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:

In the present study, a new, simple and cost-effective method for the synthesis of sulfated beta-cyclodextrin (S-β-CD), one of the most widely used chiral mobile phase additive, using sulfamic acid as sulfonating agent has been described. The method was optimized and the acquired product was characterized and compared with a marketed Sigma Aldrich sulfated beta-cyclodextrin (S-β-CD1). Beta cyclodextrin (β-CD), hydroxypropyl beta-cyclodextrin (HP-β-CD), S-β-CD1 and S-β-CD2 were evaluated as chiral mobile phase additives (CMPAs) for the enantiomeric separation of cloperastine, an antitussive agent, using reversed-phase HPLC. Under the optimized conditions, a resolution of 3.14 was achieved within 15 minutes on an achiral Kromasil C_8 (150 x 4.6 mm, 5 µ) column with a mobile phase of 5mM monopotassium phosphate containing 10mM S-β-CD3 pH 3 and 45% methanol with a run time of 15 min. The method utilizing S-β-CD3 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 S-β-CD were proven to be beneficial for chiral separation. The chiral recognition mechanism and elution order of the reported enantiomers were determined by simulation studies. It was observed that inclusion complex formation and hydrogen bonding are the major forces for the chiral resolution.

Highlights:

- Cost-effective synthesis of S-β-CD
- Rapid separation of cloperastine enantiomers by synthesized S-β-CD as chiral mobile phase additive
- Synthesized S-β-CD provided better resolution than marketed S-β-CD

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

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 investigate and evaluate activities of each enantiomer of the chiral drug individually as well as racemates and analyze the product for enantiomeric purity along with other routine analysis. [1, 2]

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

One of the most widely used CMPAs is cyclodextrins (CDs). 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 high-performance liquid chromatography (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 an 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 and its underutilization as a viable analytical tool and an alternative to commercially available expensive chiral columns. [4, 5 6]

The molecule chosen for the study of enantiomeric separation by S-β-CD is cloperastine (CP). 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 structures of both the enantiomers are given in **Figure 1**.

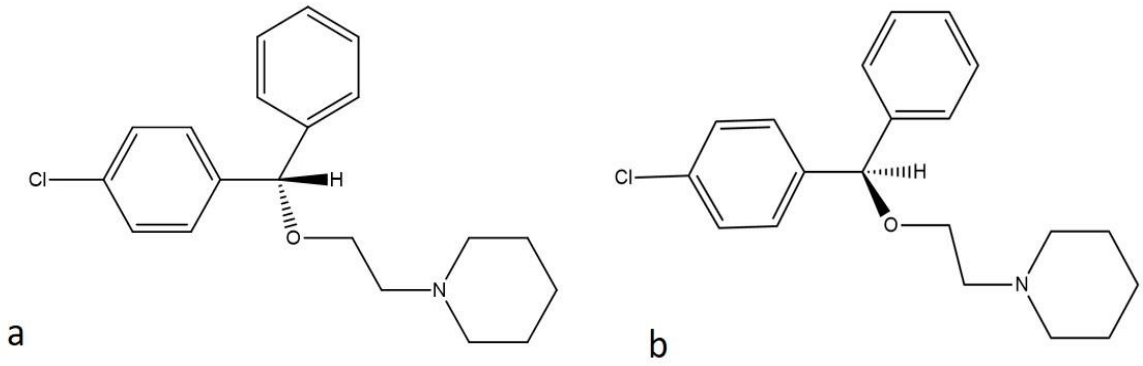


Figure 1: Structure of a. Dextrocloperastine b. Levocloperastine

A survey of the literature revealed that the enantiomeric separation of Cloperastine was reported using HPLC by employing a Chiralcel OD-H column [8] and capillary electrophoretic methods were reported using pepsin [9] and carboxymethyl-β-cyclodextrin [10] as chiral selectors in the background electrolyte. 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 enantiomeric separation using a reverse-phase column with sulfated β-cyclodextrin as chiral mobile phase additives.

In the present investigation, a simple and cost-effective synthesis of S-β-CD and its characterization by various techniques have been reported. The paper also evaluates the efficiency of marketed and synthesized S-β-CD 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 mobile phase and other solutions

2.2 Instrumentation

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 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 mm X 4.6 mm, 5 µm) was used for the study. The flow rate was maintained at 1 ml/min and the injection volume was 20 µl. Detection was carried out at 225 nm.

DSC: The Differential Scanning Calorimetric (DSC) analysis was performed using Mettler Toledo DSC 822 with a temperature range of up to 500 ^{0}C.

TGA: Thermogravimetric analysis (TGA) was performed using a Mettler TGA/SDTA 8S° with a temperature range up to 600 ^{0}C.

SEM: A JEOL 6310 Scanning Electron Microscope (SEM) fitted with an Oxford Instruments ISIS X-ray microanalysis system was used for microscopic analysis.

ICP-OES: 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 dual photomultiplier tube detector with High Dynamic Detection System (wavelength range: 160 nm- 800 nm).

NMR: Nuclear magnetic resonance (NMR) analyses of the samples were performed with a Bruker Avance III 400MHz, FT-NMR spectrometer, which includes 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.

GFC: 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.

2.3 Synthesis of sulfated beta-cyclodextrin

2.3.1 *Synthesis of S-β-CD by chlorosulfonic acid*

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**.

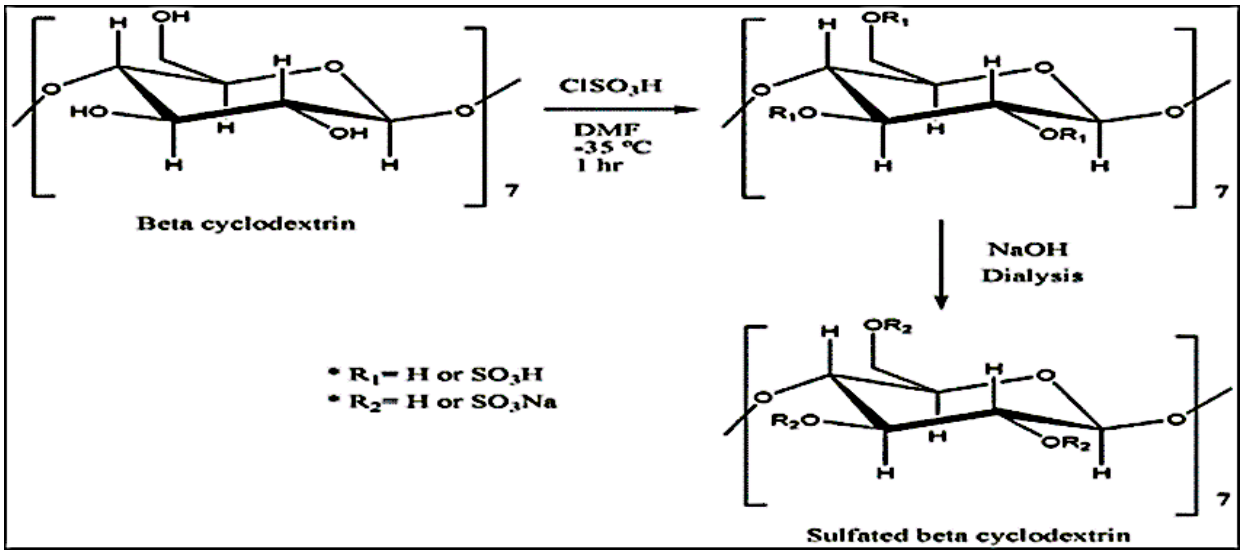


Figure 2: Reaction scheme for synthesis of sulfated beta-cyclodextrin by chlorosulfonic acid

2.3.2 ***Synthesis of S-β-CD by sulfamic acid***

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 1.5liter methanol. The resultant precipitate was filtered, dissolved in 5N NaOH and treated with activated charcoal. The charcoal was removed by filtration after 15 min 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 3**.

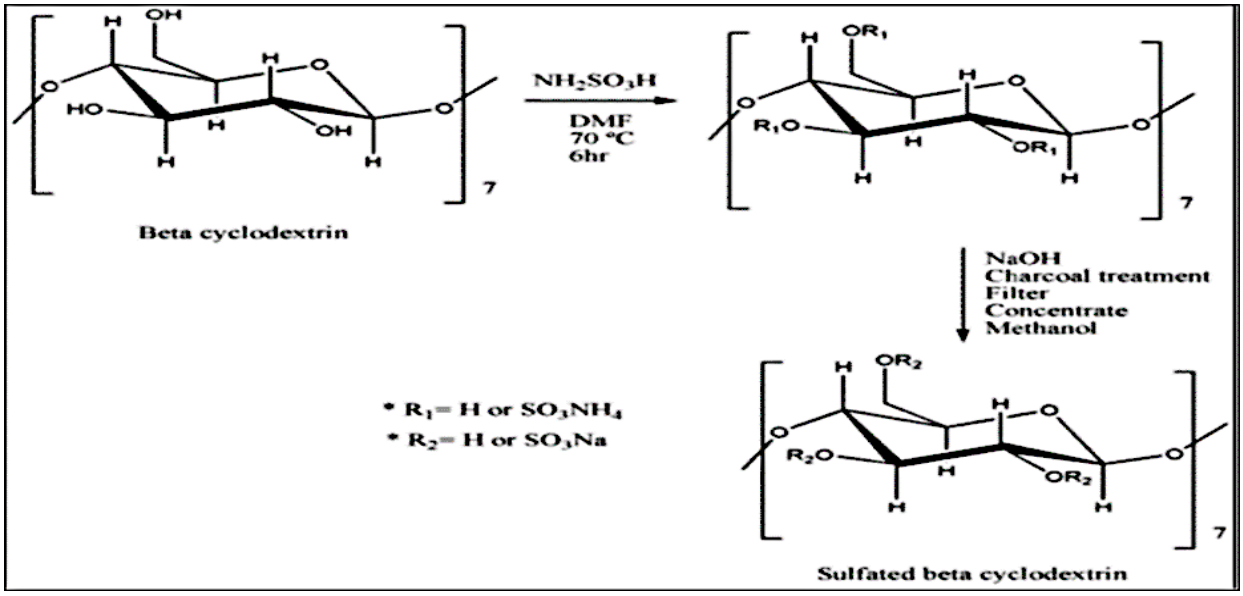


Figure 3: Reaction scheme for synthesis of sulfated beta-cyclodextrin by sulfamic acid

2.3 Molecular Modeling

Molecular modeling studies, particularly molecular docking simulations, were performed to estimate whether there is a 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 the 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.

2.4 Preparation of mobile phase for enantiomeric separation

Methanol and 5mM KH_2PO_4 containing 10mM S-β –CD3 at pH 3 (adjusted with 10% OPA) in the ratio of 45:55 was used as the mobile phase for enantiomeric separation of CP. The mobile phase was filtered through 0.45 µm nylon filter and sonicated for 5 min before use.

2.5 Preparation of stock and standard solution

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 was diluted to 100 ml with methanol to provide 50 µg/ml and this stock was further used for analysis with appropriate dilutions. Diluents used for final dilutions were 5mM KH_2PO_4 and methanol (55:45).

2.6 Assay of levocloperastine in the formulation

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

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 the 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 evaluated as sulfonating agents and the best results were obtained with sulfamic acid. Sulfonation using sulfuric acid was carried out using the procedure given in the literature. [13] The reaction was successful only at a lower scale. In the scaled-up reaction, the reaction gave a charred product. Hence, chlorosulfonic acid was employed for sulfonation of β-CD. [14] Following the procedure given in the literature, charred product was obtained. Hence, the reaction was modified, as described in the experimental section. Following this procedure, very low yield was obtained after dialysis. Hence, sulfamic acid was evaluated as a sulfonating agent. When the procedure given in literature was used, the synthesized product was dark brown in color whereas marketed sulfated beta-cyclodextrin is available as a white colored product. Therefore, it was decided to modify the procedure. During the synthesis, it was observed that reaction time, temperature and volume of DMF played a significant role. The optimized procedure is given in the experimental section. The synthesized product was found to be cheaper than the marketed Sigma Aldrich product.

3.1 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 and S-β-CD3 on S-β-CD1 is given in **Figure 3**.

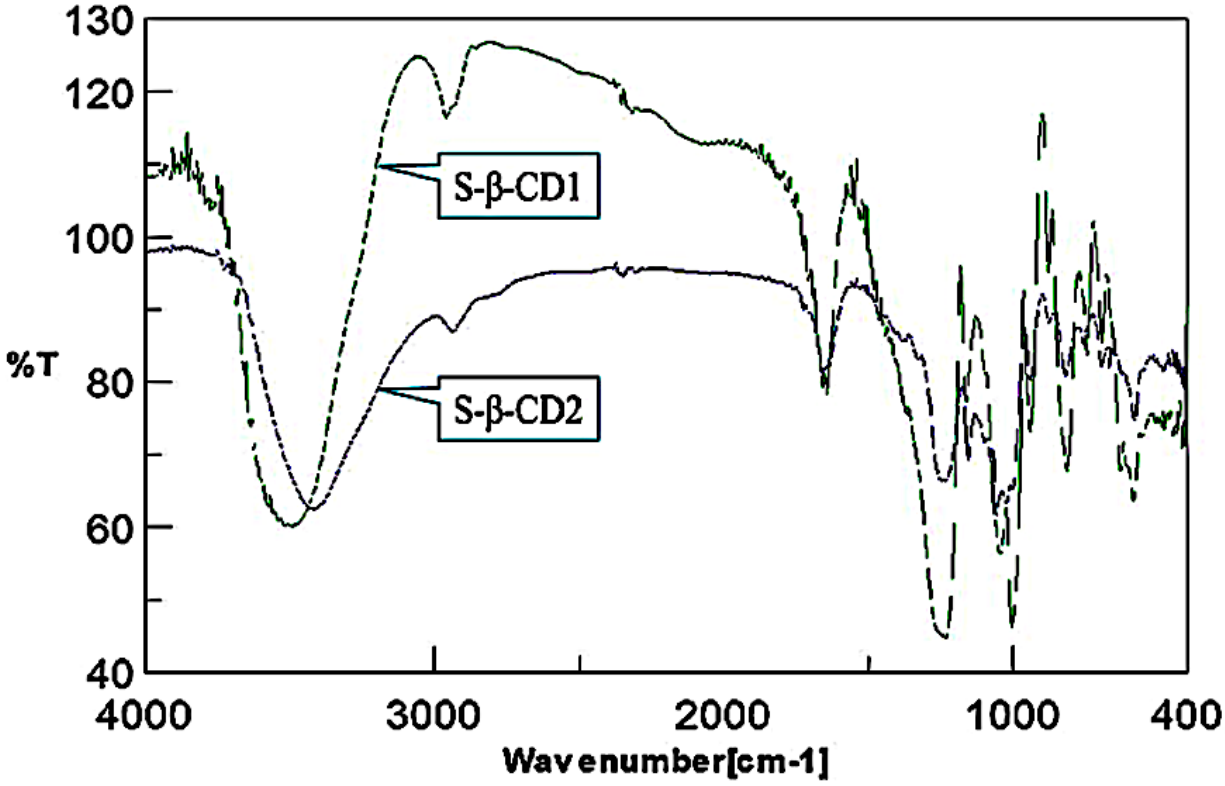


Figure 3: Overlay FT-IR spectra of marketed sulfated beta-cyclodextrin (S-β-CD1) and synthesized sulfated beta-cyclodextrin (S-β-CD2, synthesized by chlorosulfonic acid)

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

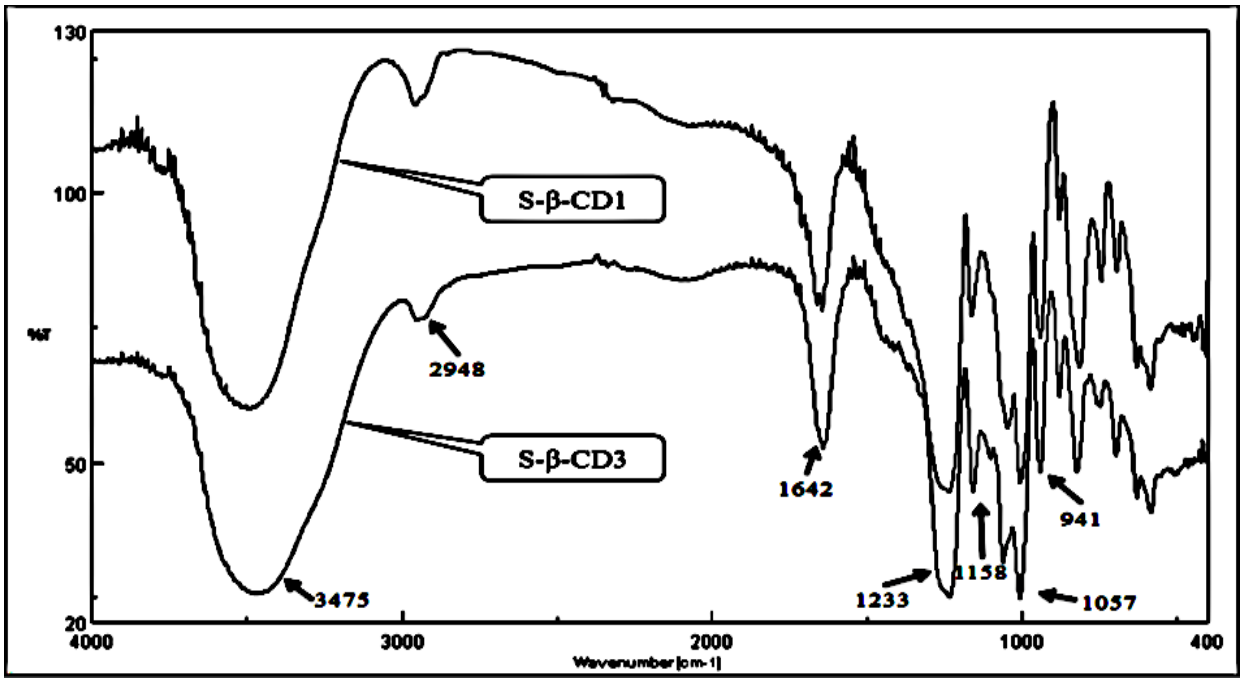


Figure 4: Overlay FT-IR spectra of marketed sulfated beta-cyclodextrin (S-β-CD1) and synthesized sulfated beta-cyclodextrin (S-β-CD3, synthesized by sulfamic acid)

IR spectrum of synthesized S-B-CD was compared with the Sigma Aldrich product. As seen in figure 3a, the spectrum of S-β-CD2 is identical with S-β-CD1 and has been discussed in our previous publication.[14] Differences were observed in the case of S-β-CD3 in the region of 1000 cm^{-1} and 500 cm^{-1} which can be seen in figure 4.

2. Nuclear magnetic spectroscopic (NMR) analysis

Beta-cyclodextrin 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**.

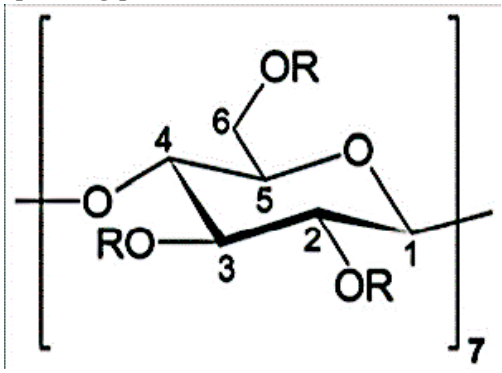


Figure 5: Structure of beta-cyclodextrin along with the numbering of carbons and corresponding protons of each monomer

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-CD3, signals were weak and the spectrum was not clear. ^{1}H and DEPT- 135 spectrum of S-β-CD2 is shown in **Figure 5 and 6** respectively.

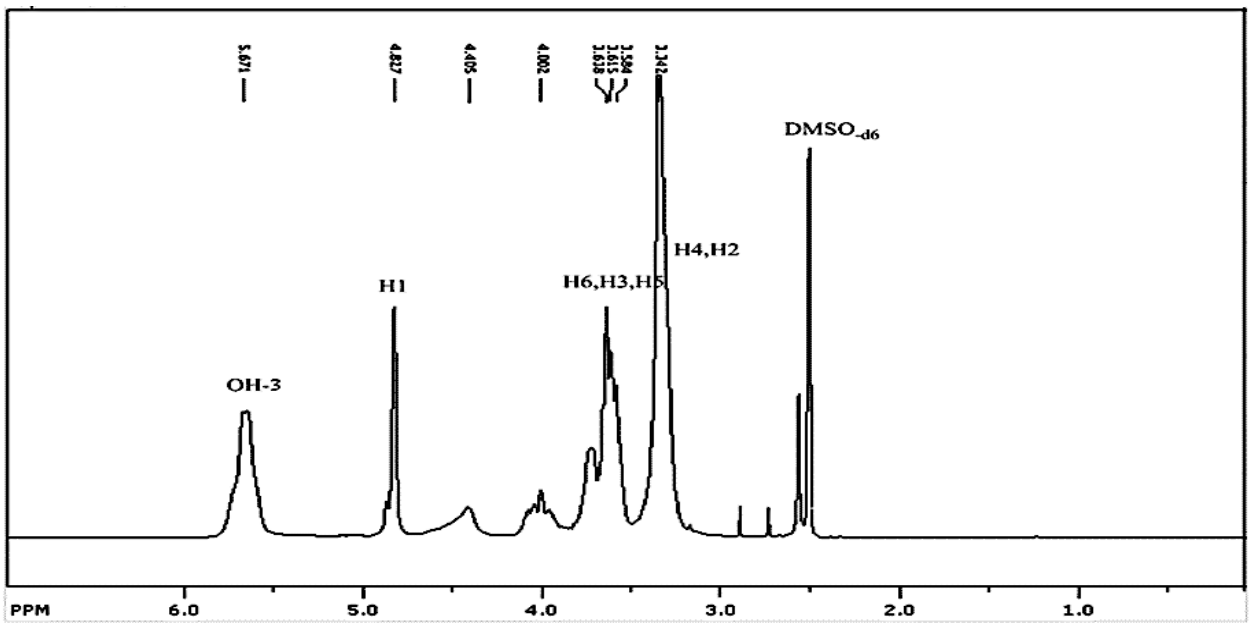


Figure 5: ^{1}H spectrum of S-β-CD2

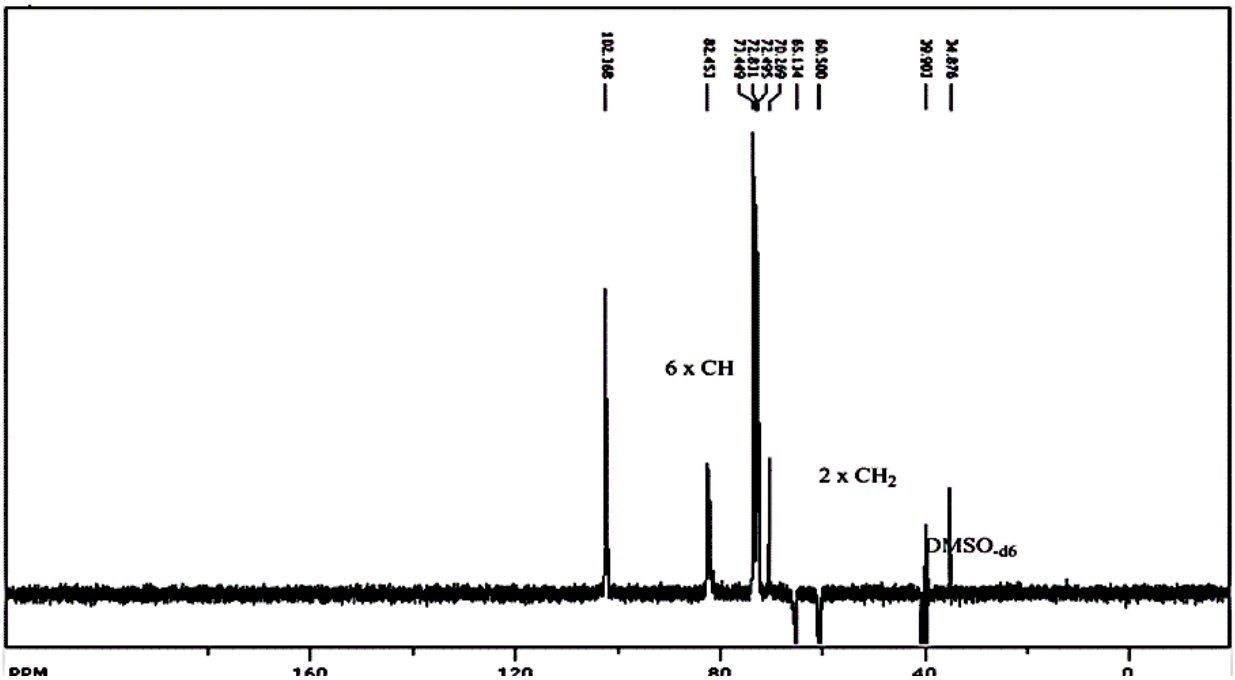


Figure 6: DEPT-135 spectrum of S-β-CD2

Positions 2, 3, and 6 of the sugar units of β-CD are possible sites of sulfonation. Poorly resolved peaks in the ^{1}H spectra demonstrate the heterogeneity of the CDs with respect to sulfonation degree. The ^{1}H NMR spectra also showed that nearly all the protons attached to carbon atoms had similar chemical shifts, in the range of 3.3 to 4.8 ppm. The DEPT-135 experiment showed the CH_2OH carbons appeared in the region of 60- 66 ppm as negative signals indicating two different chemical environments while the more crowded CHOH appeared in the region of 70 to 80 ppm and the anomeric carbon had a chemical shift around 100 ppm. HSQC spectrum showed overlapping of some signals in the case of S-β-CD3 which is shown in **Figure 7**.

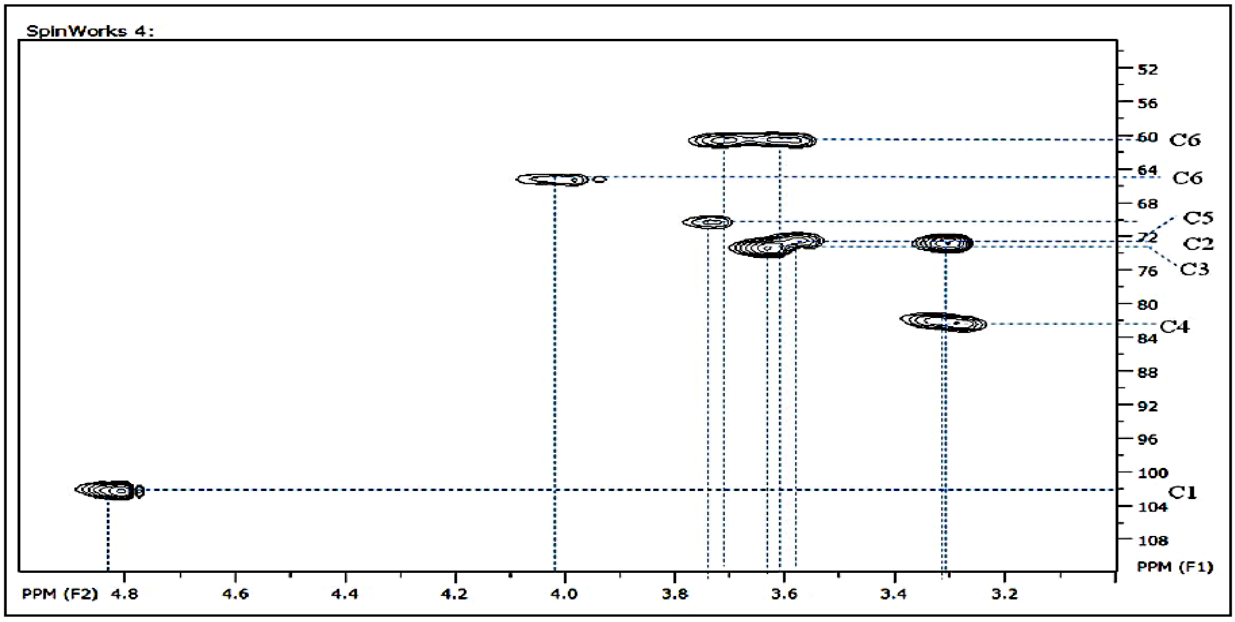


Figure 7: HSQC spectrum of S-β-CD2

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 1**.

Table 1: 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

Two columns in series [Agilent PL-aquagel OH MIXED M (molecular weight range: 1,000-150,000 Da; resin type: polystyrene/ divinyl benzene) 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, which was pumped at 1 ml/min. To check the reproducibility of the synthesis of S-β-CD2, four different batches were analyzed along with S-β-CD1 and the results are illustrated in **Table 2**.

Table 2: Comparison of various batches of synthesized sulfated beta-cyclodextrin by Gel Filtration Chromatography

Sr. no.	Sample	Average molecular weight	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

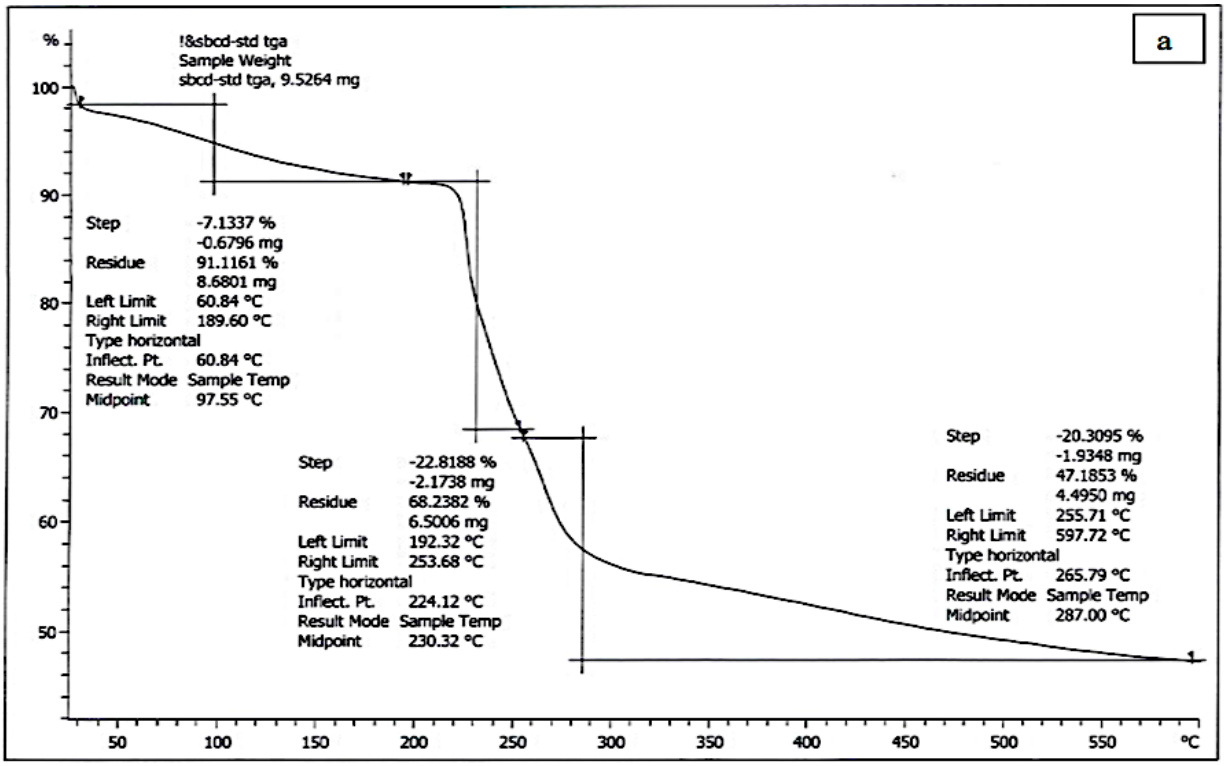
As seen in table 2, the batch to batch variation in terms of molecular weight and molecular weight distribution is minimal for S-β-CD2 indicating the reproducibility of the method of synthesis. It also shows

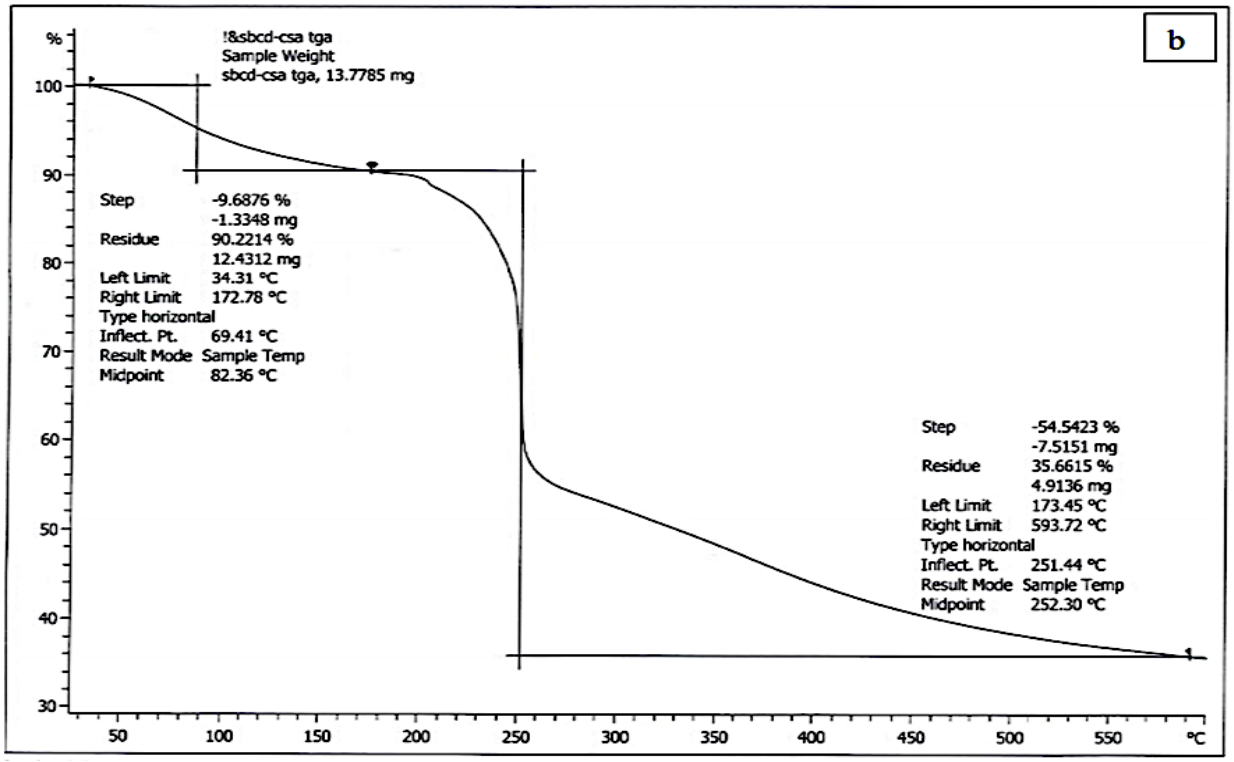
that the synthesized S-β-CD closely resembles the marketed S-β-CD. Multiple peaks of lower molecular weight in the case of S-β-CD3 indicate the fragmentation of beta-cyclodextrin ring possibly during the addition of a strong sulfonating reagent i.e. chlorosulfonic acid.

As the 3 carbons in the beta-cyclodextrin structure are 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-CD2, respectively, the molecule is sulfated mainly at the 2nd and 6th 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 8 and 9**, 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.





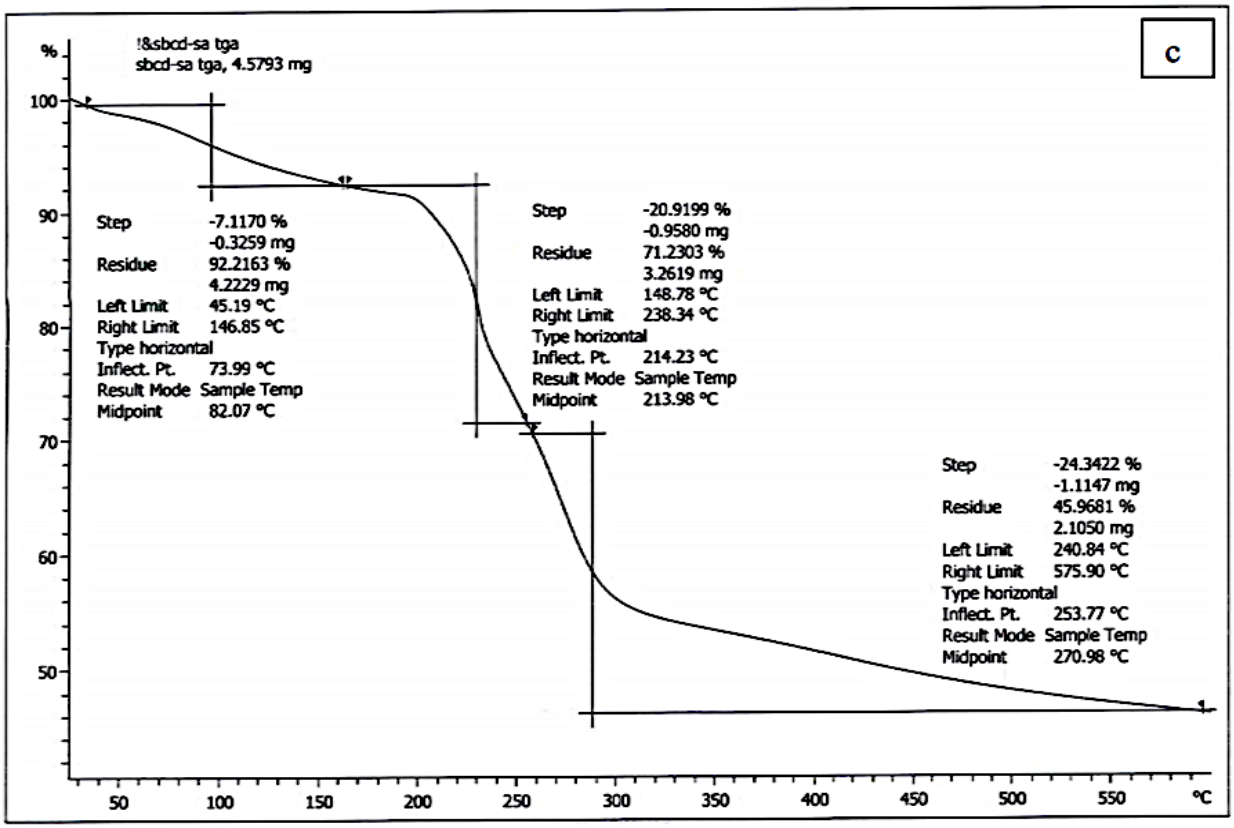
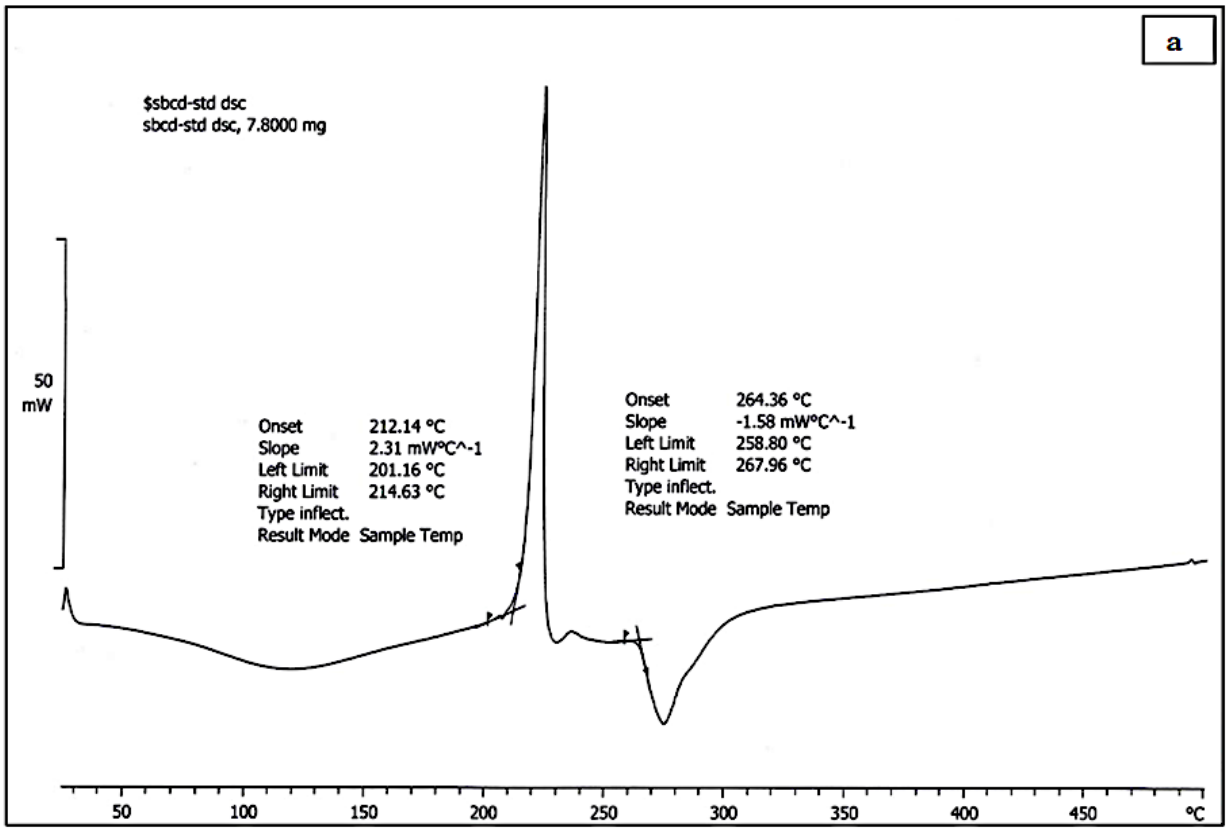


Figure 8: TGA thermogram of a. S-β-CD1 b. S-β-CD2 c. S-β-CD3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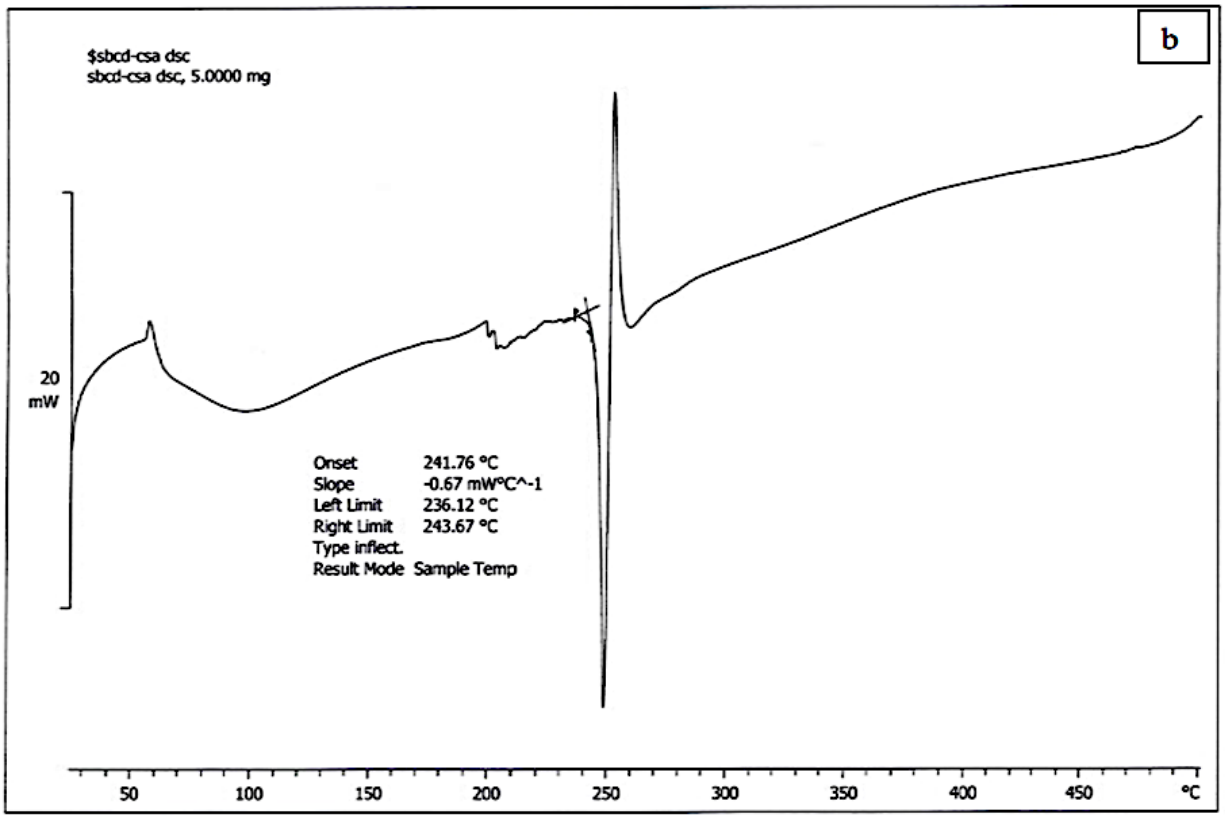
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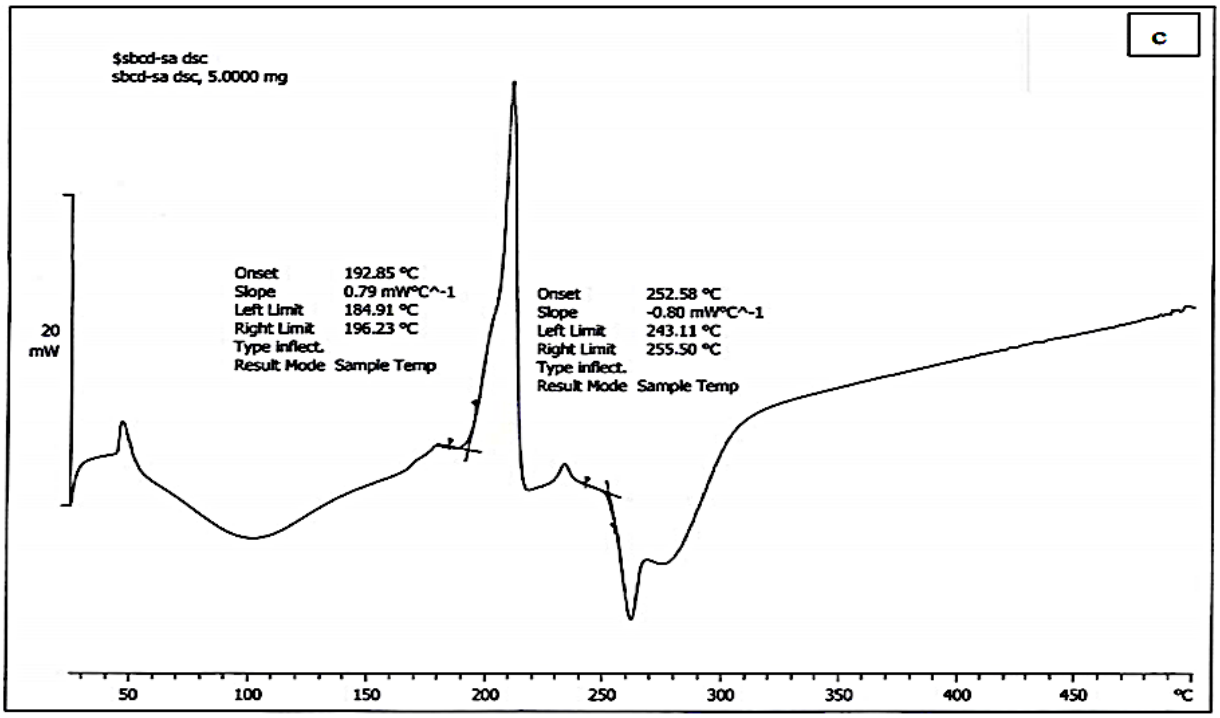


Figure 9: DSC thermogram of a. S-β-CD1 b. S-β-CD2 c. S-β-CD3

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 and followed by a rapid exothermic decomposition. Melting and crystallization temperature is given in **Table 3**.

Table 3: Melting and crystallization temperatures for S-β-CD samples

Sample	Crystallization (°C)	Melting temperature (^{0}C)
S-β-CD1	212.14	264.36
S-β-CD2	192.85	252.58
S-β-CD3	-	241.76

5. Scanning electron microscopic (SEM) analysis

SEM images of S-β-CD1, S-β-CD2 and S-β-CD3 are given in **Figure 10, 11 and 12** respectively. 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 which 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 a number of small particles of similar size interspersed with a number of 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.

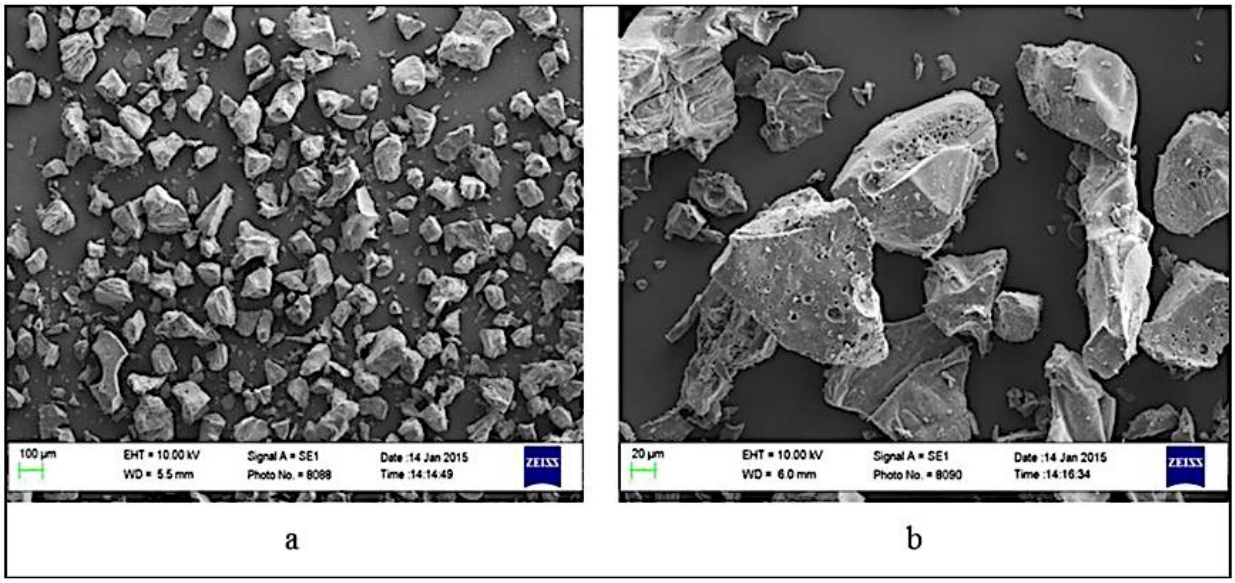


Figure 10: SEM image a. S-β-CD1 b. enlarged view of S-β-CD1

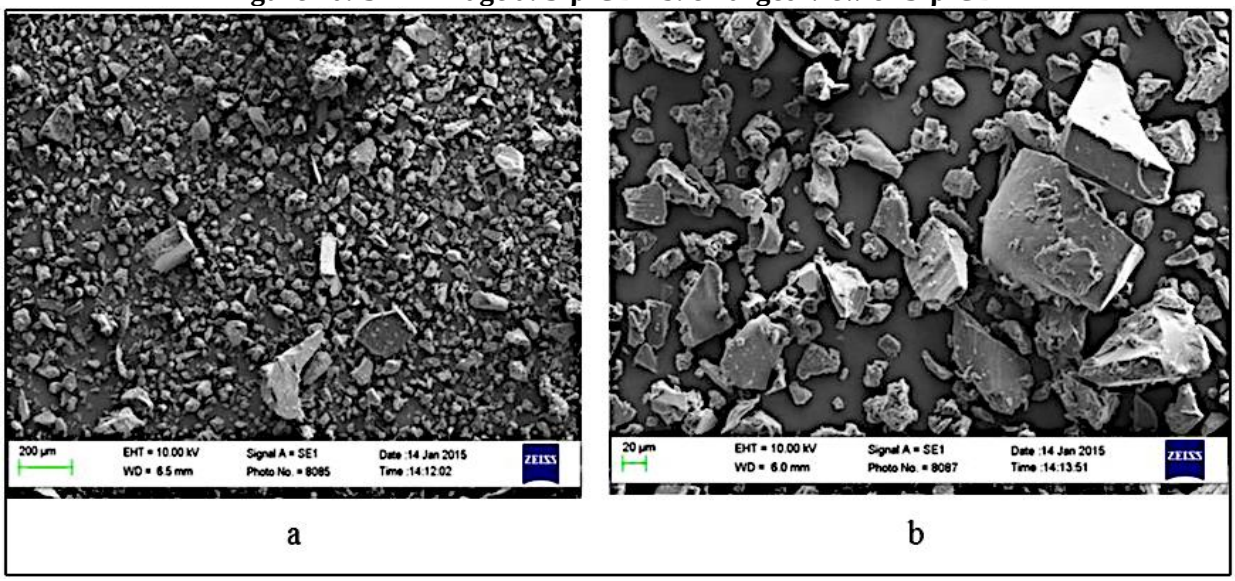


Figure 11: SEM image a. S-β-CD2 b. enlarged view of S-β-CD2

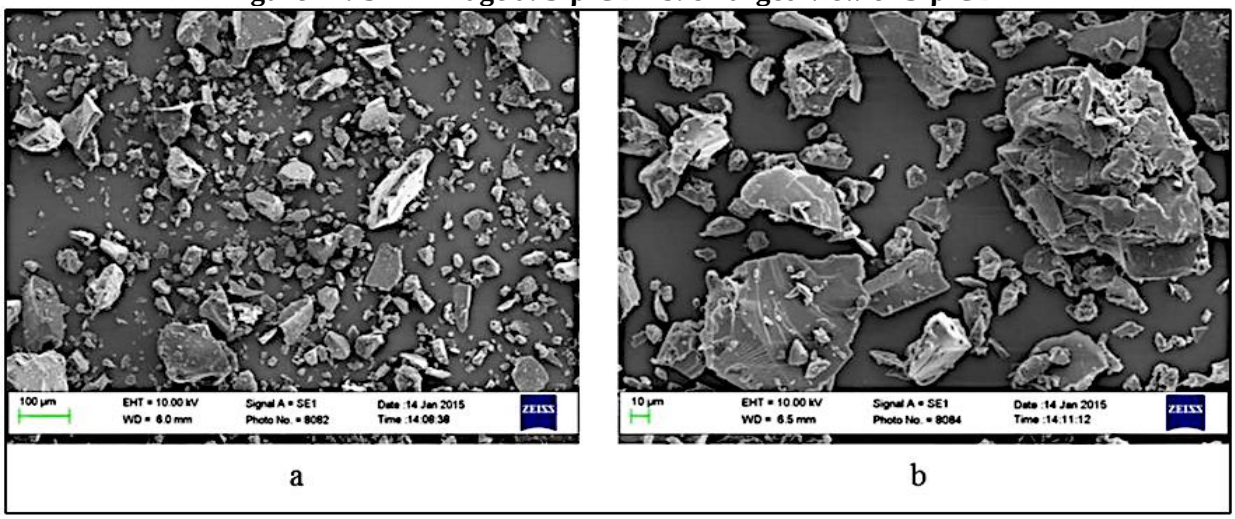


Figure 12: SEM image a. S-β-CD3 b. enlarged view of S-β-CD3

3.1 Enantiomeric separation of cloperastine by chiral mobile phase additive

In our previous studies, S-β-CD2 was successfully employed as CMPA for the enantiomeric separation of citalopram[14] and cetirizine[15]. 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, 5µm) 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.

3.3.1 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, 14-16] Hence, pH of KH_2PO_4 containing chiral additive was adjusted to 3 and methanol was used as an organic component in the mobile phase. **Table 4** shows the effect of β-CD, HP-β-CD, and S-β-CD on the resolution of cloperastine.

Table 4: Effect of beta-cyclodextrin and its derivatives on resolution and retention time of cloperastine enantiomers

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

*Other chromatographic conditions: flow rate: 1 ml/min; Column: Kromasil C8 (150 x 4.6 mm, 5^m) X: 225nm

As seen in **Table 4**, 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 15mM β-CD in the mobile phase. With β-CD as a chiral selector, the retention times for enantiomers were too long (76 and 79 min for levo and dextro enantiomers respectively). Limited aqueous solubility of β-CD restricts its concentration to 15mM in the mobile phase. Hence, HP-β-CD, a derivative of beta-cyclodextrin, which has a higher degree of aqueous solubility, was employed as CMPA. No resolution was obtained with hydroxypropyl beta-cyclodextrin. This might be because of the presence of hydroxypropyl group on either side of the cavity which results 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 β-CD structure as indicated by GFC analysis. S-β-CD3 provided better resolution than S-β-CD1 in shorter run time; hence further studies were carried out using S-β-CD3. As seen in table 4, the highest resolution was observed at 10mM concentration of S-β-CD3 in the mobile phase; hence, it was selected as the optimum concentration for resolution of cloperastine enantiomers.

3.3.2 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 10mM S-β-CD3 and 45% methanol did not give baseline resolution. Baseline resolution was obtained when KH_2PO_4 containing 10mM S-β- CD3 and 45% methanol was used as the mobile phase. The effect of 5, 10 and 20mM 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 5mM KH_2PO_4 for further analysis.

3.3.3 Effect of pH and organic component on resolution:

Chiral recognition is based on the formation of reasonably stable complexes with the guest enantiomers and pH plays a vital role in maintaining the stability of the complex between the analyte and cyclodextrin molecule.[5] The unstable complex elutes early. The influence of pH in the range of 3-5 on the resolution and retention of enantiomers was investigated. As shown in **Figure 13**, lower values of pH led to a decrease in retention time and an increase in resolution.

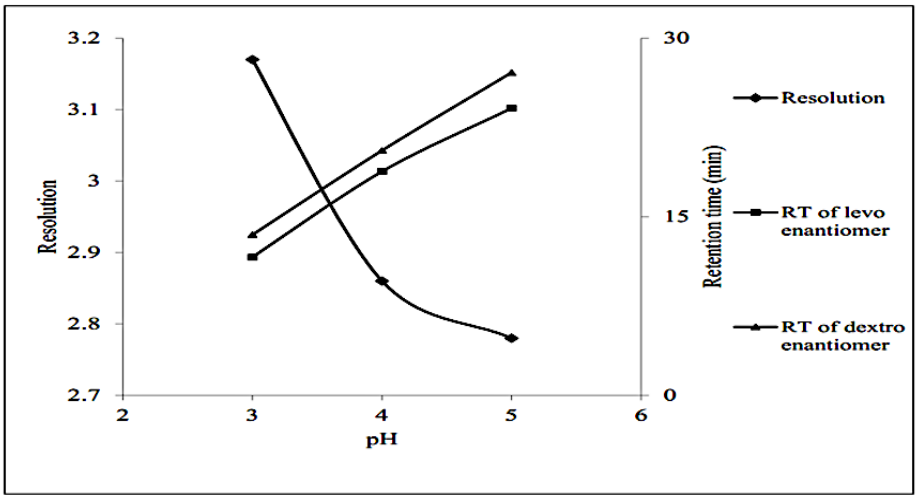


Figure 13: Effect of pH on resolution and retention time

In acidic pH, due to the presence of piperidine ring, CP mainly exists in cationic form and at the same time, S-β-CD is present in anionic form. This ensures the multipoint interactions between the cyclodextrin moiety and the enantiomers leading to higher chiral recognition. Therefore, pH 3 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 shorter run time was achieved with methanol.

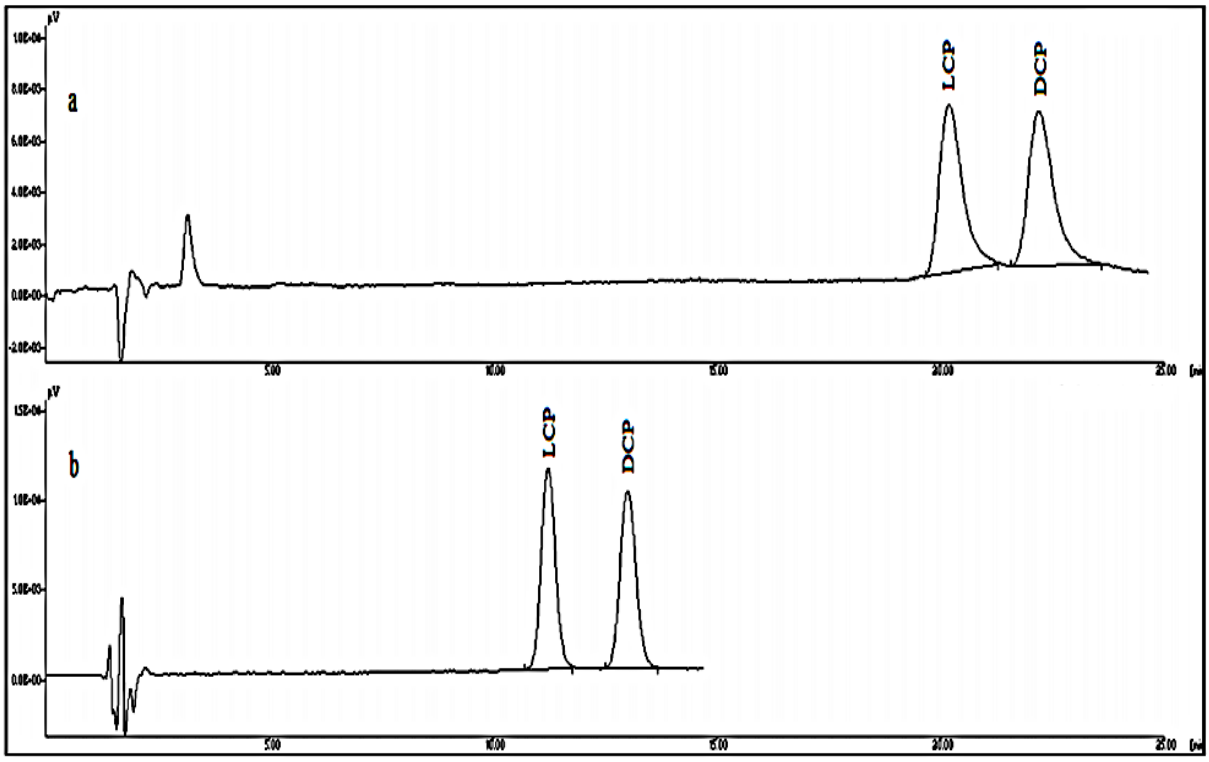
The optimized mobile phase for the enantioseparation of cloperastine was 5mM KH_2PO_4 containing 10mM S-β-CD3 (pH 3) and methanol (55:45) which showed a resolution of 3.14 with a run time of 15 min. Under this optimized condition, S-β-CD1 was evaluated as CMPA; which gave a lower resolution of 2.16 with a run time of 25 min.

3.4 Method validation:

The optimized method using S-β-CD2 was validated according to ICH guidelines.

3.4.1 *Specificity:*

Specificity was evaluated by injecting a racemic solution of 10 µg/ml of racemic CP. The resolution of 3.14 was achieved (**Figure 14b**) which confirmed the specificity of the method.



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

Figure 14: Enantiomeric separation of cloperastine a) using S-β-CD1 and b) using S-β-CD3

3.4.2 *Linearity and range:*

Linearity was evaluated by injecting racemic CP and plotting peak area (y) against concentration (x) of both enantiomers. Good linearity was obtained for both enantiomers over the concentration range of 1-20 µg/ml of racemic cloperastine. Linear regression equations were calculated as y= 46050x + 3189.4 for LCP and y= 45771x + 3744.4 for DCP. Correlation coefficients were 0.9998 for both enantiomers.

3.4.3 *Accuracy:*

Accuracy of the method was demonstrated with a good percentage recovery. The required amount of levo enantiomer was spiked in a racemic solution of 1 µg/ml to obtain a concentration of 2.5, 5 and 7.5 µg/ml for levo enantiomer. Recovery was calculated for the said concentration levels by dividing recovered concentrations with the actual concentrations. The results are shown in **Table 5**.

Table 5: Accuracy of levocloperastine (n=3)

Actual concentration of levo enantiomer (µg/ml)	Recovered concentration (^g/ml) Mean ± SD	% Recovery Mean ± RSD
2.5	2.48 ± 0.002	99.21 ± 0.08
5	4.93 ± 0.008	98.66 ± 0.16
7.5	7.44 ± 0.007	99.17 0.10

3.4.4 *Precision:*

Precision was assessed by preparing racemic samples with 5, 10 and 15 µg/ml concentration and analyzing them on two different days. Repeatability and intermediate precision was expressed in terms of % RSD for the said concentrations. (**Table 6**)

Table 6: Precision for cloperastine enantiomers (n=3)

Concentration of each enantiomer µg/ ml	(µg/ml) mean ± SD; RSD			
	Intraday		Interday	
	LCP	DCP	LCP	DCP
2.5	2.50 ± 0.02; 0.65	2.51 ± 0.02; 0.64	2.54 ± 0.01; 0.57	2.53 ± 0.01; 0.46
5	5.01 ± 0.03; 0.58	5.00 ± 0.04; 0.84	5.03 ± 0.06; 1.11	5.03 ± 0.05; 1.02
7.5	7.49 ± 0.01; 0.18	7.49 ± 0.04; 0.58	7.53 ± 0.06; 0.76	7.50 ± 0.02; 0.30

3.4.5 *LOD and LOQ:*

LOD and LOQ were calculated based on standard deviation (SD) of response and slope. A series of dilute solutions of racemic cloperastine in the concentration range of 0.5-1.25 µg/ ml was injected and their response was plotted against concentration. The results are shown in **Table 7.**

Table 7: LOD and LOQ for cloperastine enantiomers

Enantiomer	Regression equation	r^2	LOD (ng/ml)	LOQ (ng/ml)
LCP	y = 49936x - 121.28	0.9996	13.1	39.7
DCP	y = 48914x + 270.32	0.9996	12.5	38.1

* r^2: correlation coefficient

3.4.6 *Robustness.*

Robustness is the ability of a method to remain unaffected by small changes in the experimental conditions. Deliberate changes in flow rate (±0.2 ml/min), methanol content in the mobile phase (± 1%) and pH (± 0.1 unit) were made and resolution was calculated for each parameter and the resolution was found to be *>2.2* in all cases.

3.5 Application of the method

The validated method was used for the determination of enantiomeric purity in active pharmaceutical ingredient (API) and pharmaceutical formulation (Zerotuss). Assay of LCP in API and pharmaceutical formulation is given in table 8 and 9, respectively, and the representative chromatograms are shown in **Figure 13**.

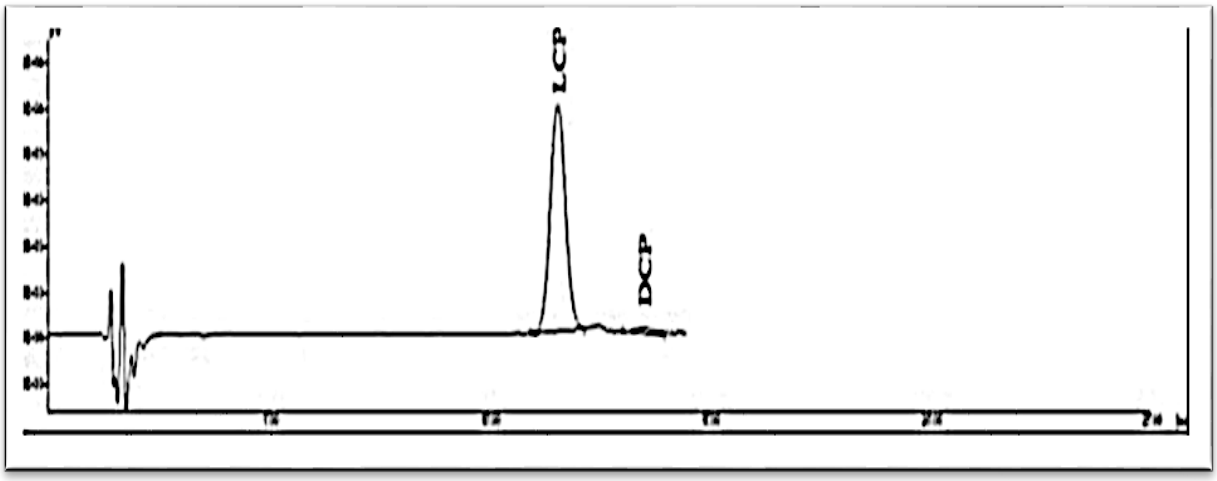


Figure 13: Assay of LCP in API and pharmaceutical formulation

Table 8: Assay of LCP in API

Concentration of LCP (µg/ml)	Recovered Concentration (µg/ml) mean ± SD		% Assay ± % RSD		Rs
	LCP	DCP	LCP	DCP	
10	9.94 ± 0.02	0.083 ± 0.001	98.96 ± 0.44	1.639 ± 0.006	3.12

Table 9: Assay of LCP in pharmaceutical formulation

Formulation	Label claim	Amount estimated (mg) mean ± SD		% Assay ± % RSD		Rs
		LCP	DCP	LCP	DCP	
Zerotuss	20 mg/ 5ml	20.45 ± 0.235	0.37 ± 0.005	102.29 ± 1.15	1.83 ± 1.47	3.1

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-CD3 leading to enhanced chiral separation. Molecular docking predicted that dextrocloperastine exhibits tighter binding than levocloperastine to S-β-CD3. 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 the only $1/20^{th}$ of the levocloperastine population could attain this binding strength **(Figure 14).** 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. The structures of complex between S-β-CD3 and enantiomers are shown in **Figure 15**.

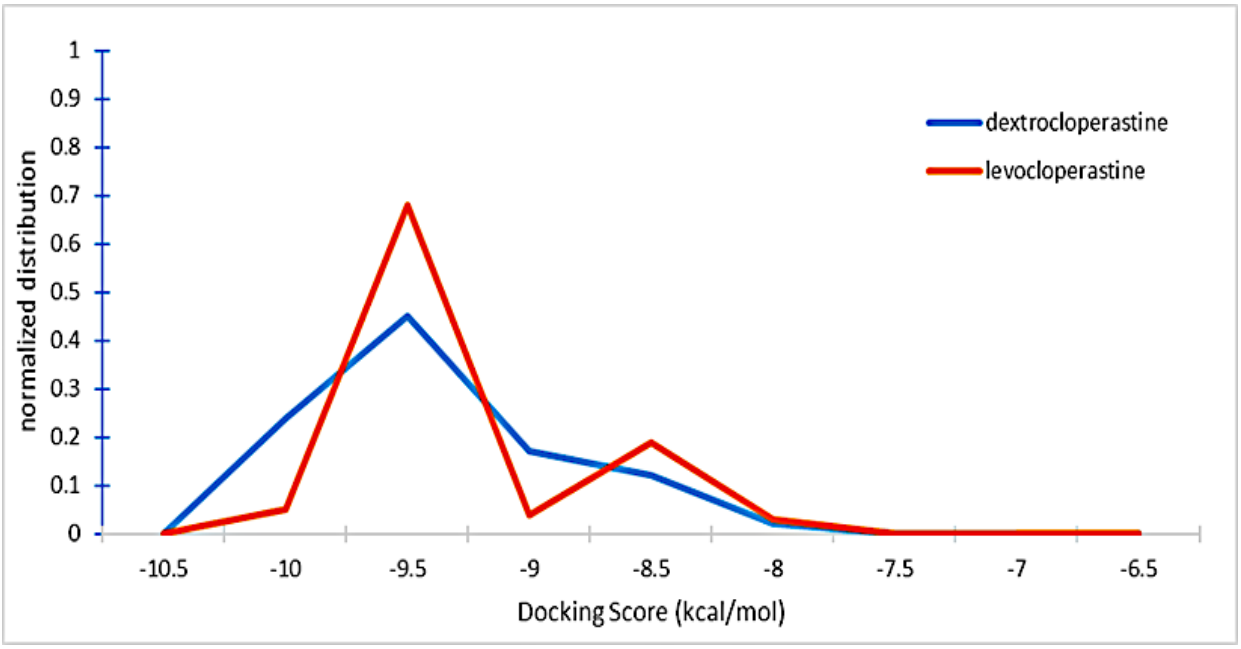


Figure 14: Analysis of molecular docking simulations using the plot of normalized distribution versus docking score.

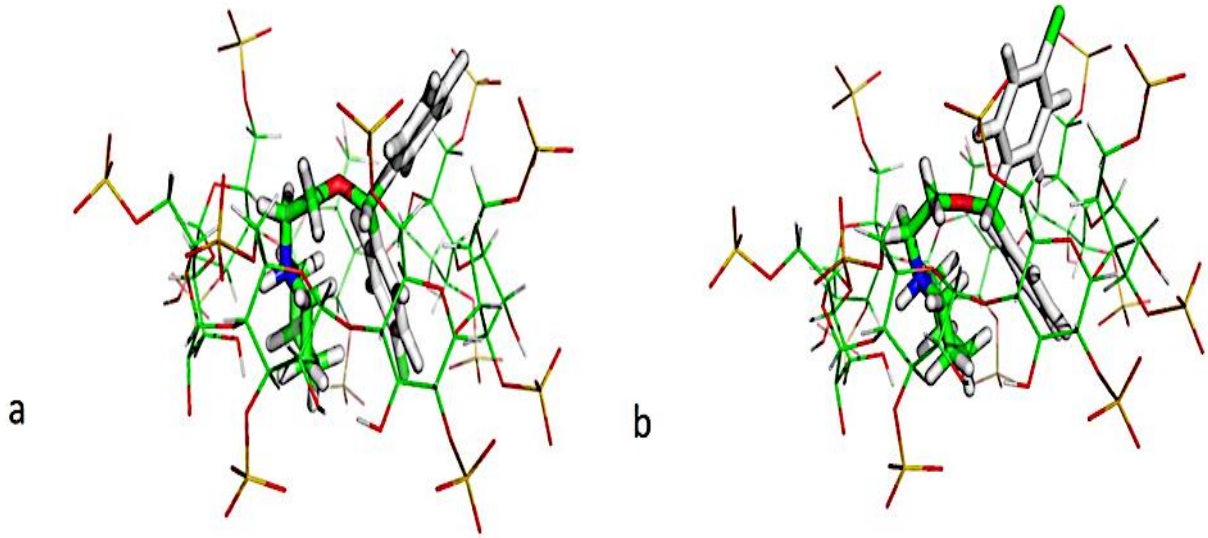


Figure 15: Docked structure of S-β-CD3 with (a) Dextro-cloperastine and (b) Levocloperastine

3 Conclusion:

A new, 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 utilizing various techniques and it was observed to be like the marketed product. The synthesized S-β-CD 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 min. Synthesized S-β-CD gave better resolution than marketed S-β-CD in shorter run time. The developed method was validated and applied for the quantitative determination of cloperastine enantiomers in active pharmaceutical ingredient and pharmaceutical formulation. The modeling studies confirmed the chiral recognition mechanism and elution orders of the enantiomers of the reported molecule. It was observed that inclusion complex formation and hydrogen bonding were the major forces for the chiral recognition. The reported chiral method may be used for the enantiomeric resolution of the two enantiomers of cloperastine of the reported molecule in any unknown matrices.

5. Acknowledgments

The authors would like to thank Precise Chemipharma Pvt. Ltd. for the gift of cloperastine and levocloperastine. The authors would also like to thank University Grants Commission (F.NO.-41-721/2012, Multicomponent Analysis of Drugs) for funding a part of this study. The authors would like to thank Prof. Evans Coutinho and Dr. Elvis Martis for helping in molecular modeling studies.

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