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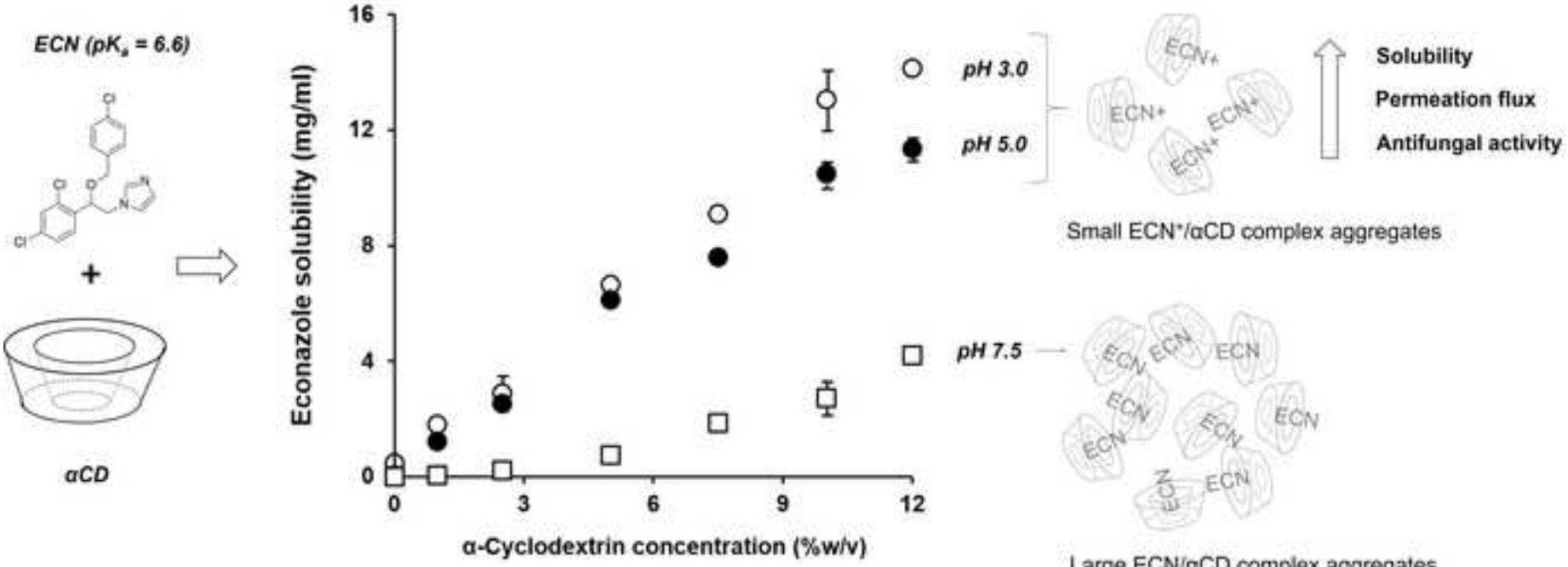
Abstract: Econazole nitrate (ECN) is a weakly basic drug with very low aqueous solubility that hampers its permeation through biological membranes and results in low ECN bioavailability. Formation of drug/cyclodextrin (drug/CD) inclusion complexes is a formulation technology that can be applied to enhance drug solubility in aqueous media. The aim of this study was to determine the effect of CD complexation and pH adjustments on the ECN solubility. The ECN pH-solubility and ECN/CD phase-solubility profiles were determined. The solubility of ECN in aqueous acidic solutions containing α-cyclodextrin (αCD) was relatively high and much higher than in aqueous γ-cyclodextrin (γCD) solutions under same conditions. The complexation efficiency of the ECN/CD complex was relatively low for the unionized drug. Formation of ECN/CD inclusion complex was verified by proton nuclear magnetic resonance spectroscopy. Formation of ECN/CD complexes enhanced the drug stability during autoclaving. γCD complexes self-assembled to form nano- and microparticles whereas αCD complexes had negligible tendency to self-assemble. Formation of CD complex nano- and microparticles was investigated by dynamic light scattering and by drug permeation through semipermeable membranes of different molecular weight cut-off. The largest aggregate fraction was observed for the unionized ECN in aqueous pH 7.5 solution containing high CD concentration, that is 10% (w/v) CD. It was shown that in acidic solutions ECN/αCD can enhance the antifungal activity to filamentous fungi. This was associated with the increased ECN solubility and increase of readily available ECN molecules in aqueous αCD solutions.

*Graphical Abstract (for review)



Phase-solubility profiles of ECN in aqueous solutions containing αCD

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Antifungal Activity of Econazole Nitrate/Cyclodextrin Complex: Effect of pH and Formation of Complex Aggregates

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Abstract

Econazole nitrate (ECN) is a weakly basic drug with very low aqueous solubility that hampers its permeation through biological membranes and results in low ECN bioavailability. Formation of drug/cyclodextrin (drug/CD) inclusion complexes is a formulation technology that can be applied to enhance drug solubility in aqueous media. The aim of this study was to determine the effect of CD complexation and pH adjustments on the ECN solubility. The ECN pH-solubility and ECN/CD phase-solubility profiles were determined. The solubility of ECN in aqueous acidic solutions containing α-cyclodextrin (αCD) was relatively high and much higher

than in aqueous γ-cyclodextrin (γCD) solutions under same conditions. The complexation efficiency of the ECN/CD complex was relatively low for the unionized drug. Formation of ECN/CD inclusion complex was verified by proton nuclear magnetic resonance spectroscopy. Formation of ECN/CD complexes enhanced the drug stability during autoclaving. γCD complexes self-assembled to form nano- and microparticles whereas αCD complexes had negligible tendency to self-assemble. Formation of CD complex nano- and microparticles was investigated by dynamic light scattering and by drug permeation through semipermeable membranes of different molecular weight cut-off. The largest aggregate fraction was observed for the unionized ECN in aqueous pH 7.5 solution containing high CD concentration, that is 10% (w/v) CD. It was shown that in acidic solutions ECN/αCD can enhance the antifungal activity to filamentous fungi. This was associated with the increased ECN solubility and increase of readily available ECN molecules in aqueous αCD solutions.

Keywords:

Cyclodextrins
Econazole
Solubility
Complexation
Antifungal

1. Introduction

Various pharmaceutical techniques can be applied to enhance solubility and permeability of poorly water-soluble drugs, such as pH adjustments, use of cosolvents, size reduction (e.g. micronization), complexation and formation of solid dispersions (Kawabata et al., 2011; Khadka et al., 2014). One of the more promising technologies is drug - cyclodextrin (CD) complexation. CDs are cyclic oligosaccharides consisting of α-D-glucopyranose units with hydrophilic outer surface and somewhat lipophilic central cavity. They increase the aqueous solubility of hydrophobic drugs through formation of water-soluble inclusion complexes. In general, uncharged hydrophobic molecules or their moieties, that is molecules that generally have poor aqueous solubility, have affinity for the CD cavity (Brewster and Loftsson, 2007; Jansook et al., 2018).

Saturated aqueous drug/CD complex solutions frequently contain mixtures of inclusion and non-inclusion complexes (Loftsson et al., 2004). Both the natural CDs and their derivatives form CD aggregates. The diameter of the parent CD aggregates (i.e. of αCD, βCD and γCD) have been reported to be between about 200 and 300 nm by dynamic light scattering (DLS) (González-Gaitano et al., 2002). Various analytical techniques, such as DLS, nuclear magnetic resonance (NMR) spectroscopy, transmission electron microscopy (TEM) and permeation through different molecular weight cut-off (MWCO) semipermeable membranes, are commonly used to detect and characterize the aggregates (Duan et al., 2005; Jansook et al., 2010a; Messner et al., 2010; Witte and Hoffmann, 1996).

Econazole nitrate (ECN) is an imidazole antifungal drug. Its antifungal activity appears to be associated with disruption of cell membranes (Dyas and Delargy, 1994). Less than 1% of applied dose is absorbed into the general blood circulation after topical administration and, thus, currently ECN is mainly used to treat skin infections (Heel et al., 1978; Kalus, 2017). The low aqueous solubility of ECN (<1 mg/ml at 20ºC) limits ECN permeation through biological membranes and results in, for example, low oral bioavailability (Al-Marzouqi et al., 2009; Dyas and Delargy, 1994). Among natural CDs, αCD has the highest solubilizing effect on ECN whereas γCD has almost negligible effect (Díaz-Tomé et al., 2018; Mura et al., 1999). Various methods to enhance the complexing efficacy (CE) have been reviewed (Loftsson and Brewster, 2012; Loftsson and Duchêne, 2007). For example, ternary drug/CD complexes with hydroxyl-acids as third component enhanced ECN solubility with consequent improved drug dissolution (Jug et al., 2014; Mura et al., 2001; Mura et al., 1999). The entrapment of ECN in chitosan/SBEβCD nanoparticles provided mucoadhesive effect and sustained antifungal activity (Mahmoud et al., 2011). However, pH adjustments or drug ionization that increases the drug solubility can result in enhanced CE of ECN/CD complex. The aim of this study was to determine the effect of pH on the CD solubilization of ECN and thermal stability of ECN, and to investigate the influence of pH on formation of ECN/CD complex aggregates. The antifungal activity of the ECN/CD complexes was also evaluated.

2. Materials and methods

2.1 Materials

Econazole nitrate (ECN) was purchased from Fagron Group (Amsterdam, Netherlands). α-Cyclodextrin (αCD) and γ-cyclodextrin (γCD) were purchased from

100 Wacker Chemie (Burghausen, Germany), and semi-permeable cellophane
101 membranes (SpectaPor®, molecular weight cut-off (MWCO) 3500, 6-8000 and 12-
102 14000 Da) from Spectrum Europe (Breda, Netherlands). All other chemicals used
103 were of analytical reagent grade purity. Milli-Q (Millipore, USA) water was used for
104 preparation of all solutions.
105
106 *2.2 pH solubility profiles*
107 Excess amount of ECN was added to the unbuffered aqueous pH medium
108 (pH from 2 to 9) without and with CD (5% w/v αCD or γCD). This pH range covered
109 the ionized and unionized forms of ECN molecules. The desired pH was obtained by
110 dropwise titration of the medium with concentrated aqueous sodium hydroxide or
111 hydrochloric acid solutions. The suspension formed was agitated at room
112 temperature (22-23°C) for 7 days and the pH was readjusted, if necessary. After
113 equilibration the samples were removed and filtered through 0.45 μm nylon
114 membrane filter. Finally, the filtrate was diluted with a mixture of acetonitrile and
115 water (30:70 v/v) and the amount of dissolved drug determined by a high-
116 performance liquid chromatographic (HPLC) method.
117
118 *2.3 Thermal stability of econazole*
119 The stability of ECN in aqueous solutions containing 5% (w/v) αCD or γCD, as
120 well as in purified water, was determined by heating in an autoclave (Loftsson et al.,
121 2005). Heating of an aqueous drug suspension can promote complex formation and
122 enhance the complexation efficiency (CE) (Loftsson and Brewster, 2012). Excess
123 amount of ECN was dissolved in the aqueous complexation media and the pH
124 adjusted with concentrated hydrochloric acid or sodium hydroxide to 3.0, 5.0 or 7.5.

125 The samples were equilibrated at 22-23°C for 24 h under constant agitation. The
126 supernatant was filtered through 0.45 µm nylon membrane filter. The clear filtrate
127 was then divided into three sealed vials that were heated in an autoclave for one,
128 two and three heating cycles, each cycle consisted of heating to 121°C for 20 min.
129 Then the ECN concentration in the vials was determined by HPLC.
130
131 *2.4 Solubility determinations*
132 Excess amount of ECN was added to aqueous solutions containing from 0 to
133 12% (w/v) αCD or 0 to 15% (w/v) γCD. The pH of the saturated drug suspensions
134 formed was adjusted with concentrated hydrochloric acid and sodium hydroxide to
135 3.0±0.2, 5.0±0.2 or 7.5±0.2 and then heated in an autoclave (121°C for 20 min)
136 followed by cooling to room temperature (Loftsson et al., 2005). Then a small
137 amount of solid drug was added to the suspensions to promote drug precipitation.
138 The suspensions were equilibrated at 22-23°C for 7 days under constant agitation.
139 The pH was monitored and readjusted if necessary. After equilibrium was attained,
140 the suspensions were filtered through 0.45 µm syringe filter, the filtrates were diluted
141 with mixture of acetonitrile and water (70:30 v/v) and analyzed by HPLC. The
142 determinations were done in triplicate. The apparent stability constants for the
143 ECN/CD complexes ($K_{1:1}$ and/or $K_{1:2}$), the CE and the ECN:CD molar ratio were
144 determined by Eqs.1-3 (Loftsson et al., 2007).
145
146 $$CE = S_0 . K_{1:1} = \frac{Slope}{1 - Slope}$$ Eq. 1
147
148 $$[S_t] - [S_o] = K_{1:1}[S_o][CD] + K_{1:1}K_{1:2}[S_o]$$ Eq. 2
149

$$\text{ECN: CD molar ratio} = \frac{1+CE}{CE} \quad \text{Eq. 3}$$

where S_0 is the ECN solubility in the aqueous complexation media when no CD is present, S_t is the total amount of dissolved ECN, slope means the corresponding slope of the phase-solubility diagrams, $K_{1:1}$ and $K_{1:2}$ are the stability constants of the 1:1 and 1:2 inclusion complexes.

2.5 Quantitative determinations

2.5.1 Econazole analysis

ECN content analysis was performed on an ultra HPLC (UHPLC) component system Ultimate 3000 Series from Dionex Softron GmbH (Germering, Germany) consisting of a DGP-3600A pump with a degasser, WPS-3000TLS well plate sampler, TCC-3100 column compartment, photodiode array detector with Chromeloen software version 7.2.8 and Phenomenex Luna C18(2) 5 µm column (4.6x150 mm) with C18 security guard cartridge. The HPLC condition was as follows:

Mobile phase:	50 mM aqueous ammonium acetate:acetronitrile (35:65% v/v)
Flow rate:	1.0 ml/min
Oven temperature:	ambient
UV detector wavelength:	220 nm
Injection volume:	20 µl
ECN retention:	6 min

2.5.2 αCD and γCD analysis

Quantitative determinations of αCD and γCD in the samples obtained from phase solubility studies were performed on a reverse-phase UHPLC. Ultimate 3000

series system from Dionex Softron GmbH (Germering, Germany) consisting of LPG-3400SD pump with a degasser, a WPS-3000 autosampler, a TCC-3100 column compartment operated at 30°C, and a Corona™ ultraRS™ CAD. Phenomenex Luna C18 (150x4.60 mm) 5 μm column with Security Guard (Phenomenex, Cheshire, UK) were used. Chromeleon™, version 7.2 SR4 chromatography data system (CDS) software (ThermoScientific) was used to analysis. The mobile phase consisted of acetonitrile and water (25:75 %v/v), the flow rate was 1.0 ml/min, and the injection volume was 10 μl.

2.6 ^{1}H-NMR spectroscopy

Solutions of the pure compounds (i.e. ECN, αCD, γCD) and the CD complexes of 1:1 stoichiometry (i.e., ECN/αCD and ECN/γCD) were prepared by dissolving in DMSO-d_6:D_2O (90:10 v/v) and equilibrated at 22-23°C under constant agitation for 24 h. Their spectrum and chemical shift values were recorded by using a 400 MHz ^{1}H-NMR spectrometer (BRUKER™ model AVANCE III HD, Brucker Biospin GmbH, Karlsruhe, Germany). The resonance at 2.5000 ppm, due to residual solvent (DMSO-d_6), was used as internal reference. ^{1}H-NMR chemical shift change ($\Delta\delta$) was calculated as $\Delta\delta = \delta_{complex} - \delta_{free}$.

2.7 Determinations of ECN/CD complexes aggregates

2.7.1 Dynamic light scattering (DLS) measurement

The particle size of ECN/CD based aggregates in solution was measured by DLS technique using Nanotrac Wave particle size analyzer (Microtrac Inc., Philadelphia, PA). The samples, that is ECN saturated aqueous αCD or γCD solutions (CD conc. 1, 5 and 10% w/v) of various pH, were placed in the cell holder.

200 The wavelength of the laser beam was set at 780 nm with the scattering angle of
201 180°. The particle size and percentage of volume were recorded. Each
202 measurement was conducted at 25 ± 0.5°C and carried out in triplicate. The mass
203 distribution was calculated, assuming that the particle of the complexes and complex
204 aggregates are spherical, according to Eq. 4:
205
206 $$M_i = \frac{A_i / R_i^a}{\sum A_i / R_i^a} x100$$ Eq. 4
207
208 where M_i is the mass distribution percentage, A_i is the intensity area, R_i is the
209 hydrodynamic radius of the size population *i* and a is the shape parameter which
210 equals to 3, assuming the spherical particles (Bonini et al., 2006; González-Gaitano
211 et al., 2002).
212
213 *2.7.2 Permeation studies*
214 To investigate the effect of pH and CD concentration on the size of ECN/CD
215 complex aggregates, the *in vitro* ECN permeation from ECN saturated 1, 5 and 10%
216 (w/v) αCD and γCD solutions at pH of 3, 5 and 7.5 through semi-permeable
217 membranes was studied in a Franz diffusion cell apparatus consisting of a donor
218 compartment and a receptor compartment (12 ml). The donor and receptor
219 compartments were separated by the semi-permeable membrane with MWCO 3500,
220 6-8000 or 12–14,000 Da. The receptor phase consists of 2.5% (w/v) αCD or γCD in
221 pure water. CD was added in the receptor phase to allow for sink condition. Two
222 milliliters of each sample were placed in the donor compartment. The receptor phase
223 was kept at ambient temperature (22-23°C) and stirred continuously (300 rpm)
224 during the experiment. A 150-µl aliquot of the receptor medium was withdrawn at 1,

2, 3, 4 and 6 h for analysis and replaced immediately by an equal volume of fresh receptor medium. The ECN content was determined by HPLC and the amount of drug permeation was calculated. Each sample was done in triplicate. The steady state flux (*J*) was calculated as the slope of linear plots of the amount of drug in the receptor chamber (*q*) versus time and the apparent permeation coefficient (P_{app}) determined from Eq. 5:

$$J = \frac{dq}{A.dt} = P_{app}.Cd \qquad \text{Eq. 5}$$

where *A* is the surface area of the mounted membrane (1.77 cm^2) and *Cd* is the concentration of dissolved drug in the donor chamber.

2.8 Antifungal susceptibility test

The methods for antifungal susceptibility testing were modified according to Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS) document M27-A3 and M38-A2 of yeasts and filamentous fungi, respectively (CLSI, 2008a; 2008b). Three isolates fungal organisms i.e., *Candida albicans* (DMST 15317), *Aspergillus flavus* (13-56-29897) *and Fusarium solani* (13-61-03470) were tested by broth microdilution assay. Briefly, the isolated organisms were subcultured onto sabouraud dextrose agar (SDA) slants and stored as suspensions at 2-8 °C. The suspensions of tested organisms were diluted with 0.85% saline to the density of $1x10^6$ - $5x10^6$ cells/ml. The turbidity of the supernatant was determined by UV-VIS spectroscopy (Model UV-1601, Shimadzu, Japan) at a wavelength of 530 nm. The tests were conducted in 96-well culture plates. Each well was inoculated with 50 µl of two-fold dilution inoculum suspension.

An aliquot of 50 µl of test samples was placed in separate wells in triplicate after appropriate dilution with the tested media. DMSO and the ECN-free medium were included as growth control. The plates were incubated at 35 °C for 24 h (*C. albicans*) and for 96-120 h (*A. flavus* and *F. solani*).

Minimum inhibitory concentrations (MICs) were read and defined as the lowest ECN concentration at which no growth could be observed. After MIC readings, 10 µl aliquots were removed from each growth-negative well and were spread on SDA petri dishes. The plates were incubated in 35°C, and the fungal colonies grown were counted after 2 days and approximately 4-7 days of incubation for yeast and filamentous fungi, respectively. The minimum fungicidal concentrations (MFCs) were defined as the lowest drug concentration from which no colonies were visible on the agar plate.

3. Results and discussion

3.1 pH solubility profiles

The pH solubility profiles of ECN in aqueous solutions without and with CD (5% w/v αCD or γCD) are shown in Fig. 1. The desired pH of the aqueous solutions was adjusted with diluted hydrochloric acid or sodium hydroxide. The aqueous solubility of ECN is less than 1 mg/ml (Abd El-Gawad et al., 2017; Pedersen et al., 1993). ECN has pK_a of 6.6. At pH ranging from 2 to 5, ECN molecules are protonated and somewhat soluble in water. In contrast, at pH above 7.5 the ECN molecules are mainly in their unionized form and very poorly soluble in water (Fig. 2). Addition of CD increases the ECN solubility. αCD is a better solubilizer of ECN than γCD. ECN had affinity to αCD resulting in significantly enhanced ECN solubility

through formation of water-soluble ECN/αCD complex. It has been reported that αCD is the best CD solubilizer for ECN of all CDs tested (Díaz-Tomé et al., 2018; Mura et al., 2001). To evaluate the effect of pH on CD complexation with ECN, the pH of solutions i.e., 3.0, 5.0 and 7.5 were selected for further studies. Of these the pH, ECN molecules exhibit fully ionized, partially ionized and unionized forms, respectively.

3.2 Thermal stability of econazole on cycles of autoclaving

The thermal stability of ECN in aqueous solutions without and with CD (αCD or γCD, 5% w/v) at the pH 3, 5 and 7.5 was determined by heating in an autoclave (each heating cycle: 121^{o}C for 20 min). Table 1 shows the drug remaining in the complexation media after zero to three cycles of autoclaving. The drug content at pH 3 with and without CD did not decrease upon heating for up to 3 cycles. At pH 5, the drug concentration in pure water or aqueous solution containing 5% w/v γCD decreased after heating for 2 cycles while it was stable in aqueous solutions containing αCD. However, upon increasing the pH to 7.5 the drug degraded rapidly in the αCD solution or from 5% to 18% after 1 to 3 cycles of autoclaving. The ECN content after heating for 3 cycles was significantly decreased when compared to that of no autoclaving (P<0.05). The protonated drug is thermally stable while the unionized drug is somewhat unstable. The degradation products of ECN would be produced by hydrolysis of the ether linkage in parent ECN molecule (Dyas and Delargy, 1994). Baker et al. (2016) have found the degradation product of ECN after heating with 5% hydrogen peroxide at 90 °C for 2 h. CD can insulate labile compounds from the environment to prevent drug hydrolysis and oxidation; for

300 examples, doxorubicin, aspirin and β-lactam antibiotics (Loftsson and Brewster,
301 1996; Popielec et al., 2016). The stabilizing effect of CD depends on the type and
302 concentration of CD, the degree of the complex formation and the rate of
303 degradation within the complex (Loftsson et al., 2005). In this case, addition of αCD
304 may shield the ECN molecules by encapsulating them against the hydrolysis
305 degradation process.
306
307 *3.3 Solubility determinations*
308 The phase solubility diagrams of ECN in αCD solutions were of type A (Fig.
309 3a) while their diagrams in γCD solutions were of B_s-type (Fig. 3b) according to
310 Higuchi-Connor classification system (Higuchi and Connors, 1965). At the pH lower
311 than the pK_a value (i.e., pH 3.0 and 5.0), the ECN/αCD phase solubility diagram is of
312 A_N-type. However, the stoichiometry of the ECN/αCD complex formed is 1:1. In
313 other words, one ECN molecule forms a complex with one αCD molecule. At pH
314 higher than the pK_a, the unionized form of ECN forms second or higher order
315 complexes with αCD and A_P-type phase solubility diagram is observed. In case of
316 γCD, the drug solubility was increased with increasing γCD concentration with a
317 maximum solubility at 3% (w/v) γCD at pH 3 and 1% w/v γCD at pH 5, with a
318 following decreased solubility at higher γCD concentrations. It indicates that the ECN
319 forms complexes and complex aggregates with γCD which have limited solubility in
320 aqueous solutions. At pH 7.5 the solubility of the ECN/γCD complex was below the
321 limit of quantitation (LOQ). Table 2 shows the apparent stability constants ($K_{1:1}$ and
322 $K_{1:2}$), the complexation efficiency (CE) which was calculated from the slopes of the
323 initial linear sections of the diagrams, and the molar ratio (ECN:CD). The determined
324 intrinsic solubilities of ECN at the pH of 3 were 10 and 1000 times higher than at pH

of 5 and 7.5, respectively. ECN is a weak base and, thus the protonized form is somewhat more soluble in water than the unionized form. Regarding to apparent stability constant (K), ECN had stronger binding affinity to αCD. The strong association is probably due to fit of ECN molecule into CD cavity. The number of αCD and γCD molecules needed to solubilize one ECN molecule (i.e. the molar ratio) was calculated from the CE values. The commercial 1% (w/w) econazole cream contains 10 mg of econazole nitrate per one gram. The results show that it is possible to develop aqueous econazole hydrogel containing CD at low pH (pH 3 or 5) by including 80-100 mg of αCD or 140-280 mg of γCD per one gram hydrogel but at pH 7.5 about 550 mg αCD/gram will be needed to solubilize ECN which is not practical.

3.4 Quantitative analysis of αCD and γCD

The presence of ECN decreased the aqueous solubility of γCD while ECN has negligible effect on the αCD solubility (Fig. 4). According to the phase-solubility profiles, precipitation of the ECN/γCD complexes resulted in limited ECN solubility at elevated γCD concentrations. If formation of 1:1 ECN/γCD complex is assumed, the number of γCD molecules in the saturated aqueous ECN solutions should be proportional to the ECN solubility profiles. However, it was found that the observed γCD solubility did slightly deviated from the theoretical values (Fig. 4b). This might indicate that γCD self-aggregates at elevated γCD concentrations. This self-aggregation strongly affected the ECN solubility. The observations were in accordance with previous reports (Jansook et al., 2010b; Messner et al., 2011). The media pH did not have any significant effect on the CD solubility in saturated ECN solutions.

3.5 ^{1}H-NMR analysis

^{1}H-NMR chemical shifts of αCD and γCD are summarized in Table 3. The H3 and H5 protons of the glucose units are facing the interior of the lipophilic CD cavity. The changes in ^{1}H-chemical shifts (Δδ) of the H3 proton of αCD and γCD in the presence of ECN were -0.176 and -0.064, respectively, displaying significant upfield shift, while the H5 proton of both CDs exhibited insignificant chemical shift (i.e. +0.028 and -0.027 for αCD and γCD, respectively). The Δδ* of the H3 proton was higher than that of the H5 proton indicating the partial inclusion of ECN into the CD cavity (Greatbanks and Pickford, 1987). In comparison, the lipophilic moiety of ECN was more efficiently inserted into αCD cavity than into that of γCD. This observation supported the obtained phase solubility data and the fact that αCD is better solubilizer of ECN than γCD. The results are consistent with the 1D and 2D NMR data obtained by Díaz-Tomé et al. (Díaz-Tomé et al., 2018). They suggested that the imidazole ring of ECN was included into the CD cavity.

3.6 Determinations of ECN/CD complexes aggregates

Table 4 shows the size and size distribution data of ECN/CD complexes in aqueous solutions at different pH. The particle size distributions varied, ranging from mono-, bi- to trimodal distribution. In most cases, no complex aggregates were seen in the saturated ECN aqueous solutions containing 1% w/v CD. In most cases, small and large aggregates were observed when the CD concentration was increased to 5% and 10% w/v. The influence of pH on the ECN/CD complex aggregation was also examined. It was noticed that the aggregate size and the size distribution increased with increasing pH. This was probably due to ECN protonation at low pH and the

consequent charge-charge repulsion. Especially, at the pH 3 there was insignificant difference among CD concentrations in the tendency to form aggregates. In other words, the unionized ECN/CD complex present at high pH has greater tendency to self-association to form complex aggregates than complexes of the ionized drug.

Permeation of drug through semi-permeable membranes of different MWCO can be used to observe aggregation of drug/CD complexes (Messner et al., 2010). According to the phase solubility profiles, the stoichiometry of ECN/CD complex can be assumed to be 1:1. Thus, the ECN/αCD 1:1 complex dimers, tetramers and octamers can pass through semipermeable membranes of MWCO 3500, 6-8000 and 12-14000 Da, respectively. γCD has higher MW and hence, the 1:1 ECN/γCD complex monomers, trimers and hexamers able to permeate these membranes, respectively. Figure 5 displays the flux and the P_{app} of ECN from ECN saturated aqueous CD solutions at various pH. As expected, both ECN permeation flux and P_{app} increased with increasing membrane MWCO. Also, the aggregate formation and their size increase with increasing CD concentration. The ECN flux from aqueous ECN saturated αCD solutions was higher in all cases than from comparable γCD solutions due to the higher ECN C_d in the αCD complexing medium. In case of ECN in saturated aqueous αCD solutions, the flux of ECN from ECN saturated αCD solutions did not increase proportionally with increasing αCD concentrations as would be expected from the observed A-type phase solubility profile. This shows that at higher αCD concentrations the solubilized ECN was partly present in soluble aggregates that could not permeate the membrane. The ratio of the flux of ECN permeated through the incremental MWCO values (i.e., 6-8 kDa/3.5 kDa and 12-14 kDa/3.5 kDa) are shown in Table 5. The fraction of small ECN/CD complex aggregates (i.e., tetramers to dimers and trimers to monomers in case of αCD and

γCD, respectively) increased with increasing CD concentrations. At pH 7.5 the small aggregates were predominant. Especially, the highest fraction of small aggregates (3-times higher) was observed in 10% αCD solution saturated with ECN (Table 5). In other words, the decreased ability to form ECN/CD complex aggregates at low pH (i.e. pH 3 and 5) was most probably due to the repulsion force between positively charged protonated ECN. This result was confirmed by the DLS data. In all cases, formation of ECN/γCD complex aggregates was less than in the case of αCD. Normally, in aqueous solutions γCD tends to self-aggregate at high γCD concentrations (Jansook et al., 2010b; Saokham and Loftsson, 2017). This phenomenon may hamper formation of ECN/γCD complexes and solubilization of ECN through formation of water-soluble complex aggregates. Thus, the saturated aqueous solutions containing complexes aggregates of ECN/αCD were selected to evaluate the antifungal activity.

3.7 Antifungal susceptibility test

The MICs and MFCs of ECN in saturated aqueous αCD solutions at pH 3 and 5 against *C. albicans*, *A. flavus* and *F. solani* were obtained by broth microdilution technique (Table 6). ECN solubilized in aqueous αCD solutions had MIC and MFC ranging between 5-45 μg/ml and 0.7-11 μg/ml against *C. albicans* and filamentous fungi i.e., *A. flavus* and *F. solani*, respectively. Aqueous 10% w/v αCD solution saturated with ECN inhibited the growth of tested pathogenic fungi at the same ECN concentration or less than solutions without αCD. ECN/αCD solutions at pH 3 and 5 did not display significantly different antimycolytic activity. In comparison to the uncomplexed ECN, the ECN/αCD inclusion complex showed higher fungicidal activity especially against *A. flavus* (MFC 0.7 μg/ml) followed by *F. solani* (MFC 11.0

μg/ml) while the CD complex did not enhance the antifungal activity of ECN against *C. albicans* (MFC ~44 μg/ml). The effect of pH on *in vitro* susceptibility of *C. glabrata* and *C. albicans* was investigated by Danby et al. (2012). It was found that *C. albicans* strains had reduced susceptibility to azole antifungal agents at pH 4. However, the exact mechanism of the pH induced reduction of the antifungal activity has not been established.

Schär et al. (1976) reported that ECN was more active against filamentous fungi such as *Aspergillus* and *Rhizopus spp*. The results indicated that the CD complexation could improve antifungal activity in terms of both MIC and MFC to filamentous fungi. Gao et al. (2019) summarized that the inclusion complex of CD and chlorothalonil had better fungicidal activity than original chlorothalonil. Thus, ECN/αCD complex is a potential therapeutic alternative that can be further developed to pharmaceutical preparation for the treatment of infectious diseases (Díaz-Tomé et al., 2018; Jacobsen et al., 1999).

In general, the ECN concentration must exceed the MIC in the epidermis and as deep as the middle region of the dermis for dermatophytes. Due to their size and hydrophilicity, CDs are not able to penetrate biological membranes. CDs enhance topical drug delivery by increasing the drug availability at the barrier surface and can only enhance topical drug delivery in the presence of water (Loftsson and Masson, 2001). In addition, the CD complex aggregates may hamper the drug permeability through the lipophilic membrane. To overcome the difficulties, ECN/CD can be incorporated into hydrogel contained water-rich structure or lipid nanoparticles; for example, liposome is possible to enhance the topical drug permeation (Chen et al., 2014).

4. Conclusions

The very low aqueous solubility of ECN is the main obstacle in ECN product development. CD complexation with pH adjustments can improve the ECN solubility. ECN has relatively high affinity to the αCD cavity, especially in acidic solutions. αCD can enhance the ECN solubility in aqueous solutions and increase the thermal stability of ECN. γCD cavity is too large to give a good fit with ENC molecule and γCD and its complexes have higher tendency to self-associate to form aggregates that limits the ECN solubility. The enhanced ECN solubility might be due to CD complex aggregates formation which was observed by DLS and permeation studies. At pH 3 and 5 the ECN saturated aqueous αCD solutions had antifungal activity against the tested filamentous fungi. The results show that αCD complexation can improve antifungal activity of ECN.

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***Conflict of Interest**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Authors' contributions

Name	Location	Role	Contribution
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CRediT author statement

Phatsawee Jansook: Conceptualization, Methodology, Investigation, Writing-Original draft preparation.: **Manisha Prajapati**: Resources; Investigation.: **Patamaporn Pruksakorn**: Resources; Investigation.: **Thorsteinn Loftsson**: Supervision; Writing - Review & Editing; Funding acquisition.

TABLE LEGENDS

Table 1. Econazole content in aqueous solution with and without 5% w/v CD at different pH after zero to three cycles of autoclaving.

Table 2. The values of the apparent stability constants (i.e. $K_{1:1}$ and $K_{1:2}$) and the complexation efficiency (CE) of econazole/CD complexes in aqueous CD solutions with different pH at 22-23°C.

Table 3. The ^{1}H-chemical shifts of αCD or γCD alone and in the presence of econazole.

Table 4. The DLS results of aqueous solution of ECN saturated in aqueous CD solutions at 25°C±0.5°C. Data reported as the means of three determinations, the hydrodynamic diameter (*d*), intensity area (*%A*) and, mass distribution (*%M*). The samples were filtered through 0.45 μm membrane filter prior to analysis.

Table 5. The fraction of size population at different cyclodextrin solutions containing saturated econazole which pass through the various MWCO of semipermeable membranes.

Table 6. *In vitro* activities of saturated aqueous ECN/αCD solutions in different pH against important medical fungi (Mean, n=3).

Table 1 Econazole content in aqueous solution with and without 5% w/v CD at different pH after zero to three cycles of autoclaving.[a]

Sample	ECN content (mg/ml) (Mean ± S.D.)			
	0 cycle	1 cycle	2 cycles	3 cycles
No CD				
pH 3	0.402 ± 0.039	0.395 ± 0.039	0.396 ± 0.039	0.402 ± 0.042
pH 5	0.160 ± 0.034	0.159 ± 0.046	0.136 ± 0.041	0.127 ± 0.042
pH 7.5	-[b]	-[b]	-[b]	-[b]
5% w/v αCD				
pH 3	2.824 ± 0.120	2.933 ± 0.108	3.019 ± 0.064	3.000 ± 0.057
pH 5	2.072 ± 0.080	2.036 ± 0.099	2.101 ± 0.056	2.099 ± 0.063
pH 7.5	0.342 ± 0.009 [c]	0.325 ± 0.016	0.285 ± 0.035	0.279 ± 0.024 [c]
5% w/v γCD				
pH 3	0.759 ± 0.021	0.761 ± 0.015	0.761 ± 0.033	0.772 ± 0.022
pH 5	0.108 ± 0.008	0.106 ± 0.011	0.094 ± 0.064	0.089 ± 0.018
pH 7.5	-[b]	-[b]	-[b]	-[b]

[a]Each cycle consisted of heating to 121°C for 20 minutes.
[b]Could not determined (ECN conc. below LOQ).
[c]$P < 0.05$. $P < 0.05$ was considered statistically significant between 3 cycles of autoclaving and no cycle of autoclaving.

Table 2 The values of the apparent stability constants (i.e. $K_{1:1}$ and $K_{1:2}$) and the complexation efficiency (CE) of econazole/CD complexes in aqueous CD solutions with different pH at 22-23°C.

Cyclodextrin	pH	S_0 (mM)	Type	$K_{1:1}$ (M^{-1})	$K_{1:2}$ (M^{-1})	CE	Molar ratio
αCD	3	1.05	A_L	354.5	-	0.371	1:4
	5	$1.13x10^{-1}$	A_L	2597.5	-	0.293	1:5
	7.5	$1.11x10^{-3}$	A_p	870.2	15.0	0.041 [a]	1:25
γCD	3	1.05	B_s	246.7	-	0.258 [a]	1:5
	5	$1.13x10^{-1}$	B_s	1032.8	-	0.117 [a]	1:10
	7.5	$1.11x10^{-3}$	- [b]	- [b]	- [b]	- [b]	- [b]

[a]Obtained from the initial linear part of the phase-solubility diagram.
[b]Could not determined (ECN conc. below LOQ).

Table 3. The ^{1}H-chemical shifts of αCD or γCD alone and in the presence of econazole

Protons	CD	ECN/CD	Δδ*
αCD			
H1	4.984	4.955	- 0.029
H2	3.563	3.546	- 0.017
H3	3.895	3.719	- 0.176
H4	3.515	3.496	- 0.019
H5	3.794	3.822	+0.028
γ*CD*			
H1	5.043	5.016	- 0.027
H2	3.572	3.581	+0.009
H3	3.835	3.771	- 0.064
H4	3.491	3.504	+0.013
H5	3.781	3.745	- 0.027

$\Delta\delta^* = \delta_{complex} - \delta_{free}$

Table 4. The DLS results of aqueous solution of ECN saturated in aqueous CD solutions at 25°C±0.5°C. Data reported as the means of three determinations, the hydrodynamic diameter (*d*), intensity area (*%A*) and, mass distribution (*%M*). The samples were filtered through 0.45 μm membrane filter prior to analysis.

Sample	pH	Peak summary		
		d (nm)	*%A*	*%M*
αCD(%w/v)				
1	3	1.07±0.04	100	100
5		1.07±0.08	100	100
10		1.05±0.12	79.93	97.78
		2.35±0.97	20.07	2.22
1	5	1.00±0.01	100	100
5		0.97±0.01	100	95.99
		1.64±1.43	78.47	4.01
		334.03±6.13	15.77	-
10		0.97±0.02	81.00	98.37
		2.04±0.41	12.40	1.63
		464.37±157.83	6.60	-
1	7.5	1.05±0.01	100	100
5		0.96±0.02	67.37	100
		239.70±47.23	3.20	-
		518.33±87.65	29.43	-
10		0.96±0.01	51.20	96.14
		1.83±0.79	13.57	3.66
		4.83±1.49	13.67	0.20
		303.90±55.18	21.57	-
γCD (%w/v)				
1	3	1.28±0.07	100	100
5		1.15±0.11	100	100
10		1.00±0.03	97.03	100
		390.93±111.97	2.97	-
1	5	1.01±0.02	98.53	99.66
		1.66±0.63	1.48	0.34
5		1.15±0.06	91.97	98.64
		2.13±0.38	8.03	1.36
10		1.20±0.02	47.71	95.38
		2.24±0.67	14.97	4.62
		335.63±42.25	37.32	-

Table 5. The fraction of size population at different cyclodextrin solutions containing saturated econazole which pass through the various MWCO of semipermeable membranes.

MWCO (kDa)	αCD conc. (%w/v)			γCD conc. (%w/v)		
	1	5	10	1	5	10
pH 3						
(6-8)/3.5	1.37	1.45	1.75	1.19	1.34	1.38
(12-14)/3.5	1.86	2.90	2.61	1.47	1.62	1.93
pH 5						
(6-8)/3.5	1.37	1.41	1.51	1.01	1.06	1.10
(12-14)/3.5	1.55	2.56	2.64	0.53	0.16	0.28
pH 7.5						
(6-8)/3.5	1.54	1.70	1.98	-	-	-
(12-14)/3.5	2.03	2.14	3.05	-	-	-

Table 6. *In vitro* activities of saturated aqueous ECN/αCD solutions in different pH against important medical fungi (Mean, n=3).

Samples	*C. albicans*		*A. flavus*		*F. solani*	
	MIC	MFC	MIC	MFC	MIC	MFC
ECN	5.0	20.0	1.25	1.25	5.0	20.0
ECN/αCD, pH 3	5.5	43.8	0.7*	0.7*	5.5	11.0*
ECN/αCD, pH 5	5.7	45.5	0.7*	0.7*	5.7	11.0*

[a] ECN: econazole nitrate; ECN/αCD: saturated ECN in aqueous solution containing 10% w/v αCD

[b] MIC, MFC (μg/ml); * more effective than ECN itself when half strength was given.

LIST OF FIGURES

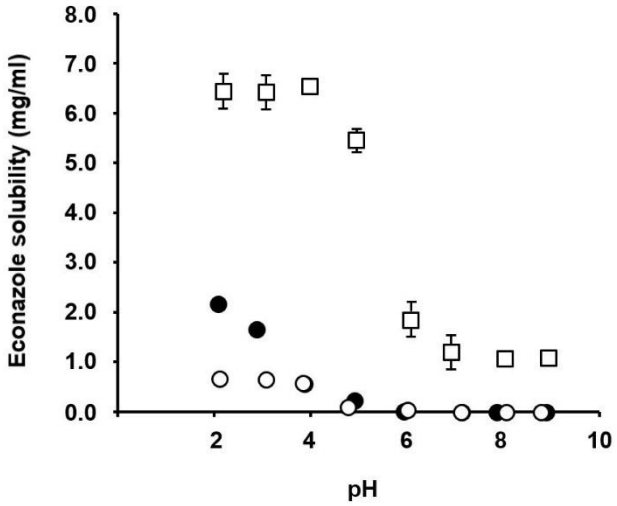
Fig. 1. The pH-solubility profiles of econazole in pure water and in aqueous solutions containing 5% w/v αCD or γCD at 22-23°C; pure water (○), αCD (□), γCD (●).

Fig. 2. The unionized and ionized form of econazole, pKa = 6.6.

Fig. 3. Phase-solubility profiles of econazole in aqueous solutions containing αCD (a) or γCD (b) at different pH (22-23°C); pH 3 (○), pH 5 (●), pH 7.5 (□).

Fig. 4. The theoretical and observed CD solubility in aqueous solution saturated with ECN at different pH. The theoretical CD concentrations means the amount of CD added to the aqueous media while the observed CD concentrations were derived from HPLC determination. Theoretical (----); observed (—); pH 3 (○); pH 5 (●); pH 7.5 (□).

Fig. 5. Permeation flux and apparent permeability coefficient (P_{app}) of econazole saturated 1, 5 and 10% w/v CD solutions at different pH through semipermeable membranes of various molecular weight cut-off (MWCO). ECN flux against CD concentrations; αCD (a); γCD (b), ECN P_{app} against CD concentrations; αCD (c); γCD (d); MWCO 3500 Da (■); MWCO 6-8,000 Da (□) MWCO 12-14,000 Da (■).



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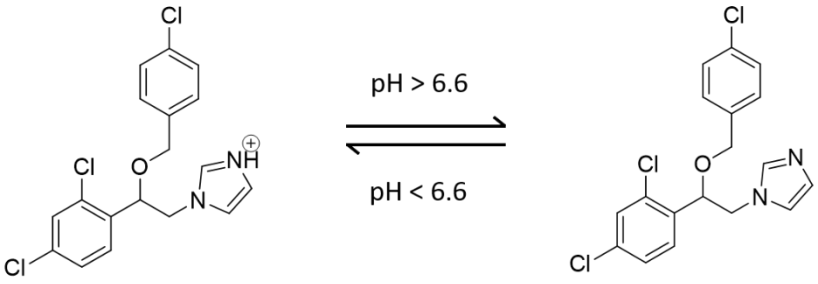

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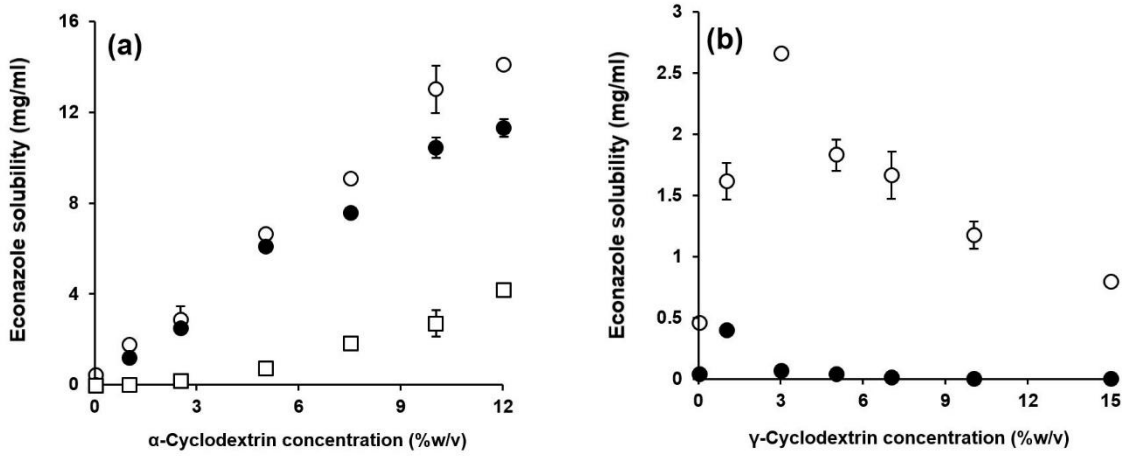


Fig. 3. Phase-solubility profiles of econazole in aqueous solutions containing αCD (a) or γCD (b) at different pH (22-23°C); pH 3 (○), pH 5 (●), pH 7.5 (□).

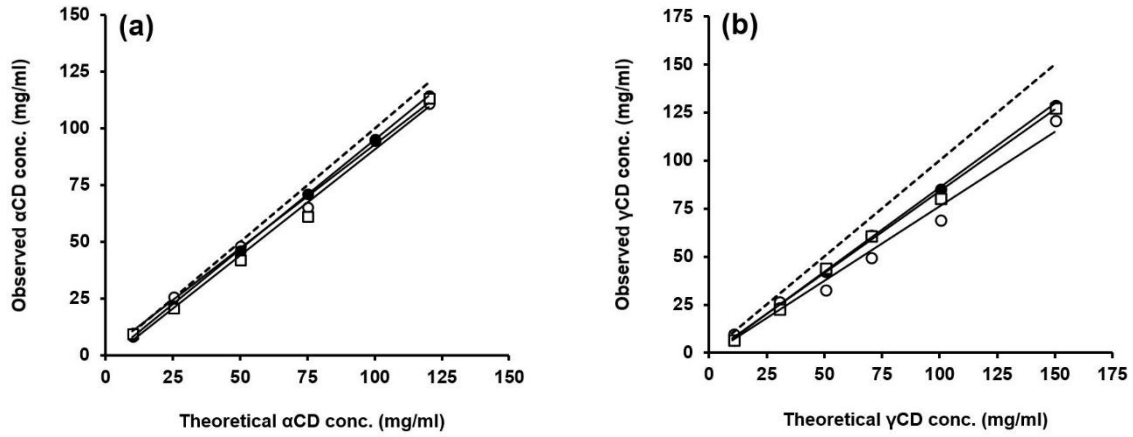


Fig. 4. The theoretical and observed CD solubility in aqueous solution saturated with ECN at different pH. The theoretical CD concentrations means the amount of CD added to the aqueous media while the observed CD concentrations were derived from HPLC determination. Theoretical (----); observed (—); pH 3 (○); pH 5 (●); pH 7.5 (□).

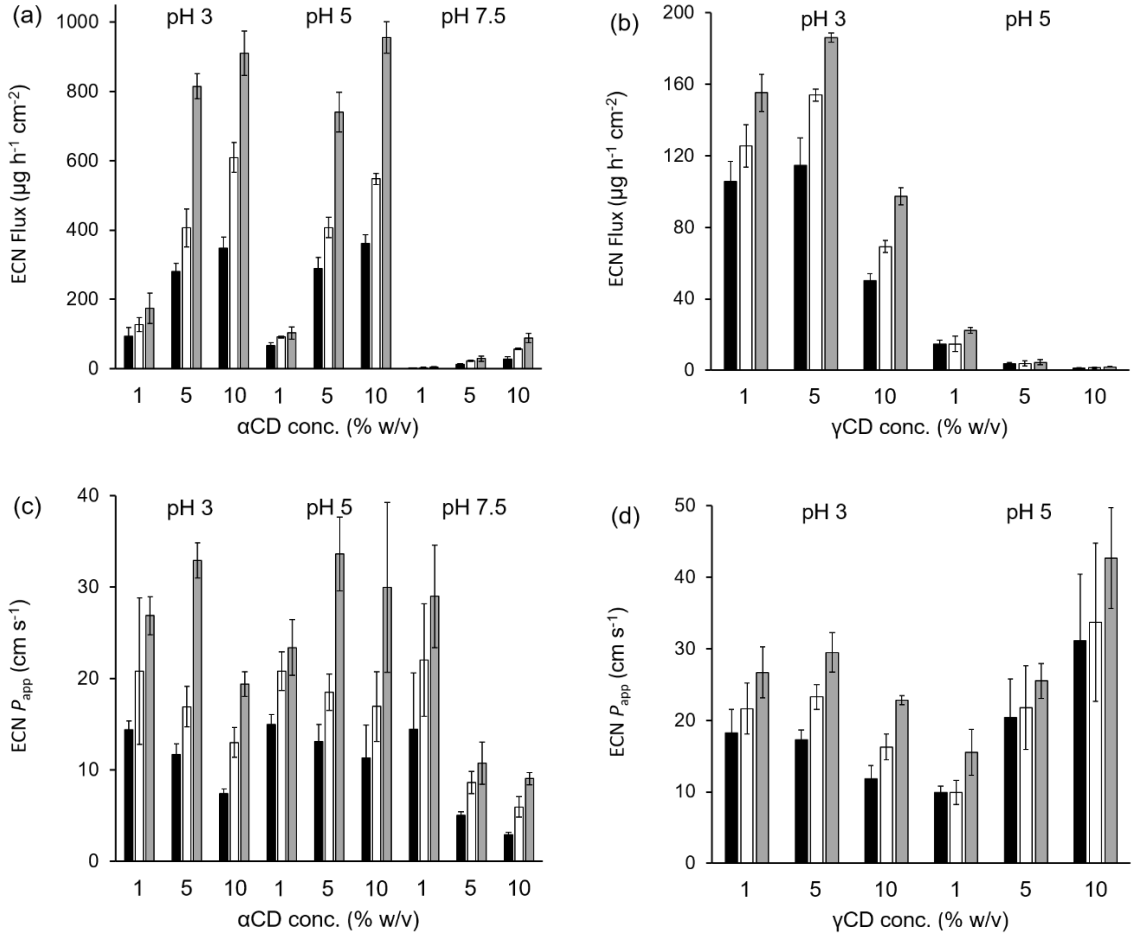
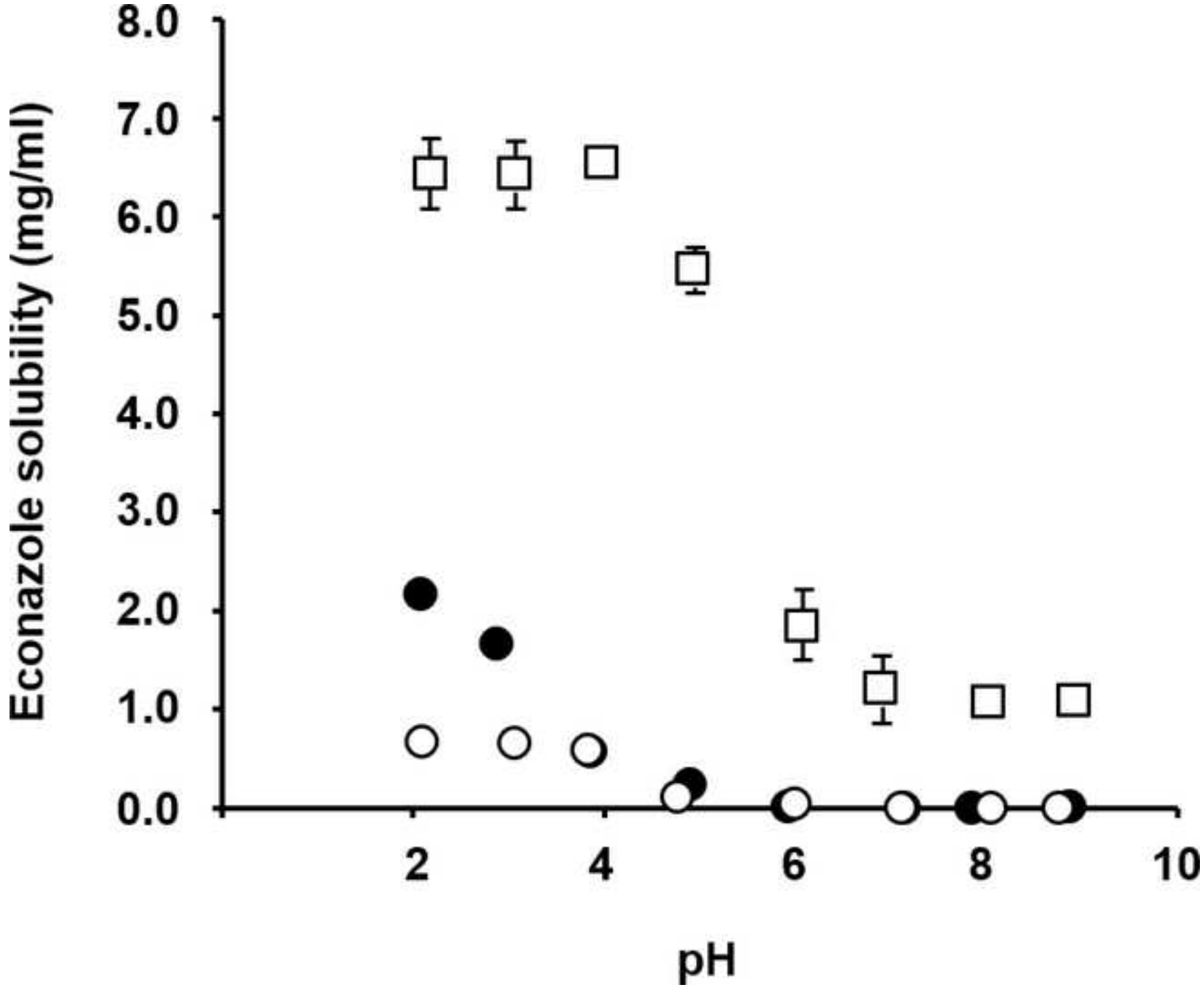
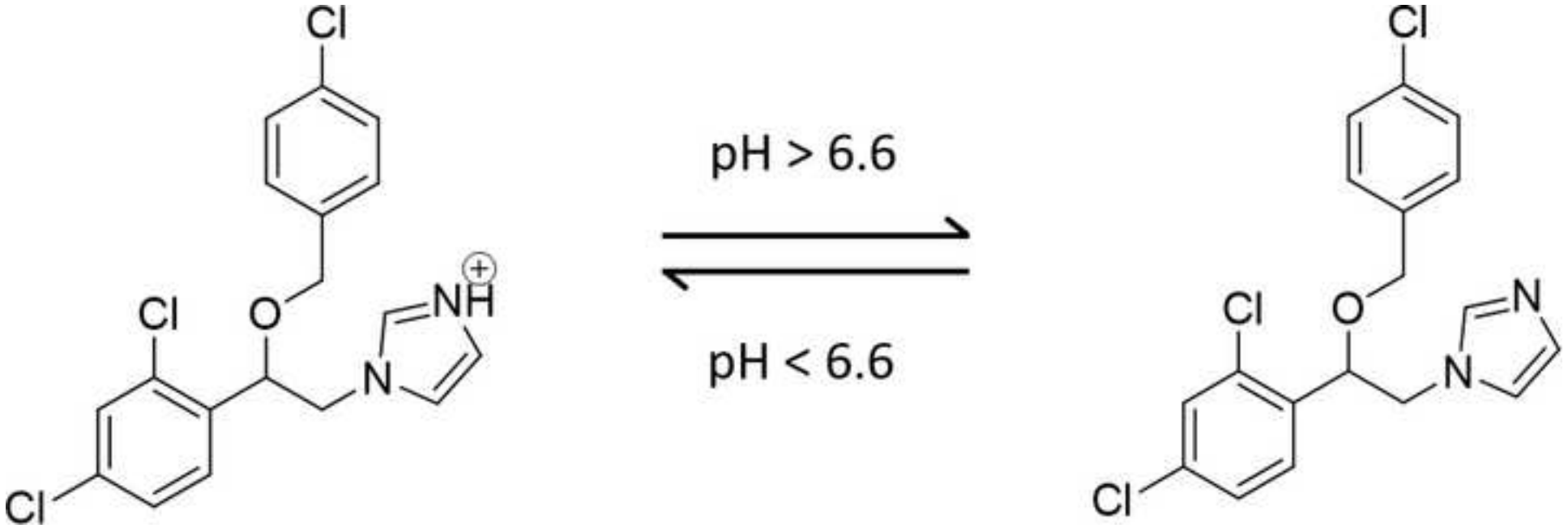


Fig. 5. Permeation flux and apparent permeability coefficient (P_{app}) of econazole saturated 1, 5 and 10% w/v CD solutions at different pH through semipermeable membranes of various molecular weight cut-off (MWCO)**.** ECN flux against CD concentrations; αCD (a); γCD (b), ECN P_{app} against CD concentrations; αCD (c); γCD (d); MWCO 3500 Da (■); MWCO 6-8,000 Da (□) MWCO 12-14,000 Da (▒)**.**

Figure(s)



Figure(s)



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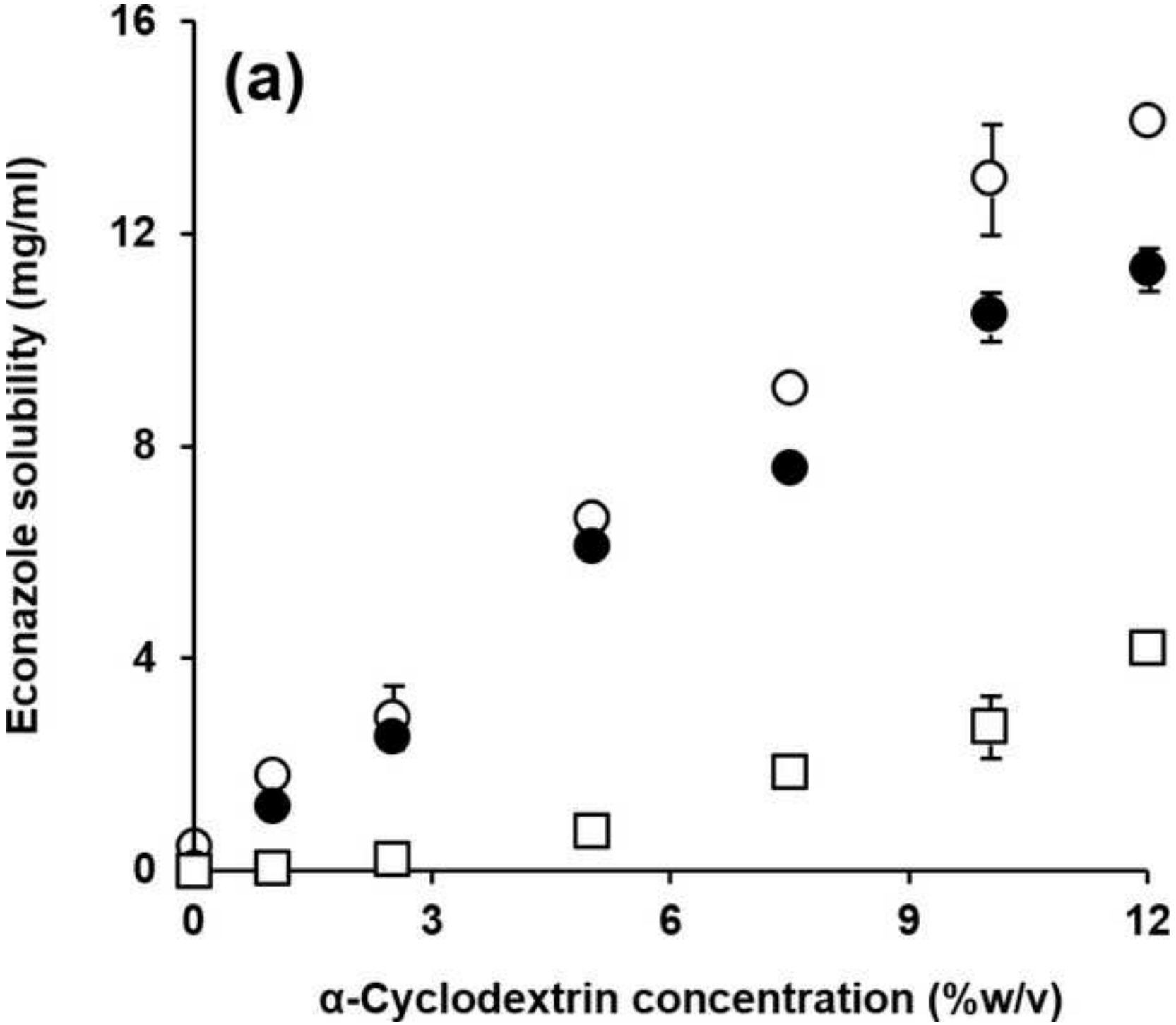
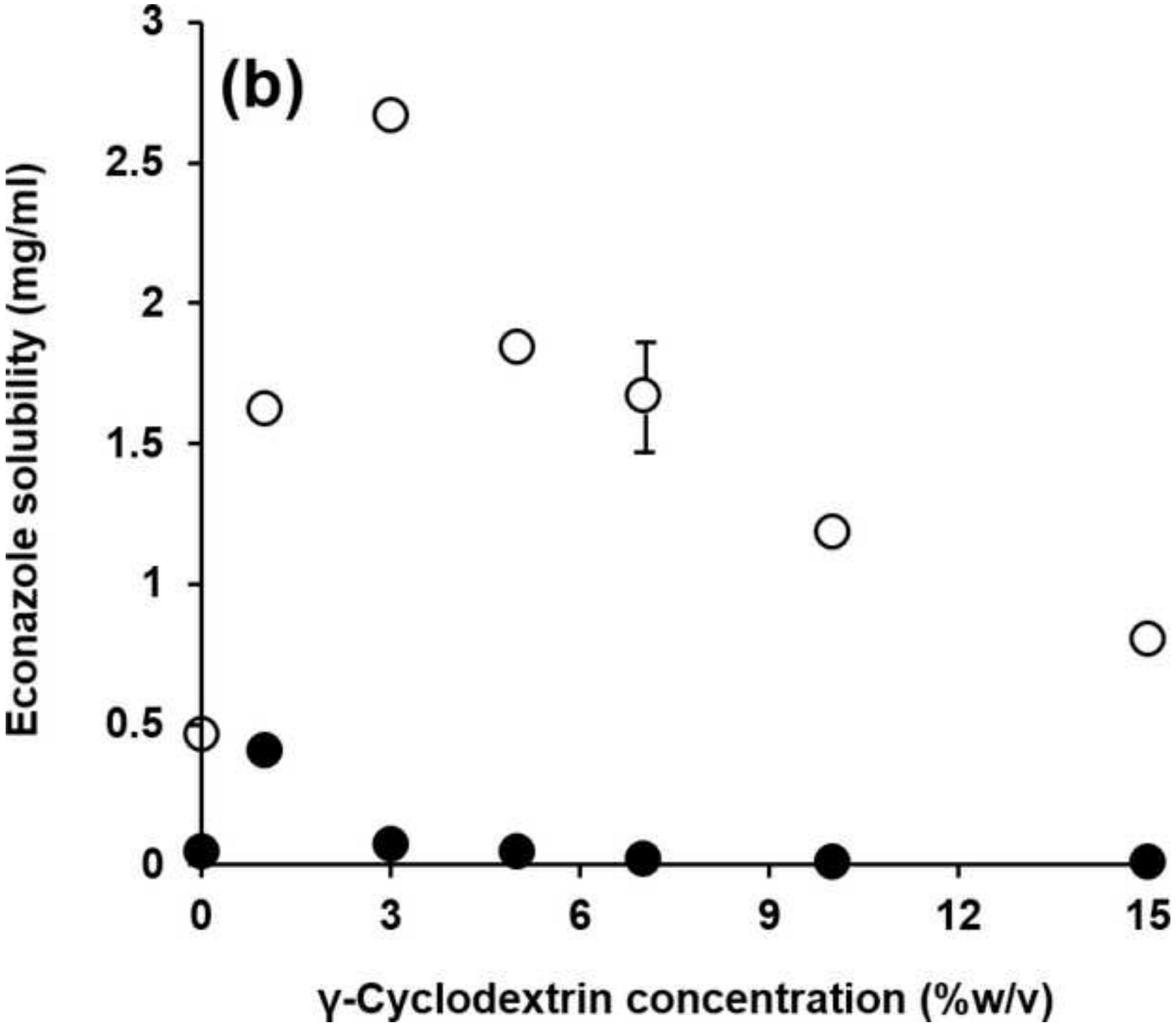
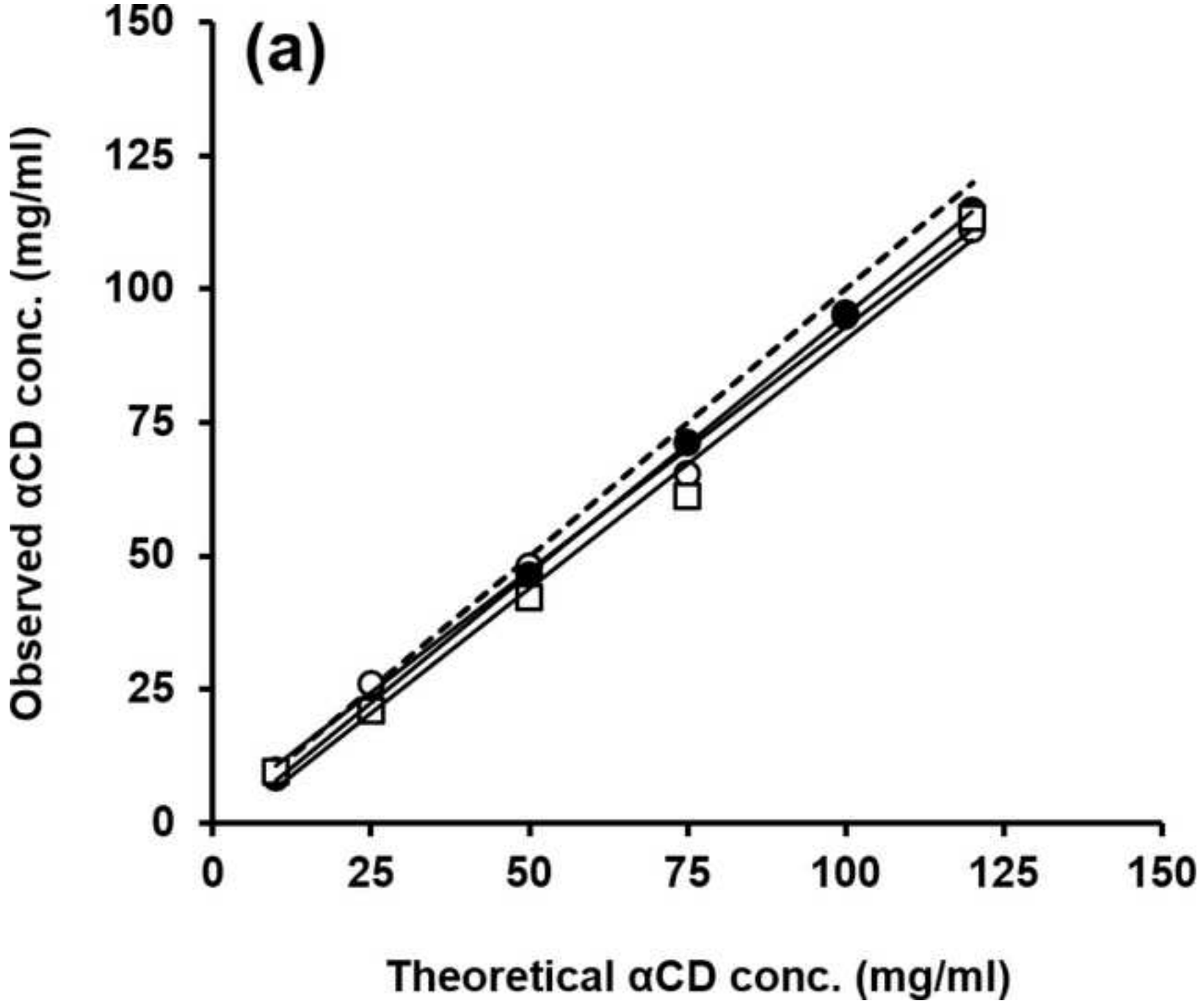

(a)
Econazole solubility (mg/ml)
16
12
8
4
0
0
3
6
9
12
α-Cyclodextrin concentration (%w/v)

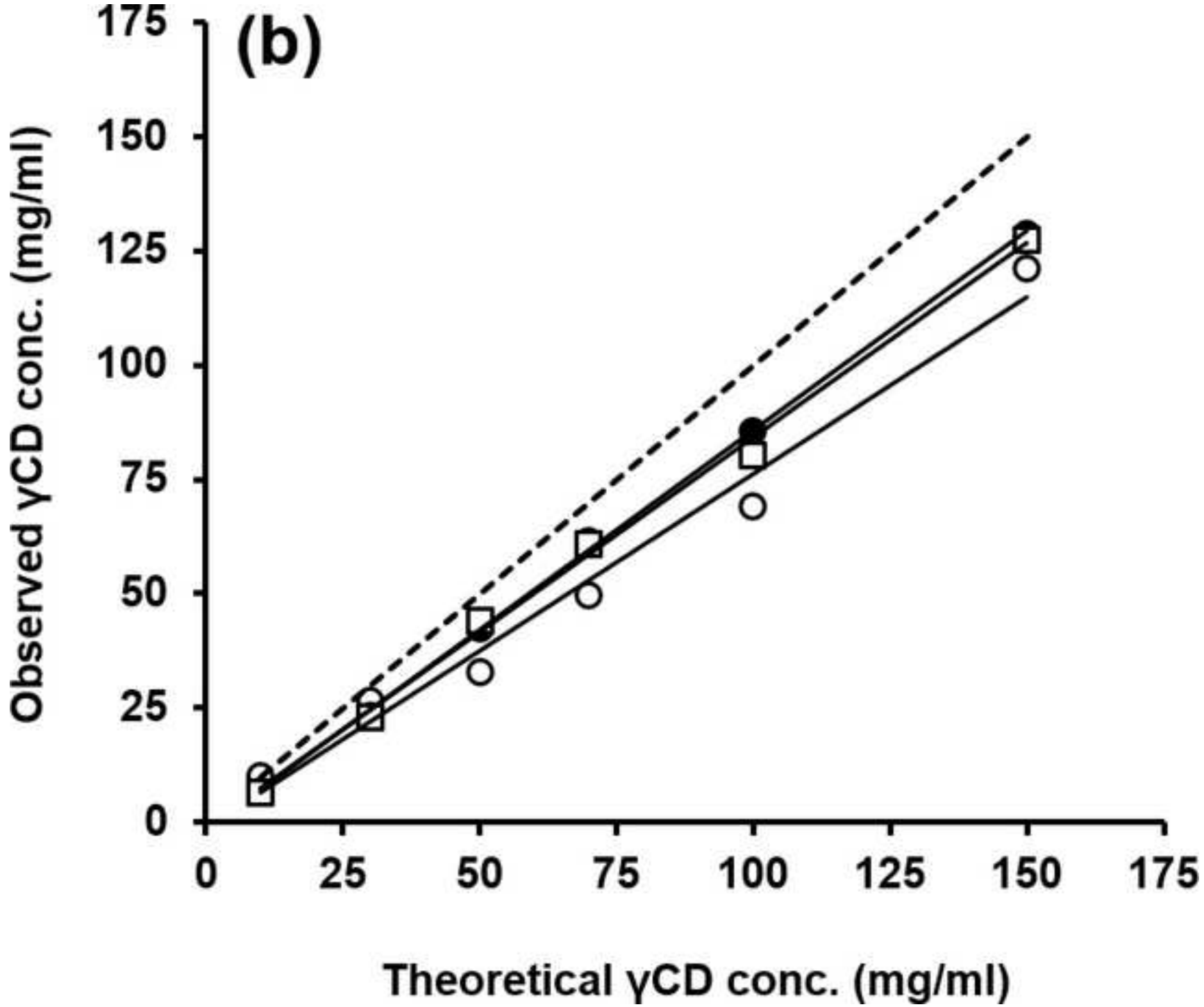
Figure(s)


(b)
Econazole solubility (mg/ml)
3
2.5
2
1.5
1
0.5
0
0
3
6
9
12
15
γ-Cyclodextrin concentration (%w/v)

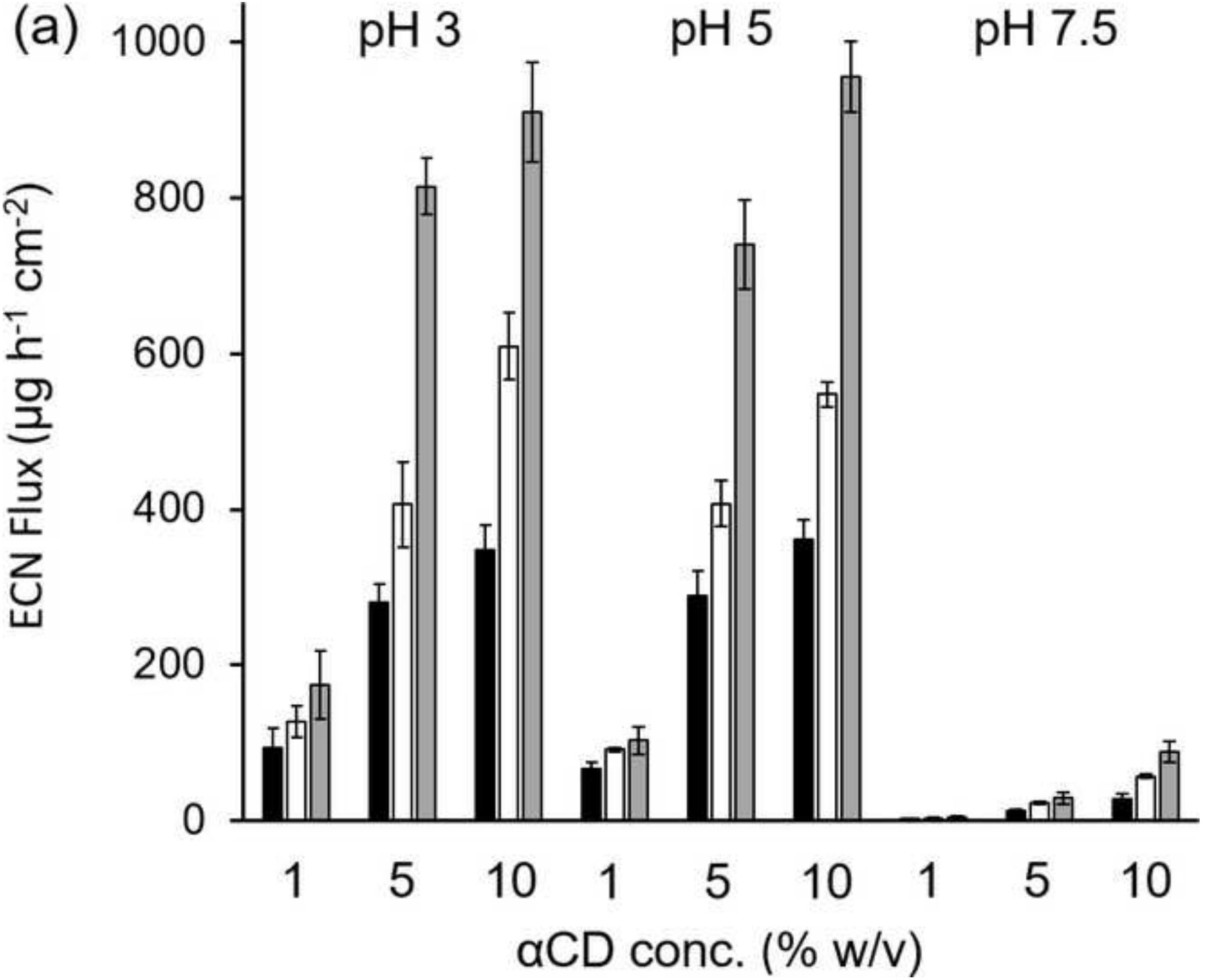
Figure(s)


(a)
Observed αCD conc. (mg/ml)
Theoretical αCD conc. (mg/ml)
0
25
50
75
100
125
150

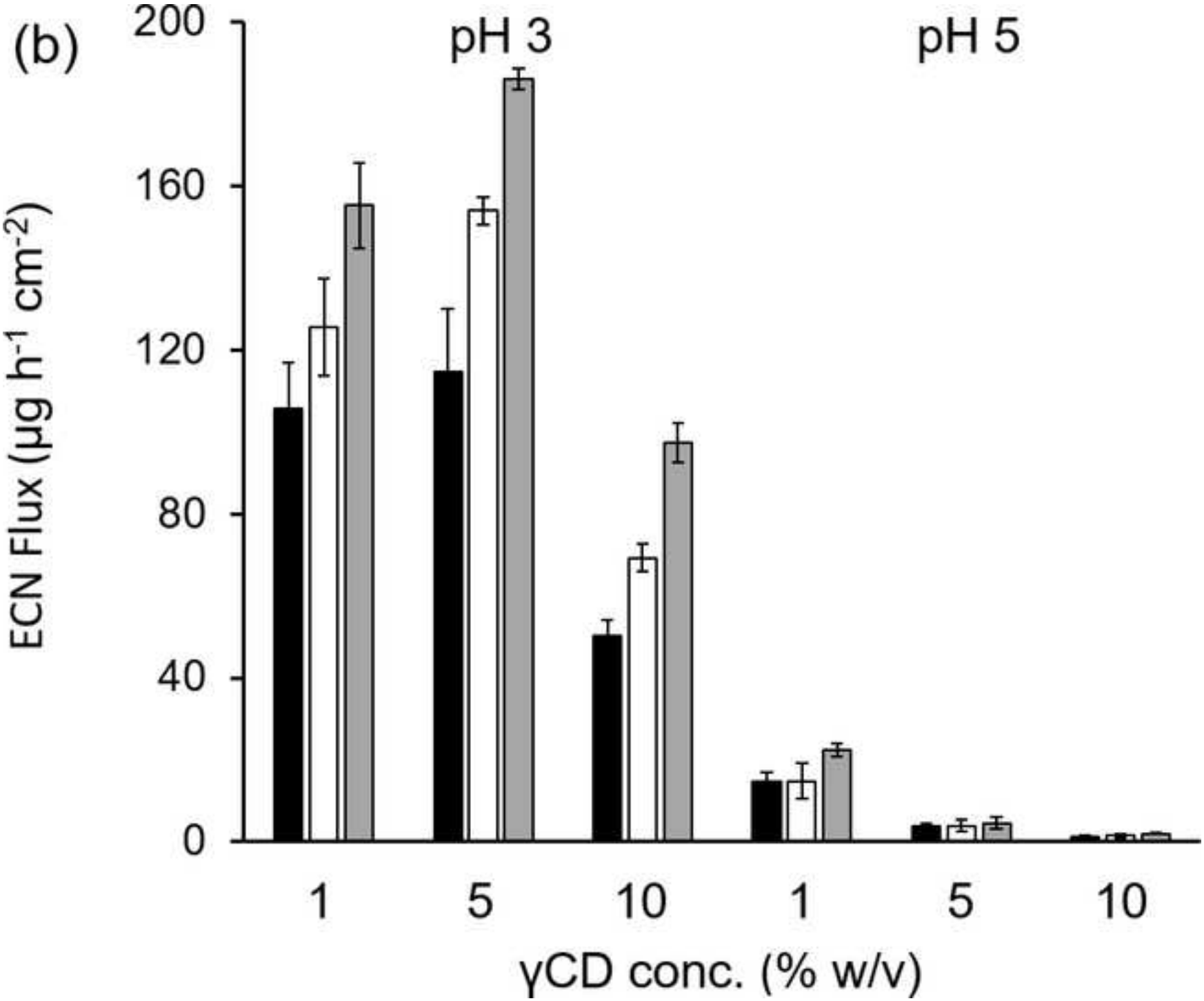
Figure(s)


(b)
Observed γCD conc. (mg/ml)
Theoretical γCD conc. (mg/ml)
0
25
50
75
100
125
150
175

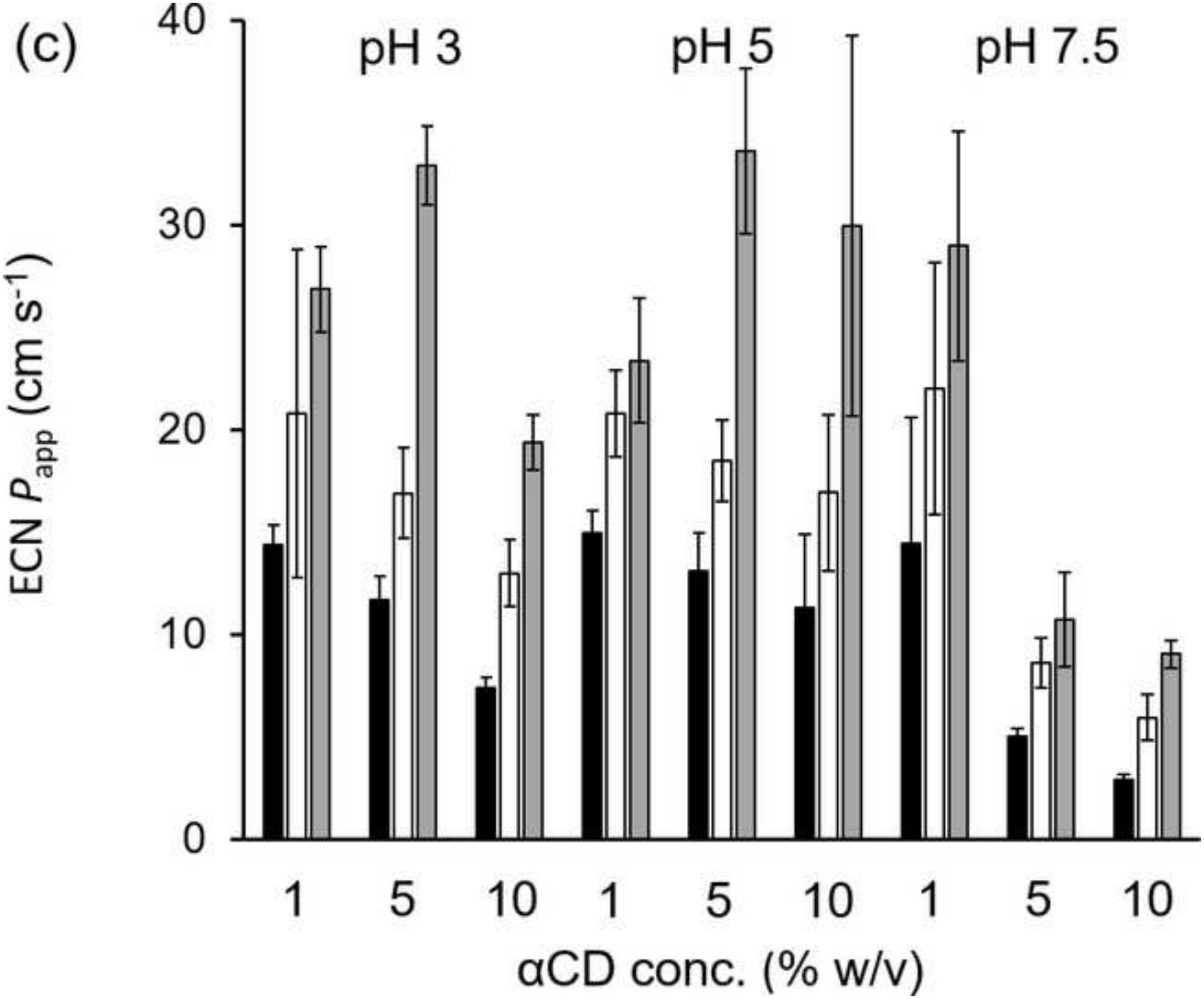
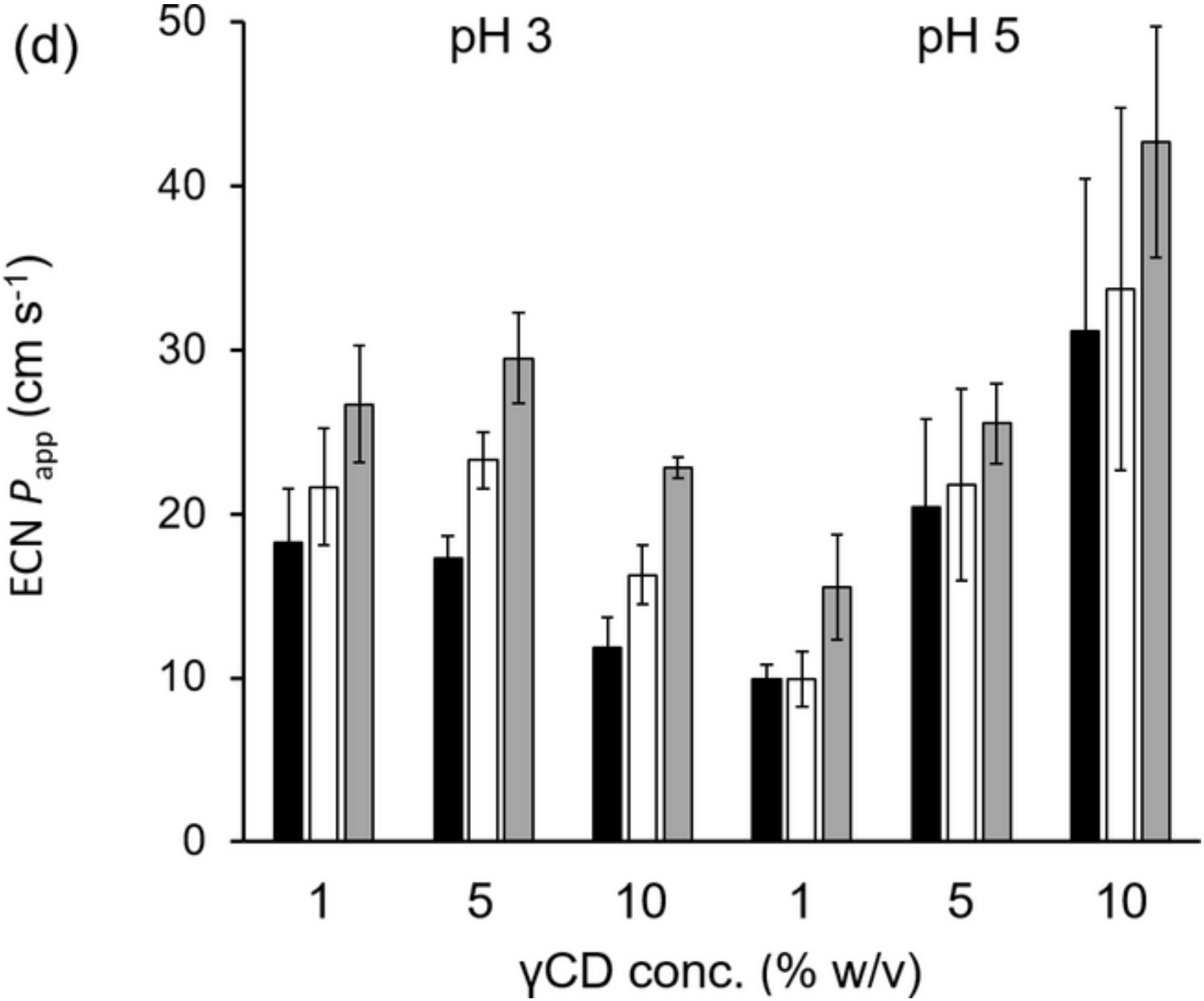
Figure(s)


(a)
pH 3
pH 5
pH 7.5
ECN Flux (µg h-1 cm-2)
1000
800
600
400
200
0
1
5
10
1
5
10
1
5
10
αCD conc. (% w/v)

Figure(s)


(b)
pH 3
pH 5
200
160
120
80
40
0
ECN Flux (µg h-1 cm-2)
1
5
10
1
5
10
γCD conc. (% w/v)

Figure(s)


(c)
pH 3
pH 5
pH 7.5
ECN P_{app} (cm s^{-1})
40
30
20
10
0
1
5
10
1
5
10
1
5
10
αCD conc. (% w/v)

Figure(s)