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Increase in aqueous solubility, stability and in vitro corneal permeability of anandamide by hydroxypropyl-β-cyclodextrin

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Abstract

Arachidonylethanolamide (AEA), an endogenous ligand for the cannabinoid receptor, has a low aqueous solubility and an instability which hinder its use in aqueous formulations. In the present study, the effect of cyclodextrins (CDs) on the aqueous solubility, stability and in vitro corneal permeability of AEA was studied. The corneal penetration of AEA in HP-β-CD formulations was investigated in vitro by using isolated corneas of rabbits. The phase solubility diagram with HP-β-CD was classified as Ap-type and stability constants ($K_{1:1}$ and $K_{1:2}$) for 1:1 and 1:2 inclusion complexes were calculated to be 39 419 M^{-1} and 12 M^{-1}, respectively. The phase solubility diagram of AEA with DIME-β-CD and HP-γ-CD were of the A_L-type, indicating the formation of 1:1-complexes. The stability constants for 1:1-complexes were 744 877 M^{-1} and 15 469 M^{-1}, respectively. The complexation of AEA with HP-β-CD markedly increased the stability of AEA. The shelf-life ($t_{90\%}$) of AEA in 10.0% HP-β-CD solution at 50°C was determined to be 166 days. The complexation of AEA with HP-β-CD increased corneal penetration of AEA compared to a suspension of the compound. Maximum permeability was achieved with the lowest HP-β-CD concentration that dissolved AEA completely. The permeability of AEA correlated well with the concentration of free AEA in solution.

Keywords: Anandamide; Cyclodextrin; Solubility; Stability; Corneal penetration

1. Introduction

Arachidonylethanolamide (AEA) was the first anandamide to be identified as an endogenous ligand for the cannabinoid receptor (Devane et al., 1992). Since 1992, several new anandamides have been synthesized and found to possess various degrees of CB1 receptor affinity (Felder et al., 1993; Hanus et al., 1993; Abadji et al., 1994; Adams et al., 1995a; Adams et al., 1995b). The pharmacological activities of anandamides are under the intensive research. AEA possesses cannabimimetic pharmacological activity (Fride and

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Mechoulam, 1993; Smith et al., 1994), including the ability to decrease intraocular pressure in rabbits (Pate et al., 1995). Anandamides are unsaturated fatty acid derivatives and show very poor aqueous solubility. So far, the low aqueous solubility of AEA has been overcome by using non-aqueous solvents (e.g. ethanol, dimethylsulfoxide and propyleneglycol), emulsifiers or mixtures of these agents (Smith et al., 1994; Adams et al., 1995a; Cabral et al., 1995; Wenger et al., 1995) which are, however, not suitable for ophthalmic formulations. In addition, AEA shows low aqueous stability which hinders its use in aqueous solutions (Cabral et al., 1995).

Cyclodextrins (CDs) are well-known for their ability to increase aqueous solubility, stability and bioavailability of many lipophilic drugs (Szejtli, 1994). In ophthalmic preparations, co-administered hydroxypropyl-β-cyclodextrin (HP-β-CD) increases the intraocular pressure lowering effect of carbonic anhydrase inhibitors (Loftsson et al., 1994c), ocular absorption of dexametasone, dexametasone acetate (Usayapant et al., 1991) and corneal penetration of diclofenac sodium (Reer et al., 1994). However, excess complexation of drugs decreases the ocular bioavailability of drugs (Reer et al., 1994; Jarho et al., 1995). It is generally assumed that only free drug, not the CD/drug complex, can penetrate across biological membranes (Nakanishi et al., 1989; Frijlink et al., 1990) and thus excess complexation of drug with CDs can decrease the bioavailability of the drug. The decreased ocular absorption of drug caused by excess formation of inclusion complexes can be hindered by increasing the viscosity of eyedrop solutions due to the fact that longer residence time of eyedrop solution will give more time for inclusion complexes to release the drug on the precorneal area (Jarho et al., 1995).

The aims of the present study were to increase the aqueous solubility and stability of AEA by CD complexation and to investigate the effect of complexation on in vitro corneal penetration of AEA. The complexation of AEA with HP-β-CD was studied by the phase-solubility method and compared to complexation with heptakis (2.6-di-O-methyl)-β-cyclodextrin (DIME-β-CD) and hydroxypropyl-γ-cyclodextrin (HP-γ-CD).

2. Materials and methods

2.1. Chemicals

Arachidonylethanolamide (Fig. 1) was obtained from Cayman Chemical (Michigan, USA). HP-β-CD (Encapsin®; mw = 1297.4, degree of molar substitution 0.40) was purchased from Janssen Biotech (Olen, Belgium), hydroxypropyl-γ-cyclodextrin (mw = 1655.1, degree of molar substitution 0.5) from Research Biochemicals International and heptakis (2,6-di-O-methyl)-β-cyclodextrin (mw = 1331.4) from Aldrich-Chemie (Steinheim, Germany). Sodium chloride and methanol (HPLC grade) were purchased from J.T. Baker (Denventer, The Netherlands), disodium phosphate dihydrate from Merck (Darmstat, Germany), hydrochloric acid from Riedel-de Haen (Seelze, Germany), sodium hydroxide from Eka Nobel AB (Bohus, Sweden) and T 61 vet. from Hoechst Veterinair GmbH (Munich, Germany).

2.2. Apparatus

Liquid chromatography (LC) was performed with a system consisting of the Beckman solvent module (Model 116), UV detector (215 nm) and System Gold data module (Beckman Instruments Inc., San Ramon, CA, USA), a Marathon autosampler equipped with column thermostat (Spark Holland, Emmen, The Netherlands) and a Rheodyne (Rheodyne, Inc., Cotati, CA, USA) 7080-080 loop injector. A deactivated Supelcosil LC8-DB (15 cm × 4.6 mm i.d., 5 μm) reversed-phase column (Supelco, Bellefonte, PA, USA) was used for the separations. Chromatographic conditions were as follows: injection volume, 20 μL; column temperature, 40°C; flow rate, isocratic at

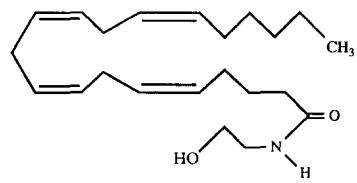

Fig. 1. Chemical structure of arachidonylethanolamide (AEA).

1.0 ml/min. The mobile phase used consisted of a 21% aqueous monobasic potassium phosphate buffer (0.02 M, pH 7.0) in methanol. An Orion SA 520 pH meter (Orion Research Inc, Boston, MA, USA) equipped with a combination pH electrode, was used for pH determinations.

2.3. Solubility studies

The complexation of AEA with various CDs was determined by using the phase-solubility method of Higuchi and Connors (1965). Excess amount of the AEA was added to phosphate buffer solutions (0.16 M, pH 7.4, ionic strength of 0.5) containing various concentrations of CDs. The CD concentration ranges were as follows: HP-β-CD (18–145 mM); HP-γ-CD (15–121 mM); DIME-β-CD (8–75 mM). The suspensions were shaken at 25°C for 24 hours and pH of each was monitored during equilibration. The pH of suspensions was adjusted to 7.4 with HCl or NaOH, if necessary. After equilibration, the suspensions were filtered through 0.45 μm membrane filters and analysed by HPLC. The solubility studies of AEA with HP-β-CD at 50°C were performed with same method. The solubility (S_o) of AEA in CD-free phosphate buffer (0.16 M, pH 7.4), was calculated as an average of six (25°C) or four (50°C) observations.

2.4. Stability studies

The stability of AEA in phosphate buffer (0.16 M, ionic strength of 0.5, pH 7.4) was studied in 2.5% and 10.0% HP-β-CD solution at 50°C, 60°C and 70°C. Solutions were prepared by dissolving 2.5 mg of the AEA in 25.0 mL of the desired CD-containing aqueous buffer. The solutions were placed in a constant temperature and the samples were taken at the appropriate intervals. The remaining AEA was determined with the HPLC method described above. The pseudo-first-order rate constants (k_{obs}) and shelf-lives ($t_{90\%}$) for the overall degradation of AEA were determined from the slopes of the linear semilogarithmic plots of remaining AEA vs. time. The degradation of aqueous AEA (CD-free) was not possible to determine due to its very poor solubility, below the sensitivity of the employed HPLC detection method.

2.5. In vitro permeability studies

The effect of HP-β-CD concentration on the permeability of AEA through isolated cornea of pigmented rabbits was studied using glass diffusion cells at 37°C. AEA (5.0 mg) was dissolved in 10.0 mL of glutathione bicarbonated Ringer's (GBR) solution (without NaCl) containing the desired amount of HP-β-CD. The pH of solutions was adjusted to 7.65 with NaOH or HCl and the solutions were made isotonic with NaCl. The rabbits (adult pigmented rabbits, weight between 2.4 and 3.7 kg) were killed by a marginal ear-vein injection of lethal dose of T 61 vet. and (within 20 min) the corneas placed between the two cylindrical compartments of the perfusion apparatus. The solution containing AEA was added to the donor cell (epithelial side of the cornea) and a similar solution, without AEA, placed in the receiver cell (endothelial side). However, the HP-β-CD concentration on the endothelial side was always at least 5% to control the solubility of penetrated AEA. Samples (200 μl) were withdrawn from the receiver side for a period of 4h. The samples were analysed by HPLC. The apparent corneal permeability coefficients of AEA with various HP-β-CD concentrations were determined by using equation 1

$$P_{app} = \frac{\Delta Q}{\Delta t \; 60 \; A \; C_o} \quad (1)$$

where Q is the total amount of AEA permeated at time t, $\Delta Q/\Delta t$ (the slope of the linear portion of the graph) is the steady-state flux of AEA to the receiver side (μmol/min), 60 is the conversion of minutes to seconds, A is the corneal surface area (1.17 cm^2) and C_o is the initial donor side drug amount. The methods used in these penetration studies have been previously described in detail (Suhonen et al., 1991).

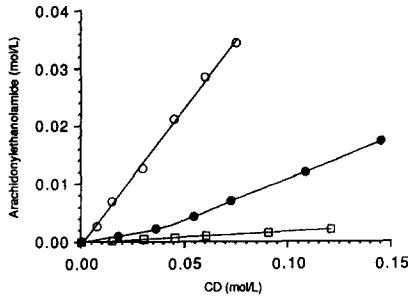

Fig. 2. The phase-solubility diagram of AEA with DIME-β-CD (○), HP-β-CD (●) and HP-γ-CD (□) at pH 7.4.

3. Results

3.1. Solubility studies

Fig. 2 shows the phase-solubility diagrams of AEA with different CDs at pH 7.4. The solubility studies showed that DIME-β-CD (10%), HP-β-CD (10%) or HP-γ-CD (10%) improves the aqueous solubility of AEA 28000-, 6000- and 800-fold at room temperature, respectively. The phase-solubility diagrams of AEA with DIME-β-CD and HP-γ-CD are of the A_L-type, indicating formation of 1:1 AEA/CD complexes at this pH and CD concentration range (Higuchi and Connors, 1965). The stability constants for 1:1 complexes ($K_{1:1}$) were calculated using equation 2

$$K_{1:1} = \frac{Slope}{[S_o](1 - Slope)} \quad (2)$$

where $K_{1:1}$ is the stability constant for the complex and $[S_o]$ is the solubility of AEA in the absence of CD. The solubility (S_o) of AEA in phosphate buffer solution (pH 7.4) at 25°C was

Table 1
The stability constants ($K_{1:1}$ and $K_{1:2}$) for 1:1 and 1:2 inclusion complex formation between AEA and HP-γ-CD, HP-β-CD or DIME-β-CD at room temperature

Cyclodextrin	$K_{1:1}$ (M^{-1})	$K_{1:2}$:(M^{-1})
HP-γ-CD	15469	—
HP-β-CD	39419	12
DIME-β-CD	744877	—

Table 2
The aqueous solubility of AEA in the presence and absence of HP-β-CD at 25°C and 50°C

HP-β-CD (g/100ml)	Solubility of AEA at 25°C(μg/ml)	Solubility of AEA at 50°C(μg/ml)
0.0	0.4	11.3
2.5	336.4	412.4
5.0	774.4	1015.4
7.5	1495.3	1854.7
10.0	2442.5	2536.9

0.413 ± 0.009 μg/mL (mean ± S.E., n = 6). This value was used in all stability constant calculations. The observed stability constants are shown in Table 1.

The phase-solubility diagram of AEA with HP-β-CD is of the A_p-type, indicating formation of 1:1 and 1:2 AEA/CD-complexes (Higuchi and Connors, 1965). The stability constants for the 1:1 and 1:2 complexes were calculated after constructing a plot by using equation 3

$$\frac{([S_t] - [S_o])}{[L_t]} = K_{1:1}[S_o] + K_{1:1}K_{1:2}[S_o][L_t] \quad (3)$$

where $[S_t]$ is the total drug concentration at total CD concentration $[L_t]$, $[S_o]$ is the solubility of AEA in the absence of CD and $K_{1:1}$, and $K_{1:2}$ represents the stability constants for the 1:1-complex and 1:2-complex, respectively.

Table 2 shows the aqueous solubility of AEA in the presence and absence of HP-β-CD at 25°C and 50°C. The aqueous solubility (S_o) of AEA increased 30-fold at 50°C (11.27 ± 2.49 μg/mL,

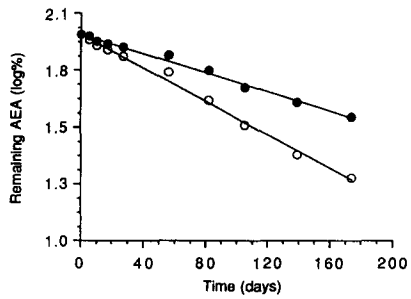

Fig. 3. First-order plots for degradation of AEA in 2.5% (○) and 10.0% HP-β-CD (●) solutions at 70°C (pH 7.4).

Table 3
Observed shelf-lives ($t_{90\%}$) for overall degradation of AEA at various temperatures in 2.5% and 10.0% solutions of HP-β-CD

HP-β-CD (g/100 ml)	Temperature (°C)	$t_{90\%}$ (Days)
2.5	70	11.3
	60	77.2
	50	138.6
10.0	70	19.8
	60	53.9
	50	165.6

Cabral et al., 1995: The half-life of AEA in aqueous solution at room temperature is 12 h.

mean $\pm$ S.E., $n = 4$) compared to the value at 25°C, but the increase of total solubility of AEA at 50°C was minor in HP-β-CD-containing solutions.

3.2. Stability studies

Fig. 3 shows the pseudo first-order plots for degradation of AEA at 2.5% and 10.0% HP-β-CD solutions at 70°C. The overall degradation of AEA followed first-order kinetics and the aqueous stability of AEA increased with increasing concentration of HP-β-CD. Table 3 shows the observed shelf-lives ($t_{90\%}$) for overall degradation of AEA at temperatures studied. Based on the linear relationship between log k_{obs} and 1/T, the half-life ($t_{1/2}$) of AEA was calculated with the Arrhenius method to be 62 years in 10% HP-β-CD solution at 25°C.

3.3. In vitro permeability studies

The apparent corneal permeability coefficients of AEA as a function of HP-β-CD concentration is shown and compared to free AEA concentration in Fig. 4. The corneal permeability of AEA increases until the maximum solubility of AEA is achieved on the epithelial side. Any further increase in CD concentration decreases corneal permeability and the concentration of free AEA.

4. Discussion

4.1. Solubility studies

These solubility studies have shown that AEA forms inclusion complexes with HP-β-CD, DIME-β-CD and HP-γ-CD. Compared to HP-γ-CD, β-CD derivatives formed more stable inclusion complexes with AEA (Table 1). This result may reflect that the size of the β-CD cavity is more suitable to form a stable inclusion complex with AEA than γ-CD. In the case of β-CD derivatives, the phase-solubility diagrams show that DIME-β-CD formed only 1:1 complexes with AEA, but HP-β-CD formed both 1:1 and 1:2 complexes (Fig. 2 and Table 1). On the other hand, AEA was found to form a much more stronger inclusion complex with DIME-β-CD than with HP-β-CD (Table 1). HP-β-CD was selected for use in stability and penetration studies because of its lower toxicity. DIME-β-CD is destructive to rabbit corneal epithelium at concentrations of 5% and 12.5% (Jansen et al., 1990) and well-known to cause a hemolytic effect (Yoshida et al., 1988), but HP-β-CD at concentrations up to 12.5% is well tolerated (Jansen et al., 1990; Freedman et al., 1993).

4.2. Stability studies

CDs are well-known to increase the aqueous stability of drugs due to formation of inclusion complexes (Loftsson, 1995). The present study shows that complexation of AEA with HP-β-CD markedly increased the aqueous stability of AEA. The half-life of AEA was calculated to be 62.5 years in 10% HP-β-CD solution at 25°C, based on the Arrhenius plot. The aqueous stability of AEA in the absence of HP-β-CD was not determined in the present study due to the very poor aqueous solubility of AEA (0.413 μg/mL), which was below the analytical sensitivity of the HPLC detector employed, but $t_{1/2}$ of AEA without CD has been reported to be about 12 h in aqueous solution (Cabral et al., 1995). In the phase-solubility studies, it was shown that HP-β-CD forms both 1:1 and 1:2

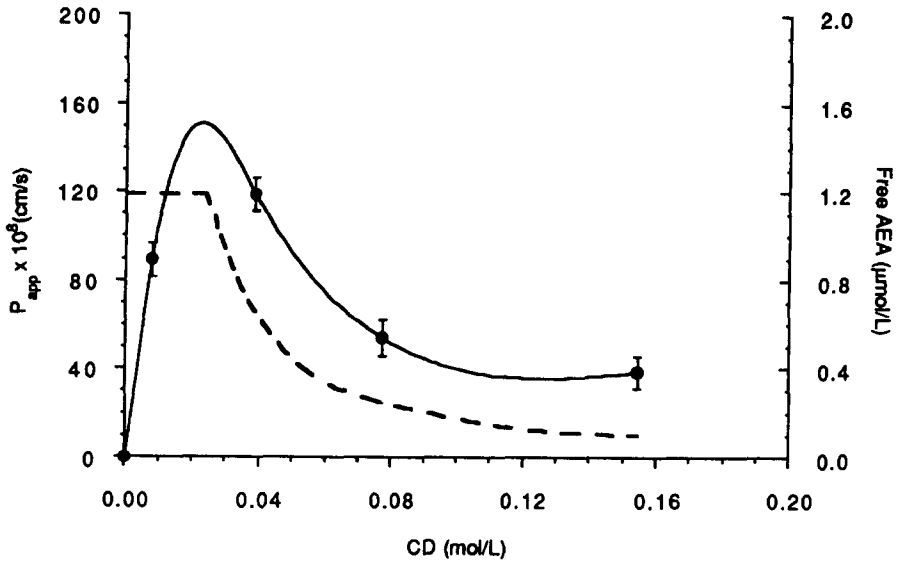

Fig. 4. The determined permeability coefficient (mean ± S.E.) of AEA through the cornea of pigmented rabbit (n = 2-6) as a function of HP-β-CD concentration (solid line) and calculated concentration of free AEA on the epithelial side as a function of HP-β-CD concentration (broken line).

inclusion complexes with AEA. The general definitions for stability constants for 1:1 and 1:2 inclusion complexes formed by substrate (S) and ligand (L) are given by the following two equations (Higuchi and Connors, 1965):

$$K_{1:1} = \frac{[SL]}{[S][L]} \tag{4}$$

$$K_{1:2} = \frac{[SL_2]}{[SL][L]} \tag{5}$$

where $K_{1:1}$ and $K_{1:2}$ are stability constants for the complexes, [SL] and [SL_2] are the concentrations of the complexes, [S] is the concentration of free substrate (AEA) and [L] is the concentration of free ligand (HP-β-CD). When total AEA and HP-β-CD concentrations are known, the concentrations of 1:1 and 1:2 complexes and free AEA in the solution can be calculated with equations 6, 7 and 8

$$[SL] = \frac{K_{1:1}[S_t][L]}{1 + K_{1:1}[L] + K_{1:1}K_{1:2}[L]^2} \tag{6}$$

$$[SL_2] + K_{1:1}[SL][L] \tag{7}$$

$$[S] = [S_t] - [SL] - [SL_2] \tag{8}$$

where [S_t] is the total AEA concentration (in the stability studies, initial concentration is 100 μg/mL). In equations 6 and 7, the assumption is made that [L] $\approx$ [L_t]. This is reasonable only if [L_t] $\gg$ [S_t]. Table 4 represents concentrations of the complexes and free AEA in 2.5% and 10% HP-β-CD solution, calculated by using values 39 419 M^{-1}, 12 M^{-1} and 0.288 mM for $K_{1:1}$, $K_{1:2}$ and [S_t], respectively. If the $t_{1/2}$ of free AEA is 12 h (Cabral et al., 1995) in aqueous solution at room temperature, the $t_{1/2}$ of AEA in 10% HP-β-CD solution can be calculated, based on concentration of free AEA, to be only 6.9 years (assuming that the degradation rate of AEA is zero within 1:1 and 1:2 inclusion complexes). The large difference in $t_{1/2}$ of AEA obtained with different methods can be explained by the relatively increased degradation rate of AEA at higher temperatures, resulting in problems for application of the Arrhenius equation. The relatively increased degradation of AEA at higher temperatures in the presence of HP-β-CD may also be due to decreased complexation of AEA

Table 4
Calculated concentrations of 1:1- and 1:2-inclusion complexes and free AEA in HP-β-CD (2.5% and 10%) solution at room temperature contaning 100 μg/mL of AEA

HP-β-CD(g/100 ml)	Free AEA (mol/l)	1:1-complex (mol/l)	1:2-complex(mol/l)
2.5	3.35×10^{-7}	2.37×10^{-4}	5.04×10^{-5}
10.0	5.97×10^{-8}	1.55×10^{-4}	1.32×10^{-4}

with HP-β-CD or a decreased relative stability of AEA within the inclusion complex. The increased aqueous solubility (S_o), but same total solubility of AEA at 50°C compared to the values at 25°C in HP-β-CD containing solutions (Table 2), suggest the decreased complexation of AEA with HP-β-CD at higher temperatures. In any case, the results show that the degradation of AEA in aqueous solution can be decreased dramatically with CDs, which may have a practical value in future AEA experiments. The HP-β-CD solution at 70°C showed a yellow/orange color after few weeks. This may indicate the degradation of HP-β-CD previously reported (Helm et al., 1994). However, the degradation of HP-β-CD was not observed to affect to the degradation of AEA.

4.3. *In vitro permeability studies*

These penetration studies indicate that the complexation of AEA with HP-β-CD increases the in vitro penetration of AEA through rabbit cornea, compared to a suspension of AEA. This result reflects the slow dissolution of free AEA from suspension compared to the fast equilibrium between complexed and free AEA in a HP-β-CD-containing solution. The results also show that increased complexation of AEA with HP-β-CD at high concentrations of HP-β-CD decreases the penetration of AEA through the cornea (Fig. 4). This result is in good agreement with earlier observations which show a CD concentration-dependent penetration of dexamethasone (Loftsson et al., 1994b) and acetazolamide (Loftsson et al., 1994c) through a cellophane membrane and of hydrocortisone through mouse skin (Loftsson et al., 1994a; Loftsson and Sigurdardottir, 1994). Fig. 4 shows the calculated concentration of free AEA on the epithelial side at different HP-β-CD concentrations. The concentration of free AEA has been calculated with equations 6, 7 and 8 by using values 39 419 M^{-1}, 12 M^{-1} and 1.439 mM for $K_{1:1}$, $K_{1:2}$ and $[S_t]$, respectively. The linear part of the diagram represents AEA vehicles where CD concentration is too low to dissolve the AEA completely (i.e. the concentration of free AEA molecules is theoretically equal to aqueous solubility (S_o) of AEA). The lowest HP-β-CD concentration (23.9 mM = 31.0 mg/mL) able to dissolve AEA completely was calculated by resolving equation 9, which can be derived from equations 4, 5 and 8 by substituting $[S] = [S_o]$.

$$K_{1:1} K_{1:2} S_O CD^2 + K_{1:1} S_O CD - S_t + S_O = 0 \quad (9)$$

The penetration of AEA through rabbit cornea is clearly dependent on HP-β-CD concentration and correlates well with the calculated concentrations of free AEA on the epithelial side (Fig. 4).

5. Conclusions

In the present study, it was found that the low aqueous stability and solubility of AEA, the first discovered endogenous cannabinoid ligand, can be increased substantially with CDs. This improved aqueous solubility also caused an increase of AEA in vitro corneal penetration. Excess HP-β-CD decreased corneal penetration of AEA due to increased complexation. In general, the results show that CDs are very useful additives for increasing the aqueous solubility and stability of anandamide.

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