and pharmaceutical formulations. The selectivity changes imparted by sulfated beta-cyclodextrin were proven to be beneficial for chiral separation. For the enantiomeric separation of cloperastine, synthesized sulfated beta-cyclodextrin provided better resolution than marketed sulfated beta-cyclodextrin.

Highlights:

1. Cost-effective synthesis of sulfated beta-cyclodextrin
2. Rapid separation of cloperastine enantiomers by synthesized sulfated beta-cyclodextrin as chiral mobile phase additive
3. Synthesized sulfated beta-cyclodextrin provided better resolution than marketed sulfated beta-cyclodextrin

Keywords: Enantiomeric separation, Sulfated β-cyclodextrin**,** Reversed-phase high-performance liquid chromatography (RP-HPLC), Chiral mobile phase additives, Cloperastine

1. INTRODUCTION

Significant differences in the biological activities between the two enantiomeric forms of many drugs have resulted in the need for enantiomeric separation of chiral drugs. The US Food and Drug Administration and other regulatory agencies have made it mandatory for the manufacturers to identify and evaluate the activities of each enantiomer of the chiral drug as well as racemates and analyze the product for enantiomeric purity along with other routine analysis [1, 2].

Chiral high-performance liquid chromatography (HPLC) is one of the most powerful techniques used in enantiomeric separation. Under chiral HPLC, enantiomeric separation can be achieved directly using a chiral stationary phase and chiral mobile phase additive (CMPA) or indirectly using precolumn derivatization. Chiral mobile phase additives offer several advantages, such as the availability of a wide range of chiral additives and the use of achiral columns that are less expensive and more rugged than chiral columns.

Cyclodextrin (CD) is one of the most widely used CMPAs. CDs are cyclic oligosaccharides and are of three main types, namely, *a,* β, and γ, comprising of six, seven, and eight glucopyranose units, respectively. The glucose units in CDs adopt a chair conformation and orient themselves in such a way that the molecule forms a truncated cone-shaped structure with a hydrophobic cavity sandwiched between hydrophilic surfaces. Due to this unique structure, CDs can form inclusion complexes with guest enantiomers and because of this property, CDs are widely applied as CMPAs for enantiomeric separation by HPLC and capillary electrophoresis (CE).

β-CD is readily available and is the lowest-priced and most widely used CD in its family. It has limited solubility in water. The introduction of sulfate groups onto the hydroxyl groups of cyclodextrins confers higher aqueous solubility and enhances the chiral recognition capability for guest enantiomers. The cation exchange and orthogonal hydrophobic interaction (i.e., via inclusion complex) by S-β-CD are responsible for the stereoselective discrimination of enantiomers that fit into the CD cavity [3]. Although S-β-CD is predominantly used in capillary electrophoresis, its successful application in HPLC as CMPA, in some reports, emphasizes the versatility of this approach, its underutilization as a viable analytical tool, and as an alternative to commercially available expensive chiral columns [4-6].

The molecule chosen for the study of enantiomeric separation by S-β-CD is cloperastine (CP, ±1-[2-[(4-chlorophenyl)-phenyl-methoxy] ethyl] piperidine); this drug exhibits antihistaminic, antitussive, and papaverine-like activity similar to codeine but without its narcotic effects [7]. Pharmacological studies have established that levocloperastine (LCP) is the active enantiomer and is also less toxic than dextrocloperastine (DCP) and its racemic mixture. LCP is marketed as a single enantiomer drug. The structure of cloperastine is given in **Figure 1**.

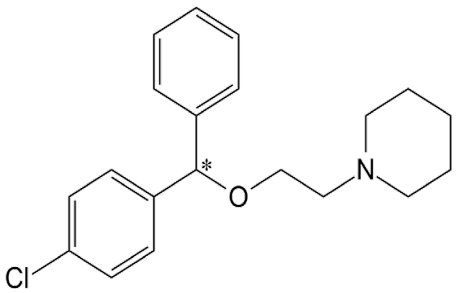


Figure 1: Structure of cloperastine

A survey of the literature revealed that the enantiomeric separation of cloperastine was reported using HPLC by Chiralcel OD-H column in normal-phase mode [8] and Chiralpak-IA in reversed-phase mode [9, 10]. However,

there is no reported method for the enantiomeric separation of cloperastine by chiral HPLC with chiral mobile phase additives. Hence, it motivated us to develop a chiral HPLC method for the enantiomeric separation of cloperastine using a reverse-phase column with S-β-CD as a chiral mobile phase additive i.e without using chiral columns. In the present investigation, a simple and cost-effective synthesis of S-β-CD and its characterization by various techniques have been reported. The chromatographic efficiency of the synthesized S-β-CD (S-β-CD3) was evaluated in comparison with marketed (S-β-CD1) as CMPA in the resolution of cloperastine enantiomers by HPLC.

2. EXPERIMENTAL

2.1 Chemicals

Cloperastine and levoclopersatine were obtained as gift samples from Precise Chemipharma Pvt. Ltd., Mumbai. Beta cyclodextrin was purchased from Jay Chem Ltd., Mumbai. Sulfamic acid was purchased from Qualigens fine chemicals. HPLC and LR grade methanol and orthophosphoric acid (OPA) were purchased from S.D. Fine Chemicals. Acetonitrile, potassium dihydrogen phosphate (KH_2PO_4), N, N dimethylformamide (DMF), and sodium hydroxide (NaOH) were purchased from Merck Ltd. Levocloperastine fendizoate syrup (brand name: Zerotuss) manufactured by Precise Chemipharma Pvt. Ltd., was used for assay studies. Sulfated beta-cyclodextrin sodium salt (degree of substitution 11-14) was purchased from Sigma Aldrich for comparison. All chemicals were of analytical reagent grade and used without further purification. Quartz double distilled water was used to prepare the mobile phase and other solutions

2.2 Synthesis of sulfated beta-cyclodextrin

Initially, for enantiomeric separation of chiral drugs, β-CD, Hp-β-CD, and S-β-CD were used as a CMPA. S-β-CD gave encouraging results, however, marketed S-β-CD (Sigma Aldrich; the degree of substitution 11-14) was priced at $ 340 for 25 g. Hence, it was decided to synthesize S-β-CD, which will further be characterized and utilized as a CMPA. For the synthesis of S-β-CD, sulfuric acid, chlorosulfonic acid, and sulfamic acid were used as sulfonating agents. The synthesized compounds were characterized by various spectroscopic, size-exclusion chromatographic, thermal, and microscopic techniques and were compared to the marketed S-β-CD. Sulfuric acid did not give a good product. S-β-CD1 is marketed S-β-CD, S-β-CD2 is synthesized S-β-CD using chlorosulfonic acid as a sulfonating agent, and S-β-CD3 is synthesized S-β-CD using sulfamic acid as a sulfonating agent.

2.2.1 Synthesis of S-β-CD by chlorosulfonic acid (S-β-CD2)

10 g β-CD was dissolved in 150 ml of DMF and transferred to a cold temperature synthesizer maintained at -35 °C. 14 ml of chlorosulfonic acid was added in a drop-wise manner and after the complete addition of chlorosulfonic acid, the reaction mass was stirred for 1h at -10 °C. The reaction mass was poured into 300 ml chilled acetone and refrigerated overnight. The precipitate was filtered, dissolved in distilled water, and pH was adjusted to 9 with 1N NaOH. The solution was concentrated under reduced pressure using a rotary evaporator and methanol was added to precipitate the product. The product was filtered and dialyzed against water (molecular weight cut off 1000 Da). After dialysis, methanol was added to the solution resulting in precipitation of the product (S-β-CD2) (Yield: 2.5g). The reaction scheme is given in **Figure 2**.

2.2.2 Synthesis of S-β-CD by sulfamic acid (S-β-CD3)

50 g β-CD and 91 g sulfamic acid were dissolved in 750 ml DMF and heated at 70 °C for 6 h. Upon completion, the reaction mass was cooled to room temperature, filtered, and poured into 1.5 liter methanol. The resultant

precipitate was filtered, dissolved in 5N NaOH, and treated with activated charcoal. The charcoal was removed by filtration after 15 minutes and the filtrate was concentrated under reduced pressure using a rotary evaporator. To the concentrated solution, methanol was added to precipitate the product (S-β-CD3). The product was filtered, air-dried, and stored in an airtight container. (Yield: 97.5 g). The reaction scheme is given in **Figure 2**.

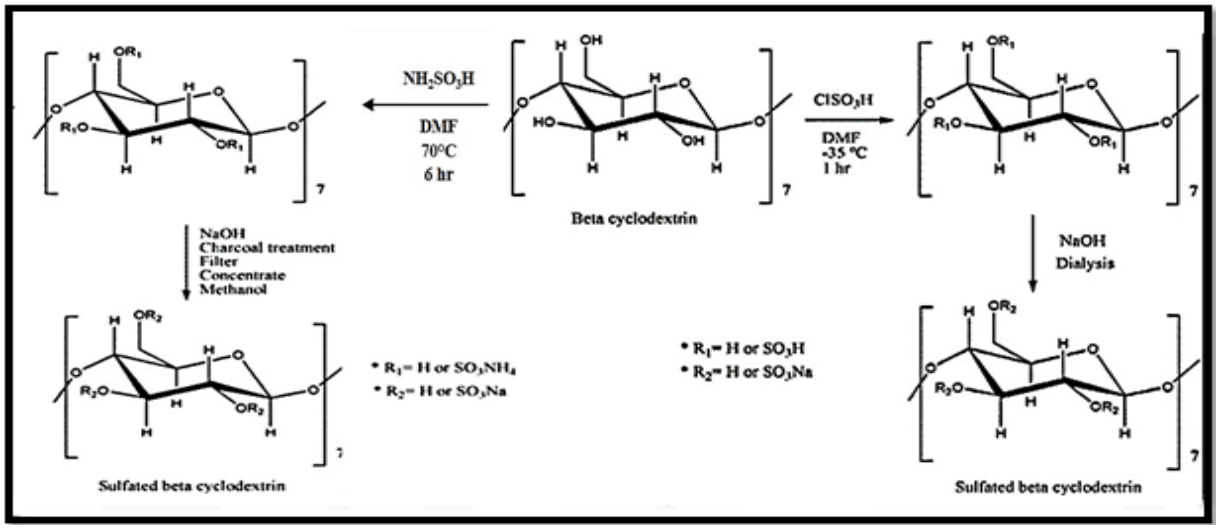


Figure 2: Reaction scheme for the synthesis of sulfated beta-cyclodextrin by sulfamic acid

The details of the scale-up of the synthesis of S-β-CD are given in Table 1.

Table 1: Scale-up of synthesis of S-β-CD

Sr. No.	Beta cyclodextrin (g)	Sulfamic acid (g)	Reaction Time (hr)	Yield (g) mean ± SD	% Yield of S-β-CD
1	5 (4.4 mM)	9.1 (93.7 mM)	4	6.9 ±1.01	84.77
2	10 (8.8 mM)	18.2 (187.4 mM)	4.5	12.6 ±0.95	77.39
3	20 (17.6 mM)	36.4 (374.8 mM)	5	24 ±0.85	73.71
4	25 (22 mM)	45.5 (468.5 mM)	6	34±1	83.54
5	50 (44 mM)	91 (937 mM)	6.5	79+1.5	97.05

Note: For calculations, degree of substitution was considered as 7.

2.3 Instrumentation for Sulfated-β-cyclodextrin characterization:

The **Differential Scanning Calorimetric (DSC) analysis** was performed using Mettler Toledo DSC 822 with a temperature range of up to 500 °C. **Thermogravimetric analysis (TGA)** was performed using a Mettler TGA/SDTA 8S° with a temperature range up to 600 °C. A JEOL 6310 **Scanning Electron Microscope (SEM)** fitted with an Oxford Instruments ISIS. The **X-ray microanalysis** system was used for microscopic analysis. A Jobin Yvon Horiba- ULTIMA 2C HR spectrometer was used for **Inductively Coupled Plasma- Optical Emission Spectroscopic (ICP-OES) analysis** of the S-β-CDs, equipped with a dual photomultiplier tube detector with High Dynamic Detection System (wavelength range: 160 nm- 800 nm). **Nuclear magnetic resonance (NMR)** analyses of the samples were performed with a Bruker Avance III 400 MHz, FT-NMR spectrometer, which includes a Microbay 2-channel console, a broadband multinuclear probe (BBFOPLUS) for observation of nuclei in the frequency range ^{15}N to ^{31}P, ^{19}F with ^{1}H decoupling, observation, and 2 H lock, B-VT 3200 variable temperature accessory for temperatures above ambient, WorkStation, and BOSS I shimmer system. Average molecular weight determination of the samples by **Gel Filtration Chromatographic (GFC) analysis** was carried out on Agilent 1200 series equipped with a refractive index (RI) detector. Two columns in series [Agilent PL-

aquagel OH MIXED M (molecular weight range: 1,000-150,000 Da; resin type: polystyrene/ divinylbenzene) and Phenomenex Biosep-SEC- s3000 (molecular weight range: 1,000 to 500,000; resin type: silica)] were used for average molecular weight determination of S-β-CD. Polystyrene sulfonate standards with different molecular weights in the range of 1830- 425000 Da were used along with a low molecular weight molecule i.e., glucuronic acid (MW 194 Da) as standards. The mobile phase used was 0.05% sodium azide in water, pumped at 1 ml/min. To check the reproducibility of the synthesis of S-β-CD3, four different batches were analyzed along with S-β-CD1.

2.4 Instrumentation for HPLC

The enantiomeric separation was carried out on a Jasco- 1500 series HPLC system comprising of an isocratic pump, a Rheodyne injector with a fixed loop of 20 µl, and a UV detector. Borwin software was used for data processing. The analysis was carried out under isocratic elution at ambient temperature. Kromasil C_8 column (150 X 4.6 mm, 5 µm) was used for the study. Methanol and 5 mM KH_2PO_4 containing 10 mM S-β-CD3 (3.696 g for 200 ml of aqueous phase) at pH 3 (adjusted with 10% OPA) in the ratio of 45:55, v/v was used as the mobile phase for enantiomeric separation of CP. The mobile phase was filtered through a 0.45 µm nylon filter and sonicated for 5 minutes before use. The flow rate was maintained at 1 ml/min and the injection volume was 20 µl. Detection was carried out at 225 nm.

2.5 Preparation of standard solutions

Stock solutions of the racemic and pure enantiomer of cloperastine were prepared by dissolving 50 mg of each drug in 50 ml methanol separately. 5 ml of the above solutions were diluted to 100 ml with methanol to provide 50 µg/ml and 2 ml of this above solutions were further diluted to 10 ml using diluent to provide 10 µg/ml, which was used for analysis. Diluents used for final dilutions were 5 mM KH_2PO_4 and methanol (55:45, v/v).

2.6 Assay of levocloperastine in the formulation

5 ml of Zerotuss syrup was transferred to a 100 ml volumetric flask and mixed with 80 ml of methanol: water (80:20, v/v). The solution was sonicated for 30 minutes and the volume was made up using the same solvent. An aliquot of the solution was filtered through a 0.45 µm nylon filter and transferred to a 10 ml standard volumetric flask to yield a concentration of 10 µg/ml.

2.7 Molecular Modeling

Molecular modeling studies, particularly molecular docking simulations, were performed to estimate whether there is clear difference in the binding affinities of the enantiomers against S-β-CD2. The coordinates for β-CD were imported from the protein data bank (PDB id: 3CGT) <DOI: 10.1021/bi9729918> [11]. The cyclodextrin glycosyltransferase protein was deleted and only β-CD coordinates were retained. The hydroxy groups in β-CD were modified to build S-β-CD such that substitution number is 13. S-β-CD was corrected by adding hydrogen atoms, adding bond orders and atom types were assigned as per AMBER force field and partial charges were computed using Gasteiger's method using AutoDock Tools-1.5.6 (MGL tools). The ligand for docking was also prepared similarly. The grid box of 25 $Å^3$ for docking was defined using the centre of mass of S-β-CD. The grid maps were generated for the ligand atom types using the autogrid program. Molecular docking simulations were performed using Autodock4.2.6 (Scripps Research Institute, USA) < DOI: 10.1002/prot.10028> [12] using Lamarckian Genetic Algorithms. The population size was restricted to 500 for 100 runs of genetic algorithm. At

each generation 25 million energy evaluations were performed for 30,000 generations. The docking run was performed in triplicate and the results were pooled for analysis.

3. Results and discussion

The purpose of the current investigation was to develop a cost-effective HPLC method for the enantioseparation of cloperastine without using expensive chiral columns. Cyclodextrins provide an effective way to develop a reversed-phase method for molecules that can form a host-guest complex with cyclodextrins. To obtain enantiomeric separation by HPLC, the structure of the analyte, cavity size of cyclodextrin, functional group present at cyclodextrin rim, and mobile phase composition play a vital role. To develop a cost-effective chiral HPLC method, beta-cyclodextrin and its derivatives hydroxypropyl beta-cyclodextrin and sulfated beta-cyclodextrin were evaluated as chiral mobile phase additives. As the marketed sulfated beta-cyclodextrin (S-β-CD1) was expensive, sulfated beta-cyclodextrin was synthesized using chlorosulfonic acid (S-β-CD2) and sulfamic acid (S-β-CD3) in our laboratory. This helped in lowering the cost of analysis.

3.1 Synthesis of sulfated beta-cyclodextrin

Sulfuric acid, chlorosulfonic acid, and sulfamic acid were tried as sulfonating agents for the synthesis of S-β-CD using reported methods [13, 14, 15]. The problems associated with sulfonation using sulfuric acid and chlorosulfonic acid were scale-up issues and charring of the product. When sulfamic acid was used as the sulfonating agent using the procedure given in the literature [15], initially a dark brown product was obtained. Therefore, it was decided to modify the reported procedure, the reaction time, temperature, and volume of DMF were modified. The optimized procedure is given in the experimental section. The synthesized product was found to be cheaper than the marketed Sigma Aldrich product.

3.2 Characterization of sulfated beta-cyclodextrin:

S-β-CD1, S-β-CD2, and S-β-CD3 were characterized by FT-IR spectroscopy, NMR spectroscopy, GFC, thermal analysis, and SEM analysis. S-β-CD1 and S-β-CD3 were also analyzed for sulfur and sodium content by ICP-OES.

1. **FT-IR analysis**

Overlay spectra of S-β-CD2 (synthesized by chlorosulfonic acid) on S-β-CD1 are given in **Figure 3**.

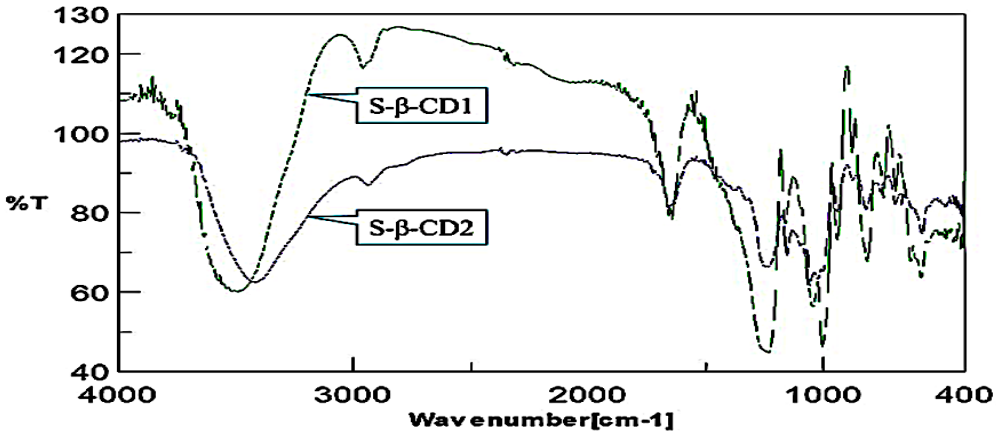


Figure 3: Overlay FT-IR spectra of S-β-CD1 (Marketed) and S-β-CD2 (Synthesized using chlorosulfonic acid)

From Figure 3, it was observed that the IR band for OH and some other bands were not similar for S-β-CD1 and

S-β-CD2. Overlay spectra of S-β-CD1 and S-β-CD3 (synthesized by sulfamic acid) are given in **Figure 4**.

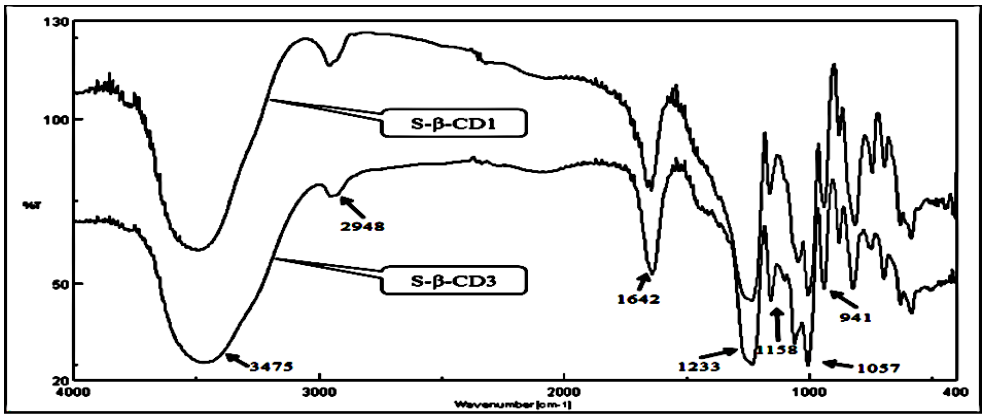


Figure 4: Overlay FT-IR spectra of S-β-CD1 and S-β-CD3

As seen in figure 4, the spectrum of S-β-CD3 is identical to S-β-CD1 and has been discussed in our previous publication [16]. Differences were observed in the case of S-β-CD3, in the region of 1000 cm^{-1} and 500 cm^{-1}, as seen in figure 5.

2. Nuclear magnetic spectroscopic (NMR) analysis

Beta-cyclodextrin monomer has three possible sites for sulfonation. The structure of beta-cyclodextrin along with the numbering of carbons and corresponding protons of each monomer unit is shown in **Figure 5**.

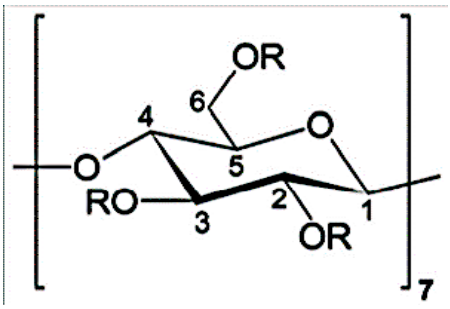


Figure 5: Structure of beta-cyclodextrin along with the numbering of carbons and corresponding protons of each monomer

Proton NMR and DEPT experiments were carried out for the synthesized S-β-CD. Both the spectra were recorded by dissolving the sample in deuterated DMSO. Due to the poor solubility of S-β-CD1 and S-β-CD2, signals were weak and the spectra were not well resolved. ^{1}H and DEPT-135 spectra of S-β-CD3 are shown in **Figures 6 and 7** respectively and the assignment is summarized in Table 2. The ^{1}H NMR showed broad peaks for the hydroxyl protons at 5.6-5.8 ppm (O<u>H</u>-2,3) and 4.4-4.5 ppm (CH_2O<u>H</u>-6). The remaining protons were assigned as follows: 4.8 ppm (H-1), 3.6-3.8 ppm (H-6, H-3, H-5), and 3.2-3.4 ppm (H-4, H-2). The DEPT-135 experiment showed peaks for the ring carbons C-2, C-3, C-5 clustered in the region 72-74 ppm while C-4 is observed at 82 ppm and the anomeric carbon C-1 at 102 ppm. The assignments were confirmed by the correlation HSQC spectrum **Figure 8** which also indicated some overlapping. The CH_2OH carbons appeared in the region of 60-66 ppm as negative signals, indicating two different chemical environments. Positions 2, 3, and 6 of the sugar units of β-CD are possible sites of sulfonation. The poorly resolved signals in the ^{1}H spectra demonstrate the heterogeneity of the CDs depending on sulfonation degree.

Table 2: ^{1}H and ^{13}C NMR peak assignments for Sulfated-β-cyclodextrin (synthesized with sulfamic acid)

^{1}H δ/ppm		^{13}C δ/ppm	
H-1	4.8	C-1	102
H-2	3.2-3.4	C-2	72-74

H-3	3.6-3.8	C-3	72-74
H-4	3.2-3.4	C-4	82
H-5	3.6-3.8	C-5	72-74
H-6	3.6-3.4	C-6	65
O<u>H</u>-2	5.6-5.8		
O<u>H</u>-3	5.6-5.8		
CH_2O<u>H</u>-6	4.4-4.5		

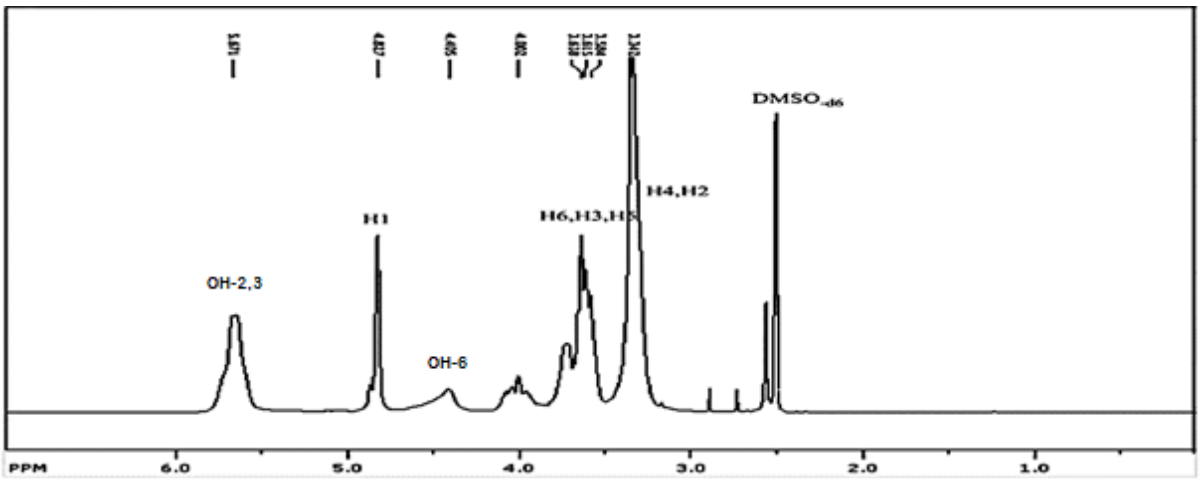


Figure 6: ^{1}H spectrum of S-β-CD3

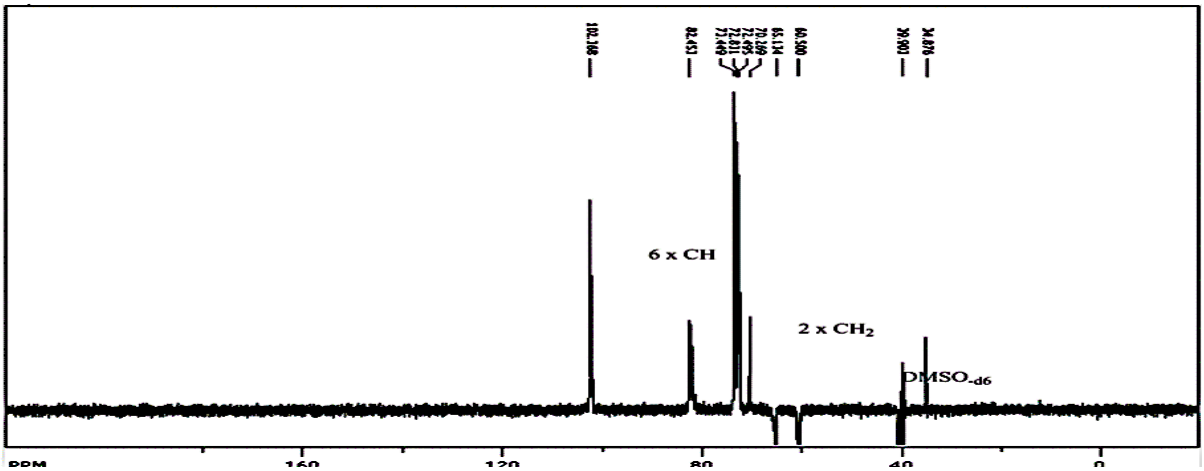


Figure 7: DEPT-135 spectrum of S-β-CD3

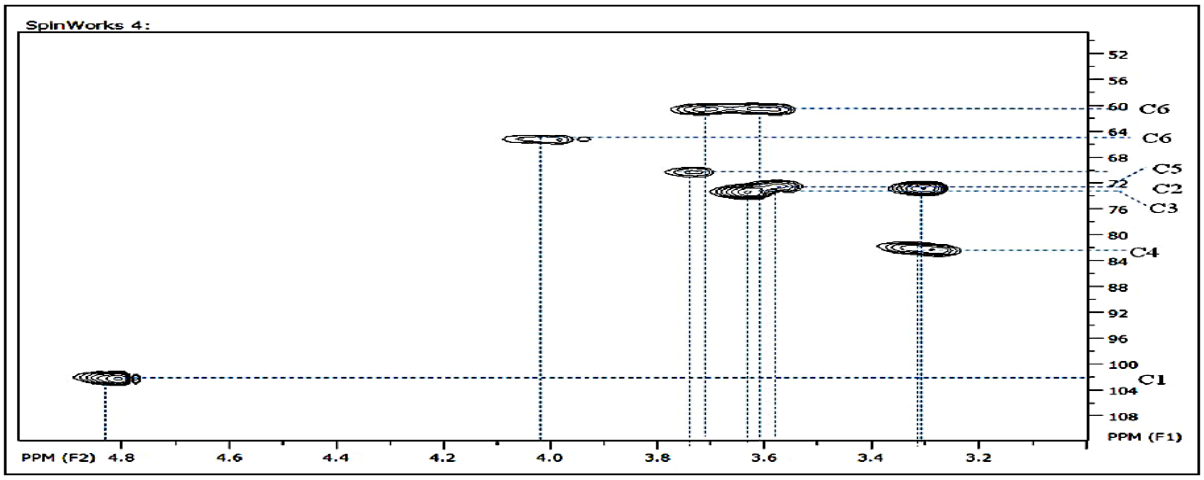


Figure 8*:* HSQC spectrum of S-β-CD3

3. Inductively coupled plasma-optical emission spectrometric (ICP-OES) analysis

ICP-OES analysis was carried out for sodium and sulfur for both samples. The instrument was calibrated with pure standards of sulfur and sodium, ahead of the analysis, and fresh solutions with a concentration of 10 g/l were prepared for both S-β-CD by dissolving 0.1 g of the samples in 10 ml of water. The results are illustrated in **Table 3**.

Table 3: ICP-OES analysis for sulfur and sodium

Sample	Mass (g)	Volume (ml)	Na mg/dm3	S mg/dm3
S-β-CD1	0.25	25	1041.5	2452.0
S-β-CD3	0.25	25	848.6	2263.8

As seen in table 1, S-β-CD1 has a higher concentration of sulfur and sodium, which indicates that the degree of sulfonation is higher in S-β-CD1.

4. Mass analysis by Gel Filtration Chromatography

The results are illustrated in **Table 4**.

Table 4: Comparison of various batches of synthesized sulfated beta-cyclodextrin by Gel Filtration Chromatography

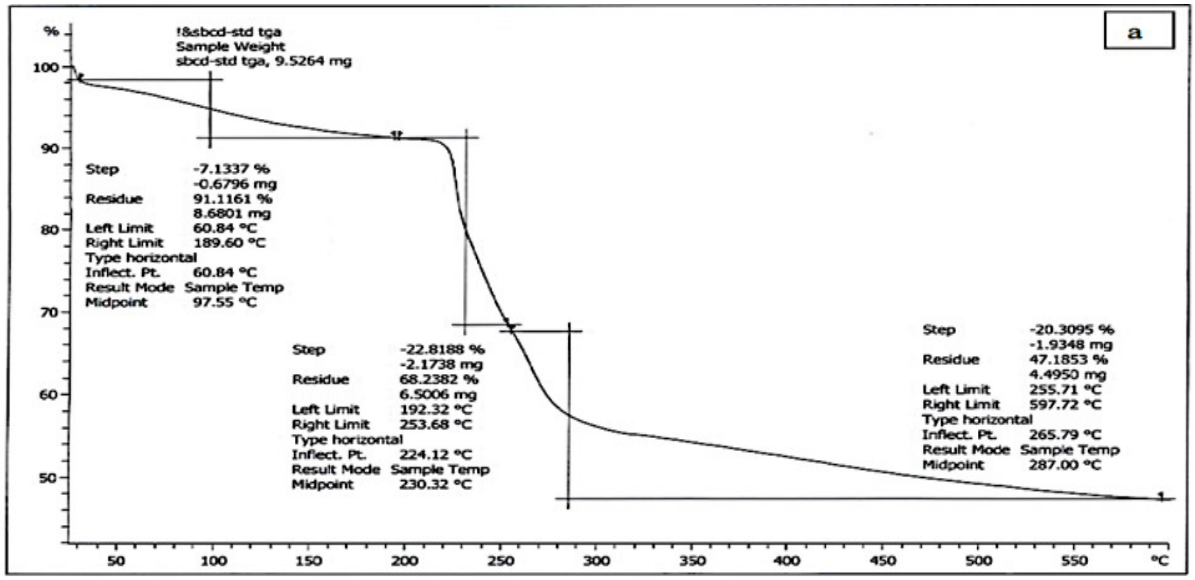
Sr. no.	Sample	Average molecular weight (Da)	Molecular weight distribution (%)			Average Degree of substitution
			0-1000 Da	1000-2000 Da	2000-4000 Da	
1	S-β-CD1	2251	0.01	16.33	83.66	14
2	S-β-CD3 batch 1	2132	0.03	33.37	66.60	13
3	S-β-CD3 batch 2	2124	0.01	34.74	65.25	13
4	S-β-CD3 batch 3	2157	0.08	29.29	70.63	13
5	S-β-CD3 batch 4	2157	0.10	29.24	70.66	13

As seen in table 2, the batch-to-batch variation in terms of molecular weight and molecular weight distribution is minimal for S-β-CD3 indicating the reproducibility of the method of synthesis. It also shows that the synthesized S-β-CD closely resembles the marketed S-β-CD. Multiple peaks of lower molecular weight in the case of S-β-CD3 indicate the fragmentation of the beta-cyclodextrin ring possibly during the addition of a strong sulfonating reagent i.e., chlorosulfonic acid. As the C-3 carbon in the beta-cyclodextrin structure is sterically hindered, at this position, chances of sulfonation are minimized. As the average degree of substitution is 14 and 13 for S-β-CD1 and S-β-CD3, respectively, the molecule is sulfated mainly at the C-2 and C-6 carbon.

5. Thermal analysis

Thermogravimetric analysis (TGA) and differential scanning calorimetric (DSC) analysis were performed for S-β-CD1, S-β-CD2, and S-β-CD3. The TGA and DSC thermograms are shown in **Figures 9 and 10**, respectively. The thermogravimetric analysis showed a fairly small mass loss (in the range of 7-9 %) for both the samples up to around 180 °C. This can be attributed to the loss of adsorbed water and/or residual solvent. Both the samples then showed a significant mass loss at around 200 °C indicative of the major thermal degradation of the sample.

The mass loss after this event was about 52 %. The samples then slowly degraded further as the temperature was taken higher.



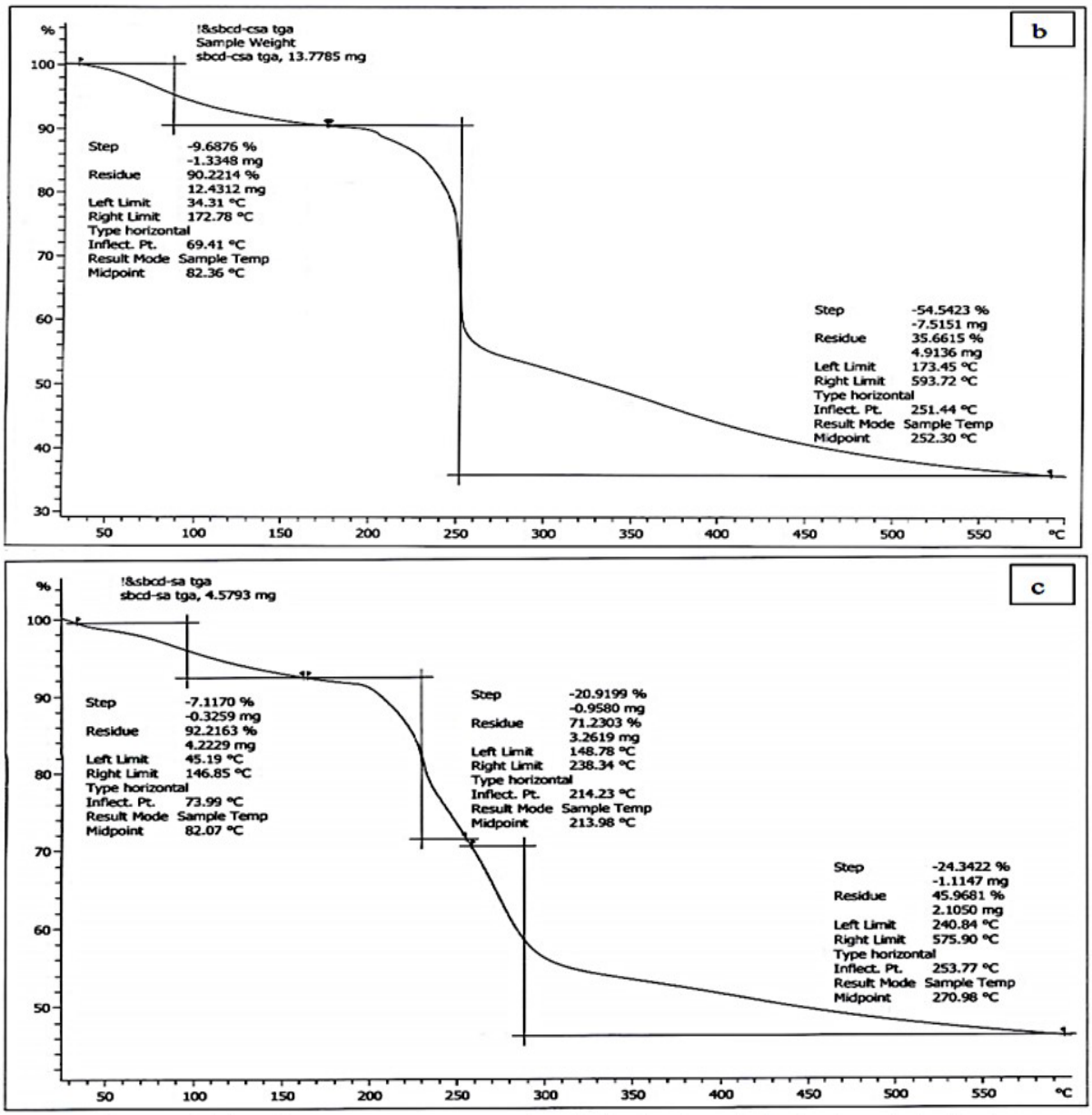
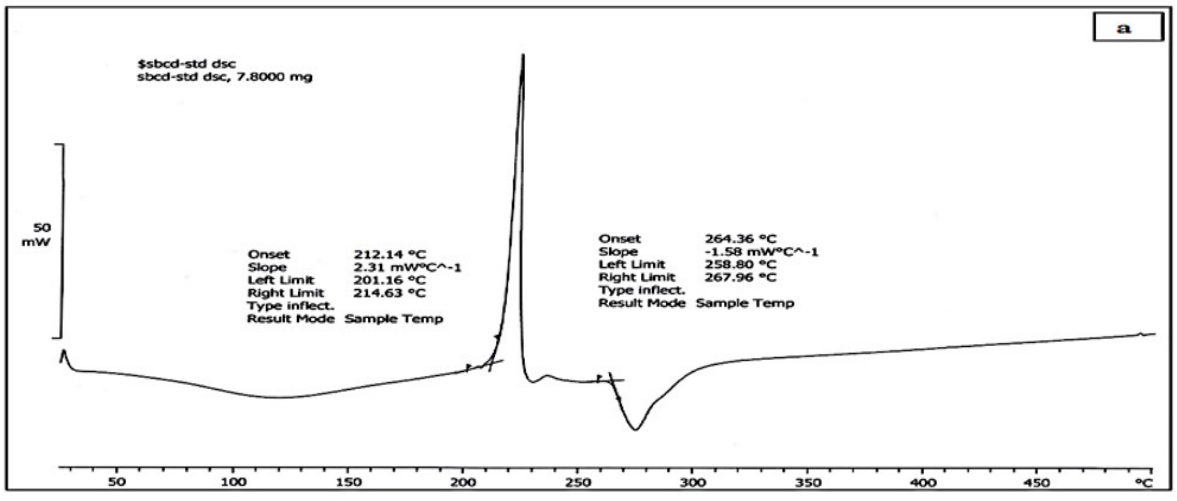


Figure 9: TGA thermogram of a. S-β-CD1 b. S-β-CD2 c. S-β-CD3



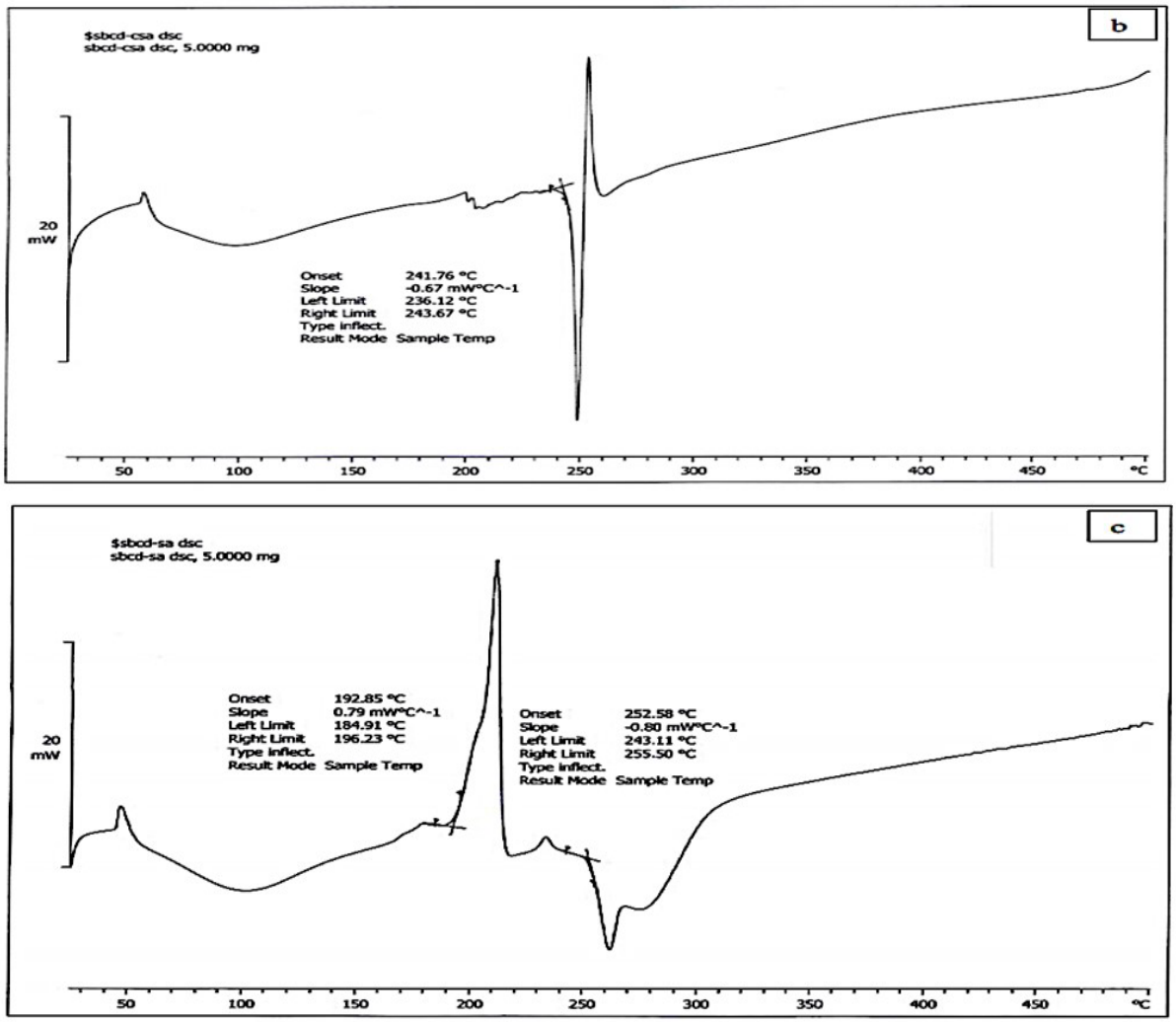


Figure 10: DSC thermogram of a. S-β-CD1 b. S-β-CD2 c. S-β-CD3

DSC thermogram showed that the melting process was overlapping an exothermal decomposition temperature. The onset of crystallization and the melting temperature was marginally higher for S-β-CD1, suggesting that it is more thermostable than S-β-CD2. The thermogram curve of S-β-CD3 showed that the endothermic melting peak was sharp followed by a rapid exothermic decomposition. Melting and crystallization temperature is given in **Table 5**.

Table 5: Melting and crystallization temperatures for S-β-CD samples

Sample	Crystallization temperature (°C)	Melting temperature (°C)
S-β-CD1	212.14	264.36
S-β-CD2	192.85	252.58
S-β-CD3	-	241.76

6. Scanning electron microscopic (SEM) analysis

SEM images of S-β-CD1, S-β-CD2, and S-β-CD3 are given in **Figures 11**. SEM images showed that the particle size distribution is more uniform in the case of the marketed sample. On a grain basis, the particles show a pumice-like appearance for S-β-CD1 that is expected to increase the surface area. The particle size range in S-β-CD2 is much more varied i.e., very small particles intermingled with large 'boulders'. The morphology is more varied with a range of different shapes and a great deal of angularity. S-β-CD3 has several small particles of similar size

interspersed with several large chunks. The smaller particles are relatively non-angular while the bigger pieces are quite angular. The pumice-like appearance was not observed in the synthesized products i.e. S-β-CD2 and S-β-CD3.

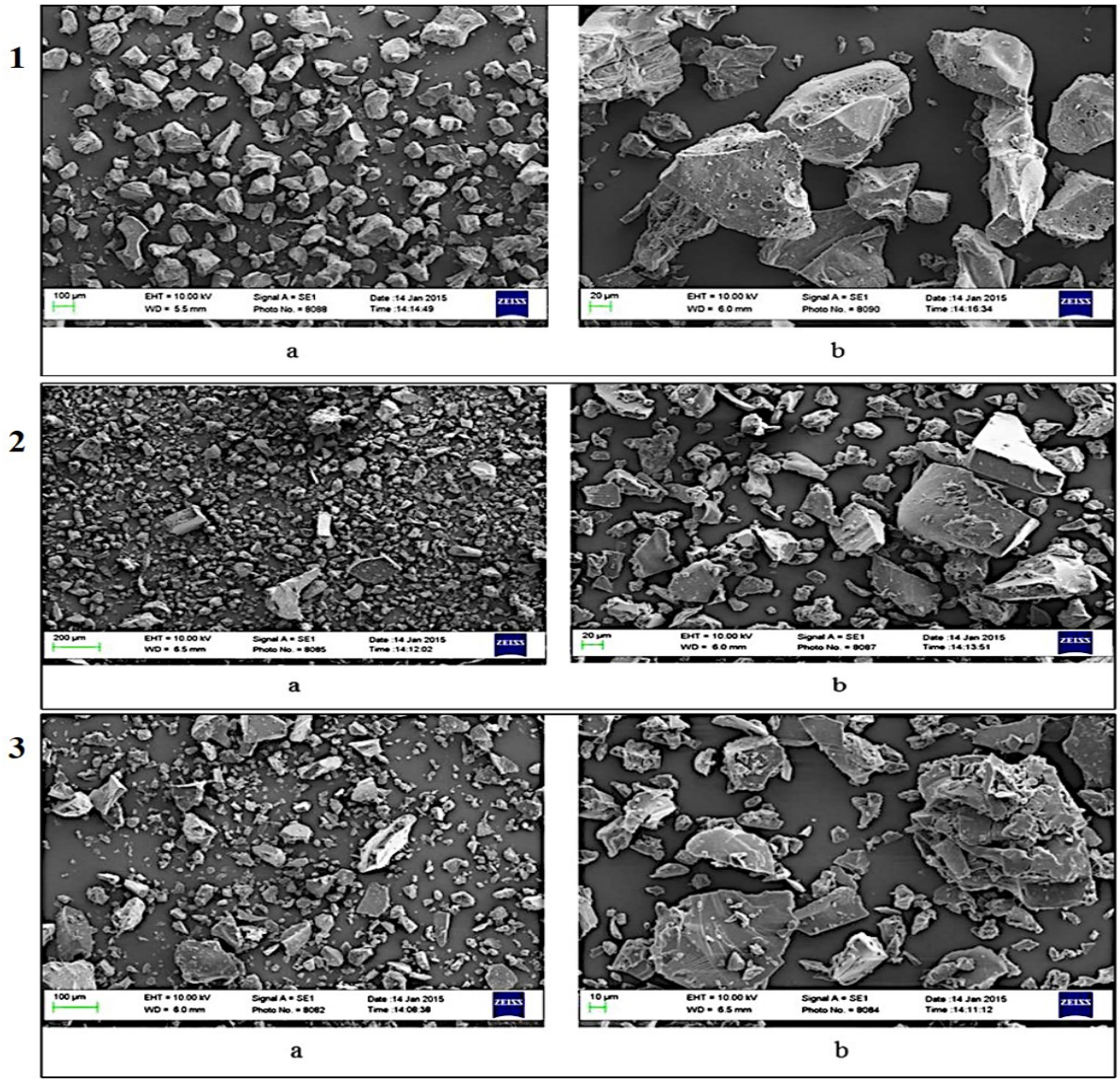


Figure 11: Scanning electron microscopic analysis a. SEM image b. enlarged view of 1.S-β-CD1, 2.S-β-CD2, 3. S-β-CD3

3.3 Enantiomeric separation of cloperastine by chiral mobile phase additive

The focus of the current study was an enantiomeric separation of cloperastine using CMPAs on an achiral stationary phase. A Kromasil C_8 (150 x 4.6 mm, 5 µm) column was used for the analysis. β-CD and its derivatives HP-β-CD and S-β-CD were evaluated as CMPAs. The parameters affecting the resolution were optimized, and the results were compared with the marketed product. For the method development, a concentration of 10 µg/ml of racemic cloperastine was used. The method development was started with the mobile phase composition of methanol: buffer (40:60) and based on results it was modified. As CMPA is added to the aqueous phase of the mobile phase, more of the aqueous phase is desired for enantiomeric separation.

1. Effect of β-cyclodextrin and its derivatives on resolution:

β-CD, HP-β-CD, and S-β-CD were evaluated as CMPAs for the enantiomeric resolution of cloperastine. The reports in the literature and our previous experience suggest that phosphate buffer with acidic pH and methanol is preferred for resolving the enantiomers of some chiral drugs [5, 16-19]. Hence, the pH of KH_2PO_4 containing chiral additive was adjusted to 3, and methanol was used as an organic component in the mobile phase. As CMPA is added in the aqueous phase of the mobile phase, more of it is required; hence, the HPLC method development was started with the mobile phase composition of methanol: buffer (40:60). It was further changed to obtain the anticipated results. **Table 6** shows the effect of β-CD, HP-β-CD, and S-β-CD on the resolution of cloperastine.

Table 6: Effect of beta-cyclodextrin and its derivatives on resolution and retention time of cloperastine enantiomers

Sr. No.	Type of chiral additive and concentration	% Methanol	Retention time of cloperastine enantiomers (minutes)		Resolution
			Levo	Dextro	
1	10 mM β-CD	40	27.3	28.0	0.83
2	10 mM β-CD	30	81.4	84.1	0.85
3	12 mM β-CD	30	78.3	80.8	0.89
4	15 mM β-CD	30	63.1	65.3	1.01
5	10 mM HP-β-CD	30	86.8		-
6	20 mM HP-β-CD	30	34.2		-
7	10 mM S-β-CD 1	30	76.2	91.4	4.72
8	10 mM S-β-CD 1	40	30.7	34.8	3.10
9	10 mM S-β-CD 1	45	20.2	22.2	2.16
10	8 mM S-β-CD 1	45	25.7	26.9	1.45
11	10 mM S β-CD 2	45	22.3	23.1	1.16
12	15 mM S-β-CD 2	45	18.1	19.5	1.39
13	10 mM S-β-CD 3	40	20.8	24.4	4.12
14	10 mM S-β-CD 3	45	11.6	13.5	3.14
15	12 mM S-β-CD 3	45	11.2	12.9	2.53
16	15 mM S-β-CD 3	45	10.9	12.1	2.23

*Other chromatographic conditions: flow rate: 1 ml/min; Column: Kromasil C_8 (150 x 4.6 mm, 5 µm) Detection wavelength: 225 nm

As seen in **Table 6**, resolution increases and retention time decreases with an increase in the concentration of the chiral additive in the mobile phase. A maximum resolution of 1.01 was obtained with 15 mM β-CD in the mobile phase. With β-CD as a chiral selector, the retention times for enantiomers were too long (76 and 79 minutes for levo and dextro enantiomers respectively). Limited aqueous solubility of β-CD restricted its concentration to 15 mM in the mobile phase. Hence, HP-β-CD, a derivative of beta-cyclodextrin that has a higher degree of aqueous solubility, was employed as CMPA. No resolution was obtained with HP-β-CD. This might be because of the presence of a hydroxypropyl group on either side of the cavity, resulting in the extension of the non-polar cavity. The cavity size plays a significant role in the formation of host-guest complex formation, and the extended cavity size in HP-β-CD may have hampered the inclusion complex formation, hence resolution was lost. The use of S-β-CD1 and S-β-CD3 in the mobile phase significantly improved the resolution of cloperastine enantiomers. S-β-CD2 failed to provide the baseline separation of enantiomers possibly due to the disruption of the β-CD structure as indicated by GFC analysis. S-β-CD3 provided better resolution than S-β-CD1 in a shorter run time; hence,

further studies were carried out using S-β-CD3. As seen in Table 4, the highest resolution of 3.14 was observed at 10 mM concentration of S-β-CD3 in the mobile phase; hence, it was selected as the optimum concentration for the resolution of cloperastine enantiomers.

2. Effect of type and concentration of buffer on resolution:

KH_2PO_4, ammonium acetate, and triethylammonium acetate buffers were evaluated for their effect on the resolution of cloperastine enantiomers. Mobile phases containing ammonium acetate and triethylammonium acetate with 10 mM S-β-CD3 and 45% methanol did not give baseline resolution. Baseline resolution was obtained when KH_2PO_4 containing 10 mM S-β-CD3 and 45% methanol was used as the mobile phase. The effect of 5, 10, and 20 mM KH_2PO_4 was evaluated. No significant difference was observed in the resolution over the concentration range. Hence, it was decided to proceed with the mobile phase of 5 mM KH_2PO_4 for further analysis.

3. Effect of pH and organic component on resolution:

Chiral recognition is based on the formation of reasonably stable complexes between the enantiomers and S-β-CD3, and pH plays a vital role in maintaining the stability of these complexes [5]. The influence of pH in the range of 3-5 on the resolution and retention of enantiomers was investigated. As shown in **Figure 12**, lower values of pH led to a decrease in retention time and an increase in resolution.

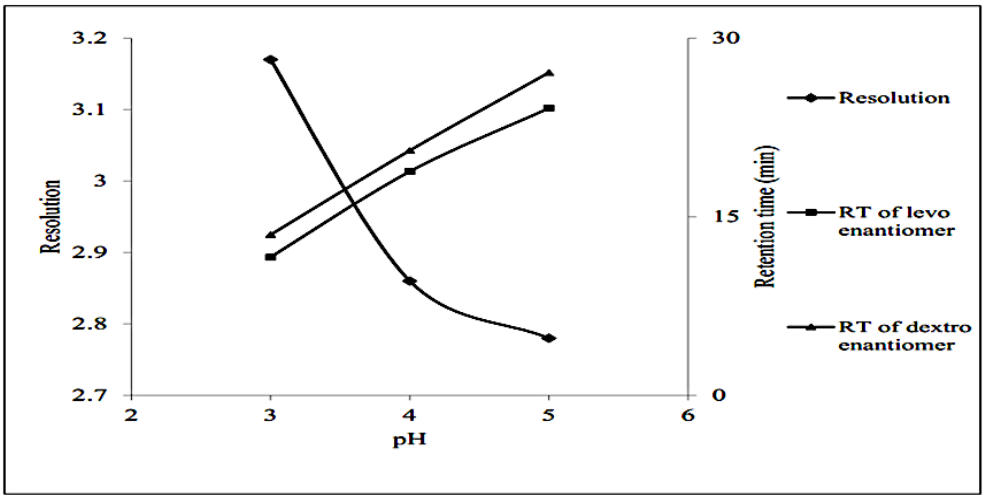


Figure 12: Effect of pH on resolution and retention time

Cloperastine is a basic drug with pKa = 8.69, which will exist in the protonated form at acidic pH. In acidic pH, due to the presence of piperidine ring, CP mainly exists in cationic form and S-β-CD exists in anionic form. This ensures the multipoint interactions between the cyclodextrin moiety and the enantiomers leading to higher chiral recognition. From figure 13, it was observed that the increase in the pH resulted in an increase in the retention times and a reduction in the resolution between the enantiomers. At pH 3.0, the highest resolution was observed with relatively lower retention times. Therefore, pH 3.0 was selected as the optimum condition for the analysis.

Acetonitrile and methanol at various compositions were evaluated as an organic component in the mobile phase and the highest resolution with a shorter run time was achieved with methanol. The optimized mobile phase for the enantioseparation of cloperastine was 5 mM KH_2PO_4, containing 10 mM S-β-CD3 (pH 3) and methanol (55:45), which resulted in a resolution of 3.14, with a run time of 15 minutes. Under this optimized condition, S-β-CD1 was evaluated as CMPA; it gave a lower resolution of 2.16, with a run time of 25 minutes (Figure 14).

4. Chiral separation mechanism:

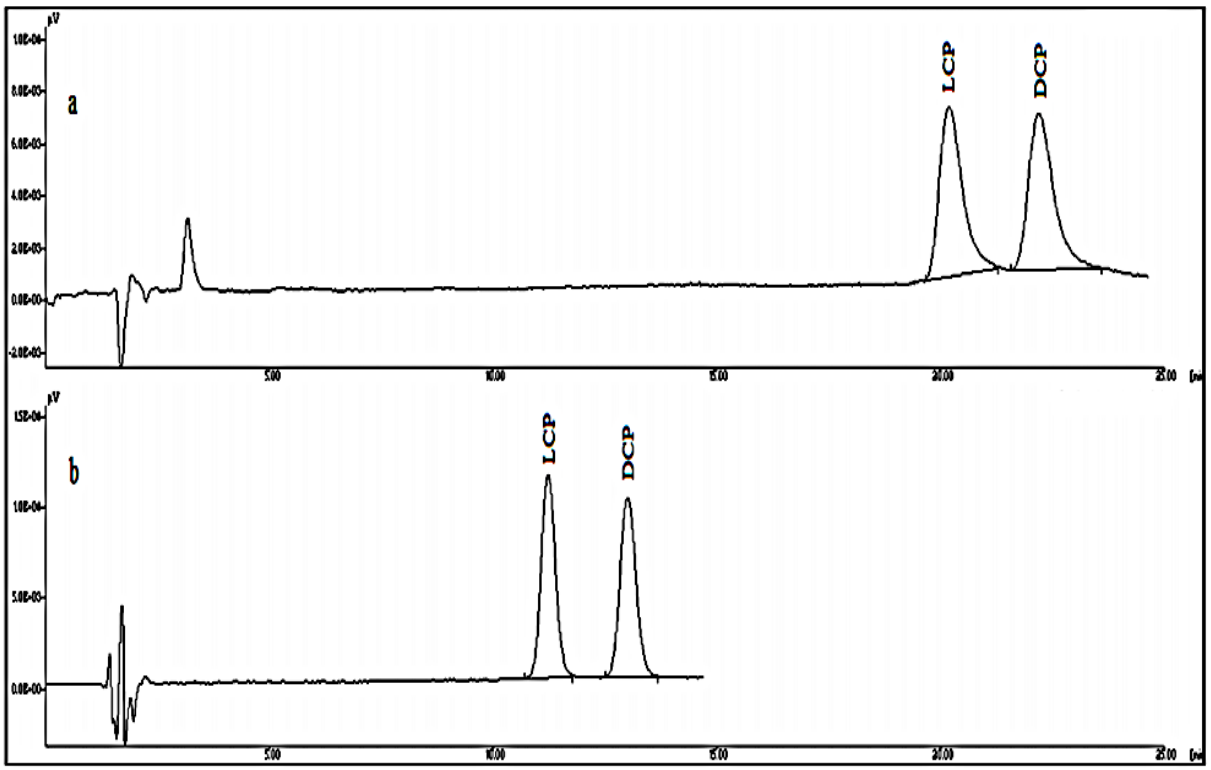
During HPLC method development, the reduction in the retention times provided evidence of inclusion complex formation between CP enantiomers and chiral selector. The inclusion of the chlorophenyl group of CP enantiomers in the hydrophobic cavity of cyclodextrin was most probably involved with β-CD, Hp-β-CD, and S-β-CD. However, β-CD and Hp-β-CD being neutral at mobile phase pH, may not form inclusion complexes with CP enantiomers, which can be separated on the HPLC system. Hence, a baseline resolution was not obtained with β-CD and/or Hp-β-CD. S-β-CD is an anionic cyclodextrin and hence, polar interactions (ionic interactions and hydrogen bonding) between the positively charged amino group of CP enantiomers and negatively charged sulfate groups lead to stable inclusion complexes. The HPLC system was able to discriminate the two diastereomeric inclusion complexes and thus a baseline resolution was obtained. The formation of diastereomeric inclusion complexes was confirmed by molecular docking studies (Section 3.6).

3.4 Method validation:

The optimized method using S-β-CD3 was validated according to ICH guidelines.

1. Specificity:

Specificity was evaluated by injecting a racemic solution of 10 µg/ml of racemic CP. The resolution of 3.14 was achieved (**Figure 13b**) which confirmed the specificity of the method. The order of the elution was determined by injecting levocloperastine.



* LCP: Levocloperastine; DCP: Dextrocloperastine, a. Using 10 mM S-β-CD1, b. Using 10 mM S-β-CD3
Chromatographic conditions: 1) Mobile Phase: Methanol: 5 mM potassium dihydrogen orthophosphate Buffer with CMPA, pH 3.0, (55:45, v/v); 2) Column: Kromasil C_8, 150 x 4.6 mm, 5 µm; 3) Column temperature: Ambient; 4) Flow rate: 1 ml/minute; 5) Detection wavelength: 225 nm

Figure 13: Enantiomeric separation of cloperastine a) using S-β-CD1 and b) using S-β-CD3

The results of HPLC method validation for the separation of enantiomers of cloperastine are summarized in Table

7.

Table 7: The summary of results of HPLC method validation for separation of enantiomers of cloperastine

Sr. No.	Validation parameters	Checkpoints	Results	
			Levocloperastine	Dextrocloperastine
1	Linearity and range	0.5- 10 µg/mL (0.5, 1, 2.5, 5, 7.5, 10)	$r^2 = 0.9998$ $y = 46050x + 3189.4$	$r^2 = 0.9998$ $y = 45771x + 3744.4$
2	Accuracy	5, 10, 15 µg/mL (Recovery studies)	99.1 %	101.0%
3	Precision	5, 10, 15 µg/mL (Intra-day and Interday)	RSD less than 2% for RT and Area	RSD less than 2% for RT and Area
4	LOD and LOQ	0.125- 0.625 µg/mL (0.125, 0.25, 0.375, 0.5, 0.625)	13.1 and 39.7 ng/mL $y = 49936x - 121.28$ $r^2 = 0.9996$	12.5 and 38.0 ng/mL $y = 48914x + 270.32$ $r^2 = 0.9996$
5	Robustness	• Flow rate (±0.2 ml/min) • Mobile phase ratio (± 1 ml methanol) • The pH of the mobile phase (± 0.1 unit)	In all cases resolution was greater than 2.2, no change in accuracy and precision was observed.	

* **r^2**: correlation coefficient, n=3 for each concentration

3.5 Application of the method

The validated method can be used for the qualitative and quantitative determination of cloperastine enantiomers from various matrices. The method was used for the determination of enantiomeric purity in pharmaceutical formulation. The sample of Zerotuss was found to contain 102.29% of the stated amount of levocloperastine and the assay chromatogram of the marketed formulation is shown in **Figure 14**.

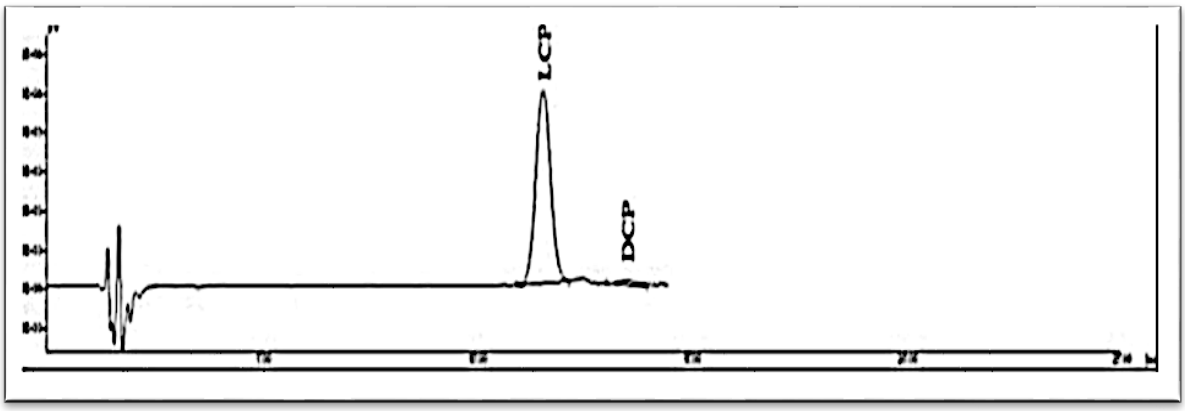


Figure 14: Chromatogram of assay of LCP in API and pharmaceutical formulation

3.6 Molecular modeling

Molecular modeling studies are generally undertaken to give directions to the experimental work and therefore, we employed molecular docking to understand the subtle differences in the binding of enantiomers to S-β-CD2 leading to enhanced chiral separation. Molecular docking predicted that dextrocloperastine exhibits tighter binding than levocloperastine to S-β-CD2. This is exemplified by the fact that approximately $1/4^{th}$ of the dextrocloperastine population (out of 300 docked conformations) showed docking score more -10.0 kcal/mol whereas only $1/20^{th}$ of the levocloperastine population could attain this binding strength. Therefore, from docking

studies we infer that levocloperastine will elute first from the column which is also seen in the experimental studies (vide infra). However, using the current docking results it was not possible to compute the resolution for separation, nonetheless the docking results corroborates with the experimental results.

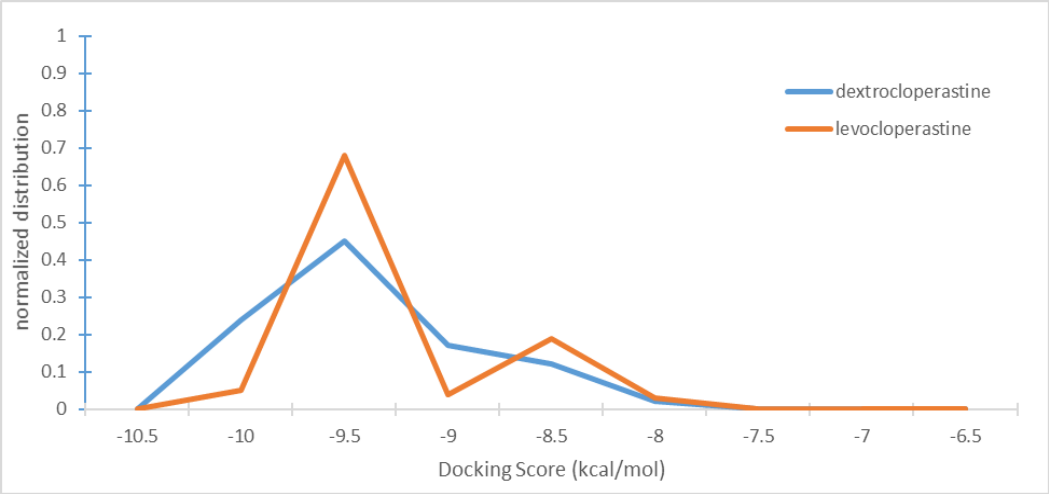


Figure 15: Analysis of molecular docking simulations using the plot of normalized distribution versus docking score.

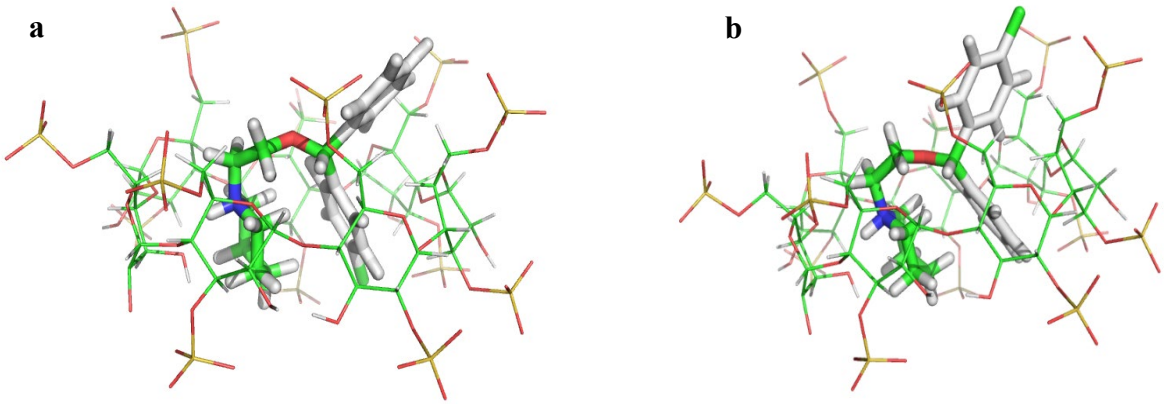


Figure 16: Docked structure of S-β-CD with (a) Dextro-cloperastine and (b) Levocloperastine

4. Conclusion:

A simple and cost-effective method was developed for the synthesis of sulfated beta-cyclodextrin using sulfamic acid as a sulfonating agent. The synthesized product was characterized using various techniques and it was observed to be like the marketed product. The synthesized S-β-CD (10 mM) was applied as a chiral mobile phase additive for the enantiomeric separation of cloperastine and the enantiomers were resolved with a resolution value of 3.14 within a run time of 15 minutes. Synthesized S-β-CD gave better resolution than marketed S-β-CD in a shorter run time. The developed method was validated and applied for the quantitative determination of cloperastine enantiomers in pharmaceutical formulation. The reported chiral method can be used for the enantiomeric purity determination of the two enantiomers of cloperastine in any unknown matrices. Most probably, inclusion complex formation and hydrogen bonding were the major forces for the chiral recognition. The limitations of the validated HPLC methods are the high consumption of chiral selector and the unavailability

of inexpensive S-β-CD commercially. To reduce the high consumption of S-β-CD, in future studies, achiral HPLC columns with different achiral support and column dimensions, for example, core-shell silica column or HILIC column, will be evaluated. The reported method of synthesis of S-β-CD can be used to obtain low-cost S-β-CD.

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