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Experimental and theoretical study on chiral recognition mechanism of ketoconazole enantiomers using heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin

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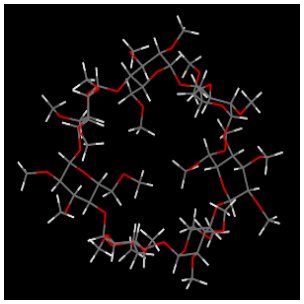
GRAPHICAL ABSTRACT



ABSTRACT

Capillary electrokinetic chromatography (EKC) has been established as a versatile and robust capillary electrophoresis (CE) method for the separation of enantiomers. One of the most attractive advantages of EKC for the separation of enantiomers is its ease of change of separation media in method development. The separation solution can easily be altered to find the optimum separation media and one can also use an expensive chiral selector because small amounts of it are required. This work aims to develop experimental and theoretical analysis of the chiral separation of ketoconazole using EKC and molecular modelling study, respectively. In the first part of the study, several cyclodextrins (CDs) as the chiral selectors (CS) namely α-cyclodextrin, sulfated β-cyclodextrin, (2-hydroxylpropyl)-β-cyclodextrin, heptakis (2,6-di-*O*-methyl)-β-cyclodextrin, and heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin were screened. CDs were initially chosen as they are easily available and cheap. Heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin (TMβCD) exhibited a higher enantioselectivity power compared with other tested CDs. The influence of TMβCD concentration, buffer pH, buffer concentration, separation temperature and applied voltage were investigated. The optimum conditions for chiral separation of ketoconazole was achieved using 10 mM phosphate buffer at pH 3.0 containing 20 mM TMβCD with an applied voltage of 30 kV at 35°C with 5 s injection time (hydrodynamic injection). The ketoconazole enantiomers were resolved in less than 7 min (R_s = 1.79). In order to understand possible chiral recognition mechanisms of ketoconazole with TMβCD, host-guest binding procedures of TMβCD and ketoconazole were studied using the semi-empirical PM3 calculations.

Keywords: Capillary electrokinetic chromatography (EKC), Chiral separation, Cyclodextrin, Ketoconazole, Molecular modelling, PM3

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1. INTRODUCTION

Ketoconazole, [IUPAC name: *1-acetyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-yl-methyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazine*], (pK_a=2.95 and 6.54) is known as a potent, orally active, broad-spectrum antifungal agent [1], which is marketed as a racemic mixture of the *cis*-(2S,4R) and -(2R,4S) enantiomers [2,3]. The *cis* configuration for these molecule means that the hydrogen and the 2,4-dichlorophenyl group at the two stereogenic (chiral) centre, respectively, are on the same side of the five-membered (dioxolane) ring [4]. Ketoconazole is an imidazole antifungal administered orally or topically. It has been given for the prophylaxis of fungal infections in immuno-compromised patients. The usual oral dose for treatment and prophylaxis of fungal infections is 200 mg once daily. Ketoconazole is applied topically as a 2% cream or a shampoo [5].

Enantioseparation by using CE offers a few advantages such as very high efficiencies and the ease of method development. Low consumption of both analyte and chiral selector and the short analysis time makes it a very good alternative for analytical separation of enantiomers compared with HPLC [6]. An important method for separating enantiomers involves cyclodextrin (CD) as a chiral selector [7]. Heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin (TMβCD) also known as permethyl-β-cyclodextrin is a derivative of cyclodextrin modified from β-cyclodextrin by substituting the 2, 3, 6-OH groups of β-cyclodextrin with methyl group. The ability of TMβCD in chiral recognition looks quite promising as compared with other permethylated CDs [8]. The growing interest in molecular modelling studies on the formation and stability of inclusion complexes of cyclodextrin with a variety of molecules has prompted us to perform a computational technique in combination with capillary electrophoresis (CE) for a deeper understanding of the chiral recognition mechanism of ketoconazole. The four stereoisomers of ketoconazole are given in Fig. 1.

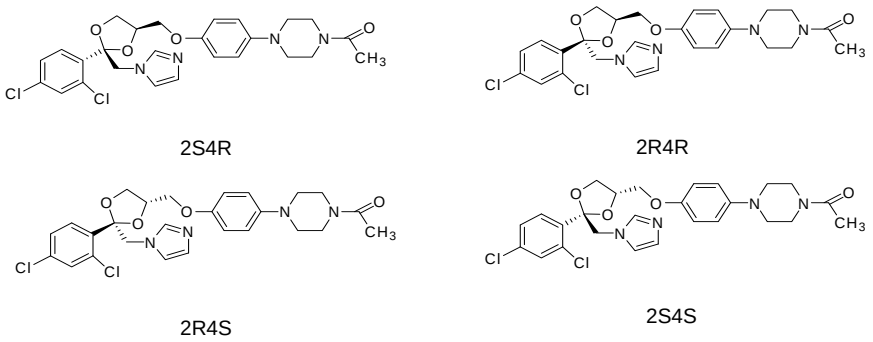


Fig. 1 Stereoisomers of ketoconazole

2. EXPERIMENTAL

2.1 Materials, method and instruments

All electropherograms were obtained with the Agilent capillary electrophoresis system from Agilent Technologies (Waldbronn, Germany), equipped with temperature control and diode array detection (DAD). Separations were performed using an untreated fused silica capillary of 64.5 cm × 50 µm i.d. (with an effective length of 56 cm to the detector window) obtained from Polymicro Technologies (Phoenix, AZ, USA). Ketoconazole and TMβCD were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide and disodium hydrogen phosphate 12-hydrate were purchased from Riedel-de Haen (Seelze, Germany). All other chemicals and solvents were common brands of analytical-reagent grade. Water used for dilutions or as buffer preparation was produced from a Water Purification System from Millipore (Molsheim, France).

2.2 Preparation of stock, working standard and background electrolyte solution

A stock solution containing 1 mg mL^{-1} of each analytes was prepared in HPLC grade methanol from Merck (Darmstadt, Germany) and stored at 4°C and protected from light. Standard solutions were prepared by diluting the stock solution. Working standards solutions for the separation of enantiomers were prepared by diluting each of the stock solutions with HPLC grade methanol. A stock of 200 mM phosphate buffer was prepared by dissolving 0.7163 g of disodium hydrogen phosphate 12-hydrate using deionized water in a 10 mL volumetric flask. The buffer solution was then diluted to the mark after solution was adjusted to pH 3.0 using 50 mM phosphoric acid. The background electrolyte (BGE) solution was prepared by diluting the phosphate buffer solution to 40 mM and addition of 20 mM TMβCD. All separation solutions were filtered through 0.20 µm nylon syringe filter from Whatman (Clifton, NJ) to remove any impurities.

2.3 Molecular modelling study

The crystalline structure of TMβCD (code: XAQJII) was obtained from Cambridge Crystal Structure Database in cif file format. The structure was converted into mol file format using the Mercury software. Then, four stereoisomers of ketoconazole were drawn using ChemSketch and converted into 3D model using MS Modelling. The complexes of ketoconazole and TMβCD were built using MS Modelling application. All the calculations were performed with Gaussian 03 software [9]. PM3 quantum-mechanical semi-empirical method was performed in order to investigate the formation of mechanism of ketoconazole and TMβCD complexes. The binding energy (ΔE) upon complexation between ketoconazole and TMβCD was calculated for the minimum energy structure using Eq. 1:

$$\Delta E = E_{complex} - (E_{KTZ} + E_{TM\beta CD}) \quad (1)$$

3. RESULTS & DISCUSSION

3.1 Optimization of the EKC conditions

Chiral separation of ketoconazole was explored using TMβCD as chiral selector (CS). Previous work by Castro-Puyana *et al*. [10,11] successfully separated two enantiomers of ketoconazole. However, the use of high concentration of phosphate buffer (100 mM) as BGE is not favourable as it generates high current and possible Joule heating [12]. The current work focused on the optimization process for the enantioseparation of ketoconazole using more favourable conditions and theoretical studies of complexation of ketoconazole with TMβCD using computer modelling. For the optimization process, the TMβCD concentration was first varied in order to achieve the best separation of the ketoconazole enantiomers by CD-EKC. The TMβCD concentration range was optimized from 3 to 30 mM in 40 mM phosphate buffer solution, pH 3.0. The best resolution (R_s = 3.80) for ketoconazole enantiomers were obtained using 20 mM TMβCD with an analysis time of 13 min (data not shown).

The effect of phosphate buffer pH and buffer concentration on the enantioseparation of ketoconazole was also investigated. Chiral separation of ketoconazole was investigated in the pH range from 2.5 to 5.0 (with 20 mM TMβCD in 40 mM phosphate buffer). The best enantioseparation was obtained at pH 3.0 (R_s = 4.66) (Fig. 2). In addition, increasing phosphate buffer concentration from 5 to 50 mM resulted in an increase of migration time of enantiomers but the resolutions were not significantly increased. Optimum concentration chosen for phosphate buffer (pH 3.0) was 10 mM, since it yielded good resolution (R_s = 2.03) with relatively short migration time (~10 min) (data not shown).

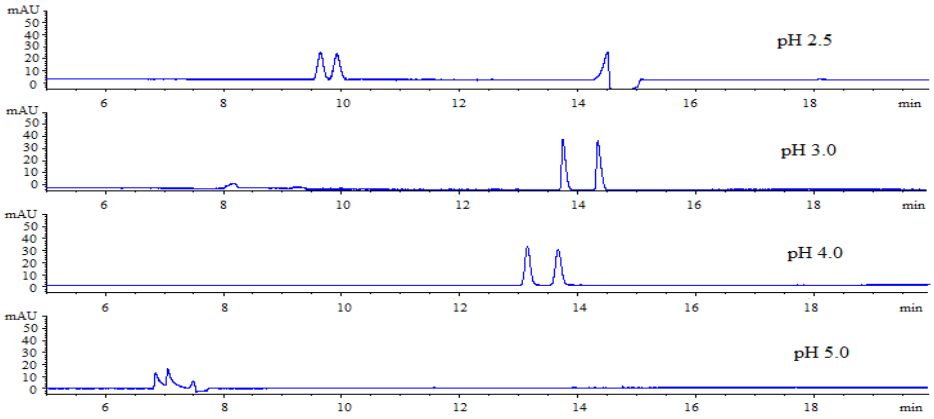


Fig. 2 Enantioresolution of ketoconazole using CD-EKC at different buffer pH (Separation solution: phosphate buffer pH 2.5-5.0, 20 mM TMβCD in 40 mM phosphate buffer; capillary, 64.5 cm × 50 µm I.D. (effective length, 56 cm); applied voltage, 25 kV; temperature, 25°C; detection wavelength, 200 nm; hydrodynamic injection, 50 mbar for 5 s; analyte concentration, 100 mg L^{-1})

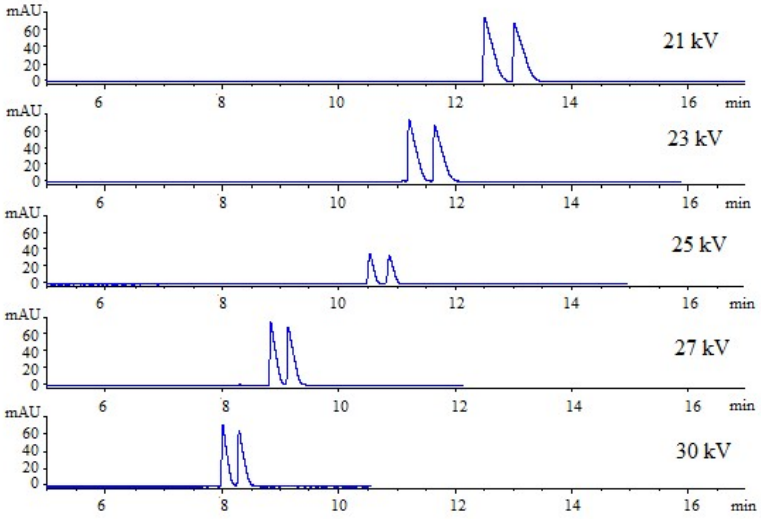


Fig. 3 Enantioresolution of ketoconazole using CD-EKC at different voltage. Separation conditions: applied voltage 21-30 kV; 20 mM TMβCD in 10 mM phosphate buffer; pH 3.0; temperature, 25°C; other condition are as in Fig. 2

In order to improve the migration time for the separation of ketoconazole enantiomers, the effect of separation voltage (25 kV to 30 kV) ((Fig. 3) and separation temperature (15°C to 40°C) (data not shown) were explored. The migration time was found to be inversely proportional to the increase in separation voltage and temperature. It is important to note that the highest voltage for the separation in EKC cannot exceed 30 kV. Therefore, 30 kV was chosen as the optimum voltage for the separation of ketoconazole. The best chiral separation of ketoconazole was obtained within 6.5 min with a BGE containing 20 mM TMβCD in 10 mM phosphate buffer at pH 3.0, applied voltage 30 kV, 35°C separation temperature and 5 s hydrodynamic injection time (Fig. 4).

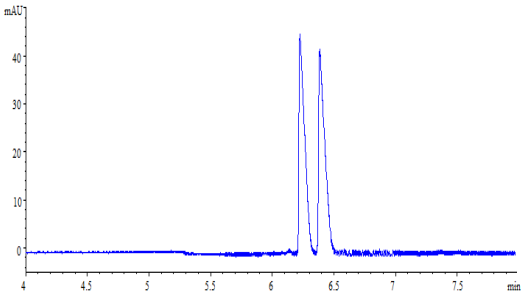


Fig. 4 Enantioresolution of ketoconazole using optimum CD-EKC conditions. Separation conditions: 20 mM TMβCD in 10 mM phosphate buffer; pH 3.0; voltage, 30 kV; temperature, 35°C; other conditions are as in Fig. 2.

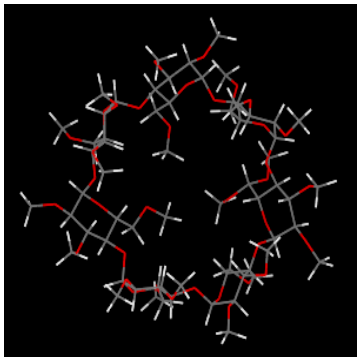
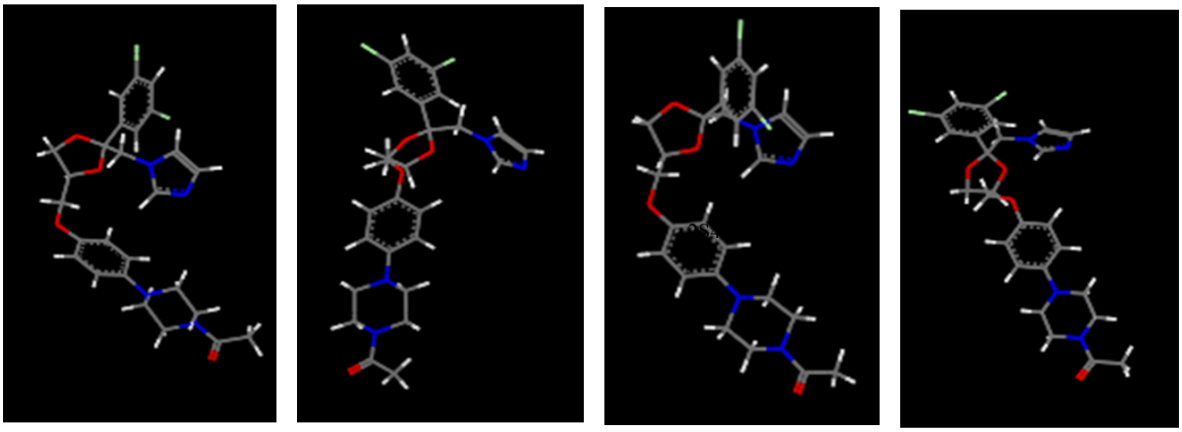


Fig. 5 TMβCD structure in 3D view with minimum energy: [grey: carbon; red: oxygen; white: hydrogen]



2S4R 2R4R 2R4S 2S4S

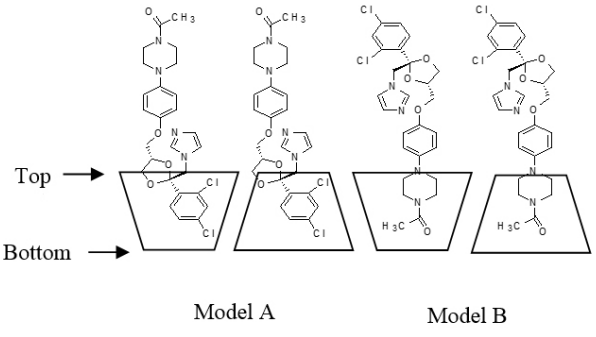
Fig. 6 Ketoconazole stereoisomers structure in 3D view with minimum energy [grey: carbon; blue: nitrogen; green: chlorine; red: oxygen; white: hydrogen]

Table 1 Optimized energies of TMβCD and ketoconazole stereoisomers

		Energy (kJ mol^{-1})			
Single TMβCD		-5684.25			
		2R4R	2R4S	2S4R	2S4S
Single ketoconazole		-314.48	-325.58	-325.15	-314.34
Complexes (Model A)	Top	-6630.62	-5840.22	-6068.26	-6002.35
	Bottom	-5777.28	-6128.77	-6235.60	-6257.91
Complexes (Model B)	Top	-5860.24	-6045.68	-6057.20	-6045.68
	Bottom	-5691.24	-6070.53	-5999.05	-6036.40

3.2 Molecular modelling calculation

Molecular modelling proved to be an excellent tool to identify the host-guest interaction in drug-cyclodextrin technology in order to calculate the binding energy (ΔE) as well as the geometrical structure of the inclusion complex [3,13]. PM3 is highly convenient for the modelling of large molecular systems and performs better than AM1 in biochemical systems due to its improved description of the interaction between non-bonded atoms [14]. The current studies were performed by using molecular mechanics calculations (PM3 semi-empirical methods and Gaussian 03) to calculate the relative energies involving the four possible inclusion forms of complexes.



Scheme 1 Four possible inclusion complexes

Fig 5 and Fig 6 shows the minimum energy structure of TMβCD and ketoconazole stereoisomers. The optimized energies of the single structure of TMβCD, ketoconazole stereoisomers and complexes of Model A and Model B in kJ mol^{-1} are summarized in Table 1.

The inclusion complexes were built from these optimized single structures and further optimization calculation of each complex was calculated with the four possible inclusion forms. The schematic diagram for the possible inclusion complexation is shown in Scheme 1.

Four possible inclusions complexes are defined as different in arrangement for the inclusion modes. For the four possible inclusions complexes, we separated into Model A and Model B. Both models have two different orientations of TMβCD; top and bottom. The difference in Model A and B is the orientation of ketoconazole structure to form the inclusion complexes into the TMβCD cavity. The binding energies for Model A and Model B were calculated and summarized in Table 2.

From Table 2, it shows that 2R4R/TMβCD complex form the most stable inclusion complex with the lowest binding energy, ΔE= -631.27 when added from the top of TMβCD cavity. However, the inclusion complex is not favourable with the other different inclusion orientation of 2R4R into TMβCD cavity. For 2R4S/TMβCD and 2S4R/TMβCD complexes, only one arrangement does not allow the complexation to occur which is inclusion from the top of cavity of the TMβCD and from bottom of cavity of the TMβCD with different orientation (Model B) of stereoisomers structure, respectively. Conversely, 2S4S/ TMβCD complex favour inclusion complexation for all arrangement and orientation of structures with the lowest binding energy, ΔE= -259.32 kJ/mol. Fig. 7 shows the most stable inclusion complex for each stereoisomer.

Table 2 Binding energies of TMβCD and ketoconazole complexes.

Complexes	Binding Energy, ΔE (kJ mol^{-1})			
	Model A (Top)	Model A (Bottom)	Model B (Top)	Model B (Bottom)
2R4R/ TMβCD	-631.27	221.45	138.49	307.49
2R4S/ TMβCD	169.61	-118.94	-35.85	-60.70
2S4R/ TMβCD	-58.86	-226.20	-47.80	10.35
2S4S/TMβCD	-3.76	-259.32	-47.09	-37.81

It can be deduced from the computational calculations that the stability of the inclusion complexes between the enantiomers and the CD allows chiral discrimination and thus leads to different migration times as observed in the experimental studies [15]. This finding is consistent with findings from previous works by Bernal *et al*. [16], Lin *et al*. [17] and Castro-Puyana *et al*. [10,11]. However, from the computer modelling study, the binding energy of the complexes proves that all the stereoisomers of ketoconazole were possible to be formed with the TMβCD. Further study is in progress to attempt to separate all four stereoisomers of ketoconazole using dual CD method. In addition, the optimization of PM3 semi-empirical method will be further simulated using different distance and angle inclusion of ketoconazole stereoisomers for more insight to the understanding the mechanism of the complexes formed and the interaction/s involved.

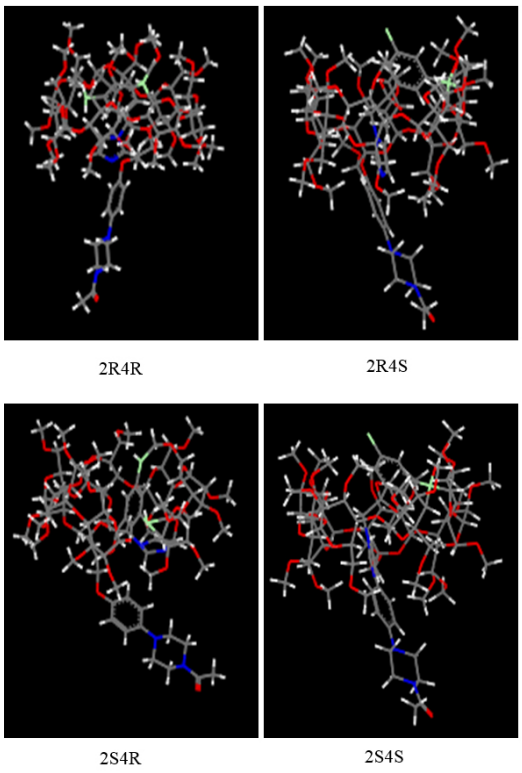


Fig. 7 Ketoconazole stereoisomers and TMβCD complexes in 3D view with the lowest binding energy [grey: carbon; blue: nitrogen; green: chlorine; red: oxygen; white: hydrogen]

4. CONCLUSION

An experimental and theoretical study of chiral separation of ketoconazole and TMβCD was explored in this current study. The enantiomers of ketoconazole were resolved within less than 7 min with R_s = 1.79 using low concentration of phosphate buffer (10 mM) in CD-EKC method. Random inclusion complexation of ketoconazole stereoisomers into the TMβCD cavity was successfully determined for the most favourable complex for each stereoisomer with the lowest binding energy using PM3 and Gaussian 03 calculations.

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