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Stabilization and solubilization of difluprednate in aqueous cyclodextrin solution and its characterization for ophthalmic delivery

Manisha Prajapati, Thorsteinn Loftsson *

Faculty of Pharmaceutical Sciences, University of Iceland, Hofsvallagata 53, IS-107, Reykjavik, Iceland

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ABSTRACT

Difluprednate is a synthetic glucocorticoid used for the treatment of postoperative inflammation and pain associated with endogenous uveitis. It is very lipophilic with limited aqueous solubility and stability. The only available marketed formulation is an oil-in-water ophthalmic emulsion that has many drawbacks. Cyclodextrin (CD) molecules are widely used to increase the solubility and stability of hydrophobic drugs through the formation of drug/CD complexes. This study aims to investigate degradation kinetics, stability and solubility of difluprednate in aqueous CD solutions in an effort to develop aqueous eye drop vehicle for ophthalmic delivery. Phase-solubility and kinetics studies were performed in presence of different CDs and polymers. Characterization of the drug/CD complexes was done using techniques like NMR, DSC, and FTIR. The results show that difluprednate has maximum stability at pH 5 in aqueous CD solution. HPγCD was found to be the best solubilizer and stabilizer among all the CDs tested. The stability was further improved with the combination of HPγCD and different polymers. Characterization of the difluprednate/HPγCD complex in solid and solution state confirmed the presence of a drug/CD complex. It was possible to solubilize 0.1% difluprednate using HPγCD and stabilize the drug using combination of CD and polymer in aqueous solution.

1. Introduction

Difluprednate (difluoroprednisolone butyrate acetate or DFBA) is a synthetic glucocorticoid that is rapidly hydrolyzed to 6α,9-difluoro-11β,17,21-trihydroxypregna-1,4-diene-3,20-dione 17-butyrate, a deacetylated metabolite of difluprednate, in aqueous humor after penetration into the eye [1,2]. It is used for the treatment of post-operative inflammation and inflammation and pain associated with endogenous uveitis [3,4]. Anterior uveitis is the inflammation of the middle layer of the eye; iris and ciliary body. Difluprednate is the first strong ophthalmic steroid to be developed in recent years and the first to be approved for both postoperative pain and inflammation [3,5].

Even though prednisolone acetate (Pred Forte 1%, Allergan, USA) was the topical standard of care for the treatment of anterior uveitis, it has not shown to be effective, even with frequent dosing of 8 times per day, and not exhibited dose uniformity [6]. Difluprednate was found to be at least equivalent at half the dosing frequency required for prednisolone and to have a comparable safety profile [1]. Also, it was found to be more potent with higher specificity and better tissue penetration. Difluprednate forms a 56 times more potent metabolite than prednisolone where the affinity for the glucocorticoid receptor has been increased by the addition of two fluorines and C-17 butyrate while the addition of acetate group to the molecule enhanced tissue penetration [7]. Moreover, it was shown that difluprednate was faster at reducing all patients' symptoms such as lacrimation, ocular pain, photophobia, and blurry vision [8] and visual rehabilitation when compared to prednisolone [9].

Difluprednate has very limited water solubility (less than 1 μg/mL) [2] and stability and hence the only commercially available formulation of difluprednate (Durezol®, Alcon, USA) is an oil-in-water ophthalmic emulsion with 0.05% difluprednate [6] (Fig. 1). Although oil-in-water emulsions can overcome the problem of suspensions like flocculation, caking and poor redispersibility by solubilizing the poorly water-soluble drug in the oil phase for better ocular bioavailability, they can still undergo flocculation, creaming, and coalescence [10,11]. Similarly, lipid emulsions frequently require the addition of surfactants in high concentrations and many other excipients for improved stability due to a relatively high volume fraction of the dispersed phase. The use of ionic surfactants can lead to irritation and even produce corneal lesions on long-term use. Also, lipid emulsions can be difficult to sterilize [3,6]. All

* Corresponding author.
E-mail addresses: map52@hi.is (M. Prajapati), thorstlo@hi.is (T. Loftsson).

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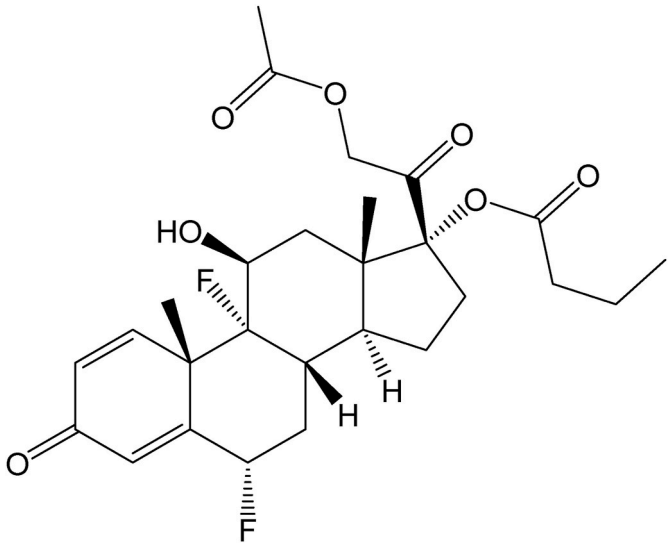

Fig. 1. Chemical structure of difluprednate.

liquid ophthalmic preparations lack a long residence time, meaning they sometimes need to be administered by the patient multiple times a day [12]. Nonetheless, the dissolved drug molecules can bypass the multiple tear layers and tissue barriers after topical administration which in turn helps the drug to reach the anterior uvea [2]. Another approach is to form simple eye drop formulation containing water-soluble difluprednate/cyclodextrin complex for enhanced solubility and ophthalmic bioavailability.

Cyclodextrins (CDs) are natural cyclic oligosaccharides with a hydrophilic outer surface and a lipophilic central cavity. They consist of α-D-glucopyranose units in the chair formation. CDs are able to form inclusion complexes with several drugs provided that their structure (or part of it) fits in the CD cavity [13]. The drug/CD complexes are readily dissociated and no covalent bonds are formed or broken during the complex formation [14]. Physicochemical properties of the drugs such as chemical stability and aqueous solubility are affected by the CD complexation process [13]. The natural CDs (i.e., αCD, βCD, and γCD) have limited aqueous solubility which limits their use as drug carriers. Hence, several hydrophilic CD derivatives have been synthesized like methylated, hydroxypropylated, and sulfobutyl ether CD derivatives [15]. These hydrophilic CD derivatives can form highly water-soluble complexes with lipophilic drugs.

The ocular barrier to topical drug delivery into the eye consists of the aqueous tear film and lipophilic epithelium and drugs permeate this barrier via passive diffusion. CDs enhance the permeation of lipophilic drugs through the aqueous tear film increasing their ocular bioavailability. In addition, CDs are able to improve the chemical stability of the drugs and reduce their local irritation [16]. Ophthalmic irritation of topically applied drugs may decrease patient compliance or, in the case of a strong irritation, may even be a reason for patients to stop their medication. The formation of drug/CD complexes is known to reduce local irritation after topical administration to the eye [17]. In rabbits, aqueous eye drop solutions containing as high as 45% of HPβCD have been found to be non-irritating [18] and were able to significantly decrease the effect of an irritant drug such as pilocarpine [19,20] and diclofenac sodium [21]. It has been shown that, in aqueous eye drop solutions, 2-hydroxypropyl-β-cyclodextrin enhances *trans*-corneal permeation of difluprednate [22].

CDs can also act as penetration enhancers while avoiding the long-term ocular toxicity that frequently is associated with traditional penetration enhancers as CDs avoid disruption of the ocular membrane barriers such as cornea and conjunctiva [23]. Application of one drop of aqueous eye drop solution containing 18% HPβCD to humans, three times a day for 28 days, was well tolerated in the eye [17]. CDs and hydrophilic CD complexes do not readily permeate lipophilic membranes and are drained via the nasolacrimal duct to the gastrointestinal tract, and are practically non-toxic due to lack of absorption from the tract [17,24]. In summary, CDs have been successfully used to formulate eye drops containing corticosteroids like dexamethasone with better ocular absorption in humans and animals compared to presently available formulations [25]. Also, they can be used at high concentrations in the formulations due to their favorable toxicological and pharmacological profiles [26].

Pre-formulation studies like evaluation of drug degradation, stability, and solubility are important during the development of pharmaceutical formulations to determine chemical degradation pathways and products as well as to estimate the product shelf-life and to avoid the toxic effects caused by the degradation products [27]. All these provide a solid foundation for stable, safe, and effective formulations. Here, we hypothesize that difluprednate can form complexes with CDs leading to better solubility and stability that allows for the preparation of the optimum ophthalmic solution.

Taking all these into considerations, the objective of this study was to investigate the solubility and chemical stability of difluprednate in aqueous CD solutions and to develop an aqueous difluprednate/CD ophthalmic formulation with the necessary characterizations.

2. Materials and methods

2.1. Materials

Difluprednate was purchased from Shanghai Huirui Chemical Technology Co., Ltd. (China). α-cyclodextrin (αCD), β-cyclodextrin (βCD), γ-cyclodextrin (γCD) and 2-hydroxypropyl-β-cyclodextrin (HPβCD) with a degree of substitution (DS) 4.2 (MW 1380) were kindly provided by Janssen Pharmaceutica (Belgium), 2-hydroxypropyl-γ-cyclodextrin (HPγCD) with DS 4.0–5.6 (MW 1540) by Chemical Marketing Concepts Europe (Netherland) and sulfobutyl ether β-cyclodextrin (SBEβCD) (sodium salt) with DS 6–7 (MW 2163) and sulfobutyl ether γ-cyclodextrin (SBEγCD) with DS 4.2 (MW 1961) by Ligand Pharmaceuticals (USA). 2-hydroxypropyl-α-cyclodextrin (HPαCD) with DS 3.6 (MW 1180) and randomly methylated β-cyclodextrin (RMβCD) with DS 12.6 (MW 1312) was purchased from Wacker Chemie (Munich, Germany). Tyloxapol reagent grade and poloxamer 407 were purchased from Sigma-Aldrich (USA), and tween 80 from Tokyo Chemical Industry Co., Ltd. (Japan). Milli-Q water was used for the preparation of all solutions and the mobile phase for UHPLC measurements. All other chemicals were commercially available products of special reagent grade.

2.2. Methods

2.2.1. Chromatographic conditions

Quantitative determination of difluprednate was performed on a reversed-phase ultrahigh-performance liquid chromatographic (UHPLC) component system from Thermo Fisher Scientific Vanquish HPLC system consisting of VF-P10-A pump, a VF-A10-A autosampler, VH-C10-A column compartment, VWD-3100 UV–Vis detector operated at 240 nm, and a Phenomenex Kinetex C18 1.7 μm 100 × 2.1 mm column with a security guard ULTRA HOLDER. The column temperature was 40 °C. The mobile phase consisted of acetonitrile (ACN) and Milli Q water containing 0.1% (v/v) ortho-phosphoric acid (50:50). The flow rate was 0.3 mL/min, sample injection volume was 5 μl and the retention time (R_t) was 3 min.

2.2.2. Buffers

Hydrochloric acid-potassium chloride buffer (pH 1–2), citrate buffer (pH 3–6), phosphate buffer (pH 7–8), and carbonate-bicarbonate buffer (pH 9) was prepared by mixing aqueous solutions of the acid with the aqueous solutions of the corresponding salt. The concentration of the

buffer salts was 0.1 M. The ionic strength of the media was not adjusted. Various amounts (expressed as % w/v) of different CDs were added to the buffer solutions when the effects of CDs were investigated.

2.2.3. Kinetic studies

The degradation was investigated by adding stock solution (100 μl) of the drug in methanol to an aqueous buffer solution (5 mL), previously equilibrated at 40 °C in a heating block and with a stirrer, and mixed thoroughly. The initial difluprednate concentration was 3.93 mM. The pH of the final reaction mixture was determined at the end of each experiment with a pH meter standardized at 40 °C. All reactions were run under pseudo-first-order conditions. Aliquots (5 μl) were injected into the column at various time intervals.

2.2.3.1. Calculation of degradation rate constant. The pseudo-first-order rate constant (k_{obs}) was determined by linear regression of the natural logarithm of the remaining drug concentration vs time plots.

The kinetic studies were performed in dilute aqueous solutions thus; it was assumed that only 1:1 complexes were formed:

$$\begin{array}{c} D = CD \overset{K_{1:1}}{\rightleftarrows} D/CD \\ \downarrow K_f \qquad\qquad \downarrow k_c \\ Degradation products \end{array} \tag{1}$$

where $K_{1:1}$ is the complex stability constant, k_c is the observed first-order rate constant for the drug degradation within the complex (D/CD) and k_f represents the observed first-order rate constant for the degradation of the free drug (D). Here D represents the drug difluprednate. The observed first-order rate constant (k_{obs}) for the drug degradation is the weighted average of k_f and k_c:

$$k_{obs} = k_f f_f + k_c f_c$$

where f_f is the fraction of drug in solution that is unbound (i.e. free) and f_c is the fraction of drug in solution that is bound in a CD complex. Further manipulation of the mathematical equations gives:

$$k_{obs} = \frac{kf + kc \,.\, K1:1\,[CD])}{(1 + K1:1\,[CD])} \tag{3}$$

where [CD] is the concentration of the free (i.e. unbound) CD in the aqueous medium. If the total CD concentration (i.e. $[CD]_T = [CD] + [D/CD]$) is much greater than the total drug concentration (i.e. $[D]_T = [D] + [D/CD]$) then $[CD] \approx [CD]_T$:

$$k_{obs} = \frac{kf + kc \,.\, K1:1\,[CD]T)}{(1 + K1:1\,[CD]T)} \tag{4}$$

Rearrangement of Equation (4) gives Equation (5):

$$\frac{1}{kf - kobs} = \frac{1}{K1:1\,(kf - kc)} \cdot \frac{1}{[CD]T)} + \frac{1}{kf - kc} \tag{5}$$

Knowing k_f, both k_c and $K_{1:1}$ can be calculated after the construction of Lineweaver-Burk plot (Fig. 4) using Equation (10). The value of k_c is obtained from the ordinate intercept and k_f, and $K_{1:1}$ is obtained by dividing the slope into the ordinate intercept.

2.2.4. Solubility studies

Solubility studies were determined by adding an excess amount of difluprednate to aqueous solutions containing various concentrations of CD at a pH of about 5 (pH of maximum difluprednate stability). The suspensions formed were sonicated in an ultrasonic bath (Edmund Buhler GmbH) at room temperature for 60 min. The vials containing these suspensions were then shaken at room temperature. After equilibrium for 7 days, aliquots were filtered through a 0.45 μm membrane filter unit (Phenomenex, UK), diluted with 50% aqueous acetonitrile solution (whenever necessary), and analyzed by UHPLC. Initial studies showed that virtually no difluprednate degraded during the solubility studies.

Higuchi and Connors (1965) have described different phase-solubility profiles: A-type phase-solubility profiles can be related to the water-soluble CD derivatives and the B-type profiles to the less soluble natural CDs [28]. The drug solubility increases with increasing CD concentration in the A-type profiles due to the formation of water-soluble drug/CD complexes. While B-type profiles are related to the formation of complexes that have limited solubility in water. For B-type profiles, an initial increase in drug solubility is observed with increasing CD concentration, then a plateau is formed, where the dissolved drug concentration is at its maximum, followed by a decrease in the total concentration of dissolved drug [29]. The most common complex observed is the 1:1 drug/CD complex where one drug molecule (D) forms a complex with one CD molecule:

$$D + CD \leftrightarrow^{K_{1:1}} D/CD \tag{6}$$

Such a 1:1 complex display A_L-type phase-solubility profiles and the stability constant of the complex ($K_{1:1}$) can be calculated from equation (7) where S_0 is the apparent intrinsic solubility of the drug in the complexation media when no CD is present. The value of $K_{1:1}$ is frequently between 50 and 2000 M^{-1} with a reported mean value of 490 M^{-1} for βCD [30,31].

$$K_{1:1} = \frac{Slope}{S_0\,(1 - Slope)} \tag{7}$$

When a drug molecule forms a complex with more than one CD molecule, a consecutive complexation is assumed, thus stability constants of higher-order complexes ($K_{1:n}$) should be calculated using a different model [32]. The most common stoichiometry is the formation of 1:2 D/CD complexes, where one drug molecule forms a complex with two CD molecules:

$$D/CD + \overset{K_{1:2}}{\rightleftarrows} D/(CD)_2 \tag{8}$$

A_P phase-solubility types are usually observed under such conditions. Equation (9), which is a quadratic model allows the estimation of both stability constants ($K_{1:1}$ and $K_{1:2}$). The value of $K_{1:2}$ is often in the range of 10–500 M^{-1} or significantly lower than that of $K_{1:1}$ [30,32].

$$[S_t] = [S_0] + K_{1:1}\,[S_0]\,[CD] + K_{1:1}K_{1:2}\,[S_0]\,[CD]^2 \tag{9}$$

Determination of the complexation efficiency (CE) can be a better alternative to $K_{1:1}$ to compare the solubilizing effect of CDs [14].

$$CE = [S_0]\,K_{1:1} = \frac{[D/CD]}{[CD]} = \frac{Slope}{1 - Slope} \tag{10}$$

The CE determination (Equation (10)) has less variation because it can be calculated from only the slope of the linear phase-solubility diagram [31–33]. And, the drug-cyclodextrin molar ratio in a particular complexation media saturated with the drug can be calculated from the CE given in equation (11) [34].

$$D : CD\ molar\ ratio = 1 : \frac{(CE + 1)}{CE} \tag{11}$$

2.2.5. Effect of polymers and surfactants on the stability of difluprednate in CD solution

Poloxamer 407, tween 80, and tyloxapol polymers were used in these studies. Aqueous solutions containing 15% (w/v) HPγCD and polymers (from 0 to 4% w/v) were prepared with difluprednate concentration of 0.1%(w/v). These solutions were subjected to one cycle of autoclaving and the remaining drug concentration was measured by using the previously described UHPLC method.

2.2.6. Characterization of drug/CD and drug/CD/polymer complexes

2.2.6.1. Preparation of inclusion complexes. Samples were prepared using a freeze-drying method [35,36]. Briefly, clear supernatant

solutions from phase solubility studies of difluprednate in HPγCD solutions, that had displayed A_L-type profiles and solutions containing difluprednate, HPγCD, and poloxamer 407 were used to confirm the presence of difluprednate/CD complexes in the binary and ternary system. 10 mL of the samples were placed in the vials and freeze-dried at −55 °C for 3 days in a Snijdersscientific 2040 Freeze dryer (Snijders Labs, Tilburg, The Netherlands).

2.2.6.2. H NMR spectroscopy. Solutions of the pure compounds (i.e. difluprednate and HPγCD), difluprednate/HPγCD, and the difluprednate/HPγCD/poloxamer complexes were prepared by dissolving the freeze-dried solid complex in D_2O. Their spectrum and chemical shift values were recorded by using a 400 MHz 1H NMR spectrometer (BRUKER™ model AVANCE III HD, Brucker Biospin GmbH, Karlsruhe, Germany). The resonance at 4.8000 ppm, due to residual solvent (D_2O), was used as internal reference. ^{1}H NMR chemical shift change (Δδ*) was calculated as

$$\Delta\delta = \delta_{complex} - \delta_{free} \quad (12)$$

2.2.6.3. Fourier transform infra-red (FTIR) spectroscopy. The FTIR spectra of pure difluprednate, pure CD, and their freeze-dried complexes were measured with an FTIR spectrometer (Thermo Fisher Scientific model Nicolet iS10, Waltham, MA, USA) using an Attenuated Total Reflectance (ATR) technique. Data were obtained in the range of 500–4000 cm^{-1}. Analyses were performed at room temperature.

2.2.6.4. Differential scanning calorimetry (DSC). The DSC thermograms of pure difluprednate, pure CD, and their freeze-dried complexes were recorded on a Netzsch DSC 214 polyma (Netzsch GmbH, Germany). Samples were weighed in an aluminum closed pierced crucible and an identical empty was one used as reference. Using a constantly purged nitrogen atmosphere, samples were heated up at a rate of 10 °C/min over 25–200 °C temperature range.

2.2.6.5. NanoSight wave for particle size measurement. The laser-based light scattering analysis of difluprednate/HPγCD and difluprednate/HPγCD/poloxamer aggregate particles was performed with NanoSight NS300 (Malvern, Worcestershire, UK), fitted with an O-ring top-plate. Nanoparticle tracking analysis (NTA) software was used to capture images and process data, representing the concentration, size distribution, and intensity of particles in the sample. Sample measurement was done in static mode using a capture time of 60 s and five repeats. The camera level was adjusted to 11 so that all particles were visible. The same camera level was used for all the samples. A suitable detection level was selected for data analysis to limit the detection of non-particles and was between levels 4 and 12. The result for each sample was based on the average of five measurements obtained from the NTA and represented by the average particle concentration, average particle size (i.e., mean size), and mode size (i.e., the size that displays the highest peak).

2.2.6.6. Transmission electron microscope (TEM) analysis. The morphology of difluprednate/HPγCD and difluprednate/HPγCD/poloxamer nanoaggregates was studied visually by TEM. Samples were prepared using 4% of uranyl acetate as a negative staining agent. Firstly, 3 μl of each sample was loaded into a coated grid on Parafilm® located inside a petri dish and left to dry for 30 min at 37–40 °C. After centrifugation of uranyl acetate at 10,000 rpm for 5 min, a drop of 26 μl of the dye was transferred to another petri dish containing a Parafilm® flip-loaded grid onto uranyl acetate and left for 5 min. The excess dye was removed and the grid was dried with filter paper and left at room temperature for 12 h. Finally, the samples were analyzed using a Model JEM 1400 TEM (JEOL, Tokyo, Japan).

3. Results and discussion

3.1. Effect of pH on difluprednate degradation in cyclodextrin solutions

The influence of pH on the degradation of difluprednate in aqueous 5% (w/v) HPβCD buffer solutions was investigated over the pH range of 1–9. The initial concentration of the drug was 3.93 mM. The ionic strength of the buffer solutions was not controlled. The degradation was shown to follow pseudo-first-order kinetics in aqueous buffer solutions at constant pH and temperature. A linear relationship was obtained in all cases between the logarithms of the remaining drug concentration and time. The slope obtained from each of these plots is the observed drug degradation rate constant (k_{obs}) at a given pH value (Fig. 2).

The pH-rate profile for the observed first-order degradation of difluprednate in an aqueous solution containing 5% (w/v) HPβCD at 40 °C is shown in Fig. 3.

The drug degradation in CD solution was found to be sensitive to the medium acidity as shown in the pH rate profile in Fig. 3. The pH rate profile was consistent with a V-shaped profile in the pH range of 1–9. The maximum stability was obtained at pH 5 with the sharp inflection below or above pH 5. The drug (pka 13.55) is in its unionized form at all pH values tested.

While at lower and higher pH values, the slope of the curve markedly increased, suggesting efficient catalysis by hydronium ion at low pH and by hydroxide ion at high pH and the experimental results could be fitted to the following equation:

$$k_{obs} = k_H[H^+] + k_o + k_{OH}[OH^-] \quad (13)$$

where k_H is the second-order rate constant for specific acid catalysis, k_o denotes the first-order rate constant for solvent catalysis (also called non-catalysis), and k_{OH} is the second-order rate constant for specific base catalysis. k_H is dominant below pH 4 where the slope of the curve is negative and k_{OH} dominant pH values greater than 6 where the slope of the curve is positive, and k_o is dominating at pH between 4 and 5. These three rate constants were determined to be $k_H = 0.109\ M^{-1}\ h^{-1}$, $k_{OH} = 2.6 \times 10^4\ M^{-1}\ h^{-1}$ and $k_o = 2.94 \times 10^{-5}\ h^{-1}$, and inserting these values into equation (13) gives:

$$k_{obs} = 0.109\ [H^+] + 2.6 \times 10^{-5} + 2.6 \times 10^4\ [OH^-] \quad (14)$$

3.2. Effects of different cyclodextrins on difluprednate stability and solubility

3.2.1. Stability studies with different cyclodextrins

The pH rate profile above in aqueous HPβCD solutions showed that difluprednate is most stable at a pH of about 5 with a higher degradation rate both below and above pH 5. To test the effect of different CDs on the stability of difluprednate in aqueous solutions, we calculated the observed rate constant under both acidic (pH 1) and basic (pH 9) conditions.

Observed rate constants, acid-catalyzed rate constant, and basic-catalyzed rate constant, were calculated for each CD at 30 mM concentration except for βCD (maximum solubility used, equivalent to 13.2 mM). Different rate constant values with k_H and k_{OH} are listed in Table 1.

All the CDs were able to stabilize difluprednate in aqueous solution except γCD in basic media where the rate constant is almost 3 times higher than the observed rate constant when no CD is present. It seems like γCD is catalyzing difluprednate degradation under basic conditions which is supported by a very high k_{OH} value.

HPγCD gave the lowest k_H value followed by the natural γCD, and HPβCD gave the lowest k_{OH} value meaning that the HPγCD was able to better stabilize the drug under acidic conditions and HPβCD under basic conditions. Then, the value of kc (degradation constant from within the CD complex) for these two CDs under acidic and basic conditions was

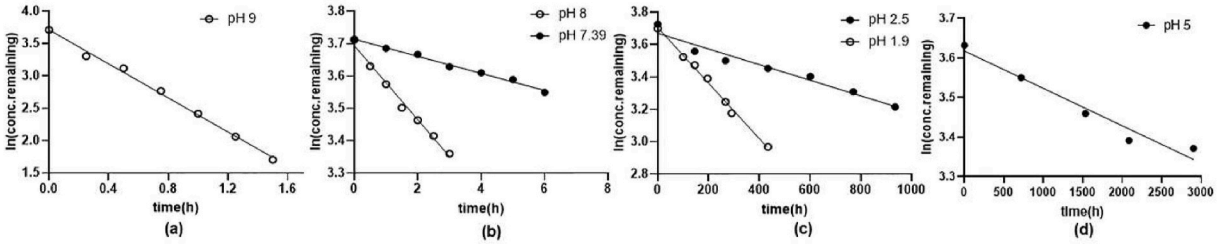

Fig. 2. Representative first-order plots (ln (drug concentration remaining) against time) for the degradation of difluprednate in aqueous 5% (w/v) HPβCD solution at 40 °C: (a) pH 9, (b) pH 8 and pH 7.39, c) pH 2.5 and pH 1.9 and, d) pH 5.

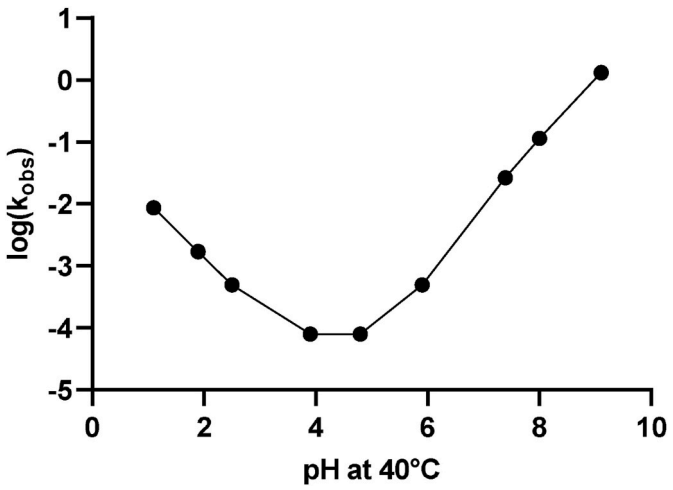

Fig. 3. pH rate (k_{obs} h^{-1}) profile for difluprednate in 5% (w/v) HPβCD solution at 40 °C.

calculated using the Lineweaver plot given by equation (5).

The value of k_c is lower than that of k_f at all conditions for both CDs suggesting that the degradation within the complex is slower in all cases. In basic media (pH 9), difluprednate degraded more than 3- and 1.2-fold slower within HPβCD and HPγCD complex, respectively, but $K_{1:1}$ for the difluprednate/HPβCD complex is 14-fold higher than that of the difluprednate/HPγCD complex. In acidic media (pH 1), difluprednate was degraded more than 1.4-fold slower within HPβCD complex and more than 3-fold slower within HPγCD complex shown by the higher k_f/k_c and stability constant values for HPγCD (Table 2). The stabilizing effect of CDs depends on both the k_f/k_c ratios and the value of $K_{1:1}$. Under acidic conditions, HPγCD is clearly the best stabilizer but under basic conditions, HPβCD can offer better stability as observed (i.e., the k_{obs} values) in Table 1. Since our drug is most stable at pH 5 (i.e. under acidic conditions), HPγCD seemed like a better host for difluprednate in terms of stability.

3.2.2. Phase solubility studies

Different CDs were used to determine the solubility of difluprednate in aqueous solutions by the phase-solubility method of Higuchi and Connors (1965) [28]. Our preliminary studies revealed that the drug degraded during the autoclaving process so the sonication method was used for the phase solubility studies. The solubility studies were carried out by sonicating the aqueous CD media containing an excess of difluprednate for 60 min and equilibration in a rotary shaker at room temperature for 7 days. Fig. 4 shows the phase-solubility diagrams of

Table 1
Observed rate constant (k_{obs}), acid-catalyzed rate constant (k_H), and basic-catalyzed rate constants (k_{OH}) of difluprednate in different CDs at 30 mM concentration, except βCD at 13.2 mM.

Samples	Acidic conditions			Basic conditions		
	pH at 40 °C	k_{obs} (h^{-1})	k_H (M^{-1} h^{-1})	pH at 40 °C	k_{obs} (h^{-1})	k_{OH} (M^{-1} h^{-1})
No CD	1	0.028	0.28	9	2.70	91,000
HPβCD	1.06	0.022	0.25	9.07	1.24	35,200
HPγCD	1.04	0.017	0.18	9.13	2.49	62,400
ACD	1.01	0.026	0.26	9.09	1.85	51,000
BCD	1.00	0.025	0.26	9.11	1.67	43,800
ГCD	1.01	0.021	0.21	9.09	7.25	200,000

Table 2
Values of k_c, k_f, and $K_{1:1}$ of difluprednate at 40 °C and pH 1 and 9 in aqueous CD solutions.

PH	1		9	
CD	HPβCD	HPγCD	HPβCD	HPγCD
k_f (h^{-1})	0.028	0.028	2.70	2.70
k_c (h^{-1})	0.019	0.009	2.40	0.85
k_f/k_c	1.4	3.1	1.2	3.2
$K_{1:1}$ (M^{-1})	18.16	52.73	193.17	13.88

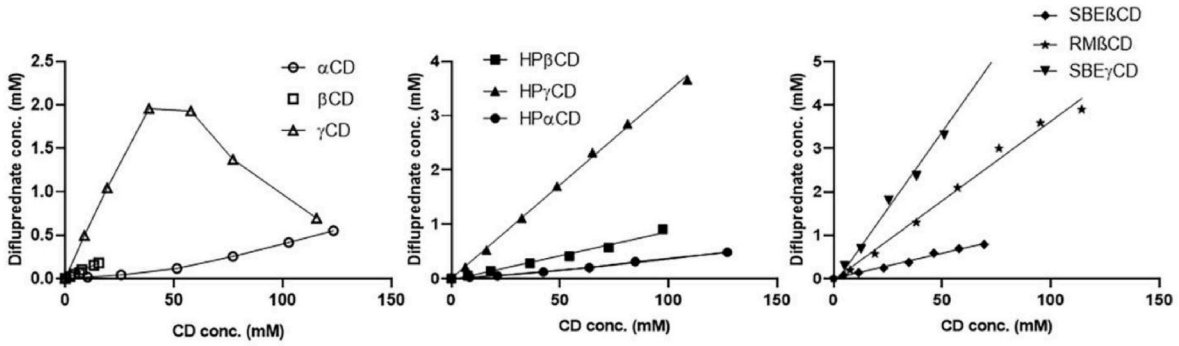

Fig. 4. Phase-solubility diagrams of difluprednate in CD in pure water at room temperature (pH about 5). Each point represents the mean of triplicate experiments. Key: (○) αCD; (□)) βCD; (△) γCD; (■) HPβCD; (●) HPαCD; (▲) HPγCD; (*) RMβCD; (▼) SBEγCD and (◆) SBEβCD.

difluprednate in various CD solutions in pure (i.e. unbuffered) water at pH 5 at room temperature.

Difluprednate has very low water solubility (less than 1 μM in water at 25 °C) [37]. We observed three different types of phase-solubility diagrams, that is A_L-type diagram where a strict linear relationship is observed, the A_P-type diagram with positive deviation from the linearity, and the B_s-type diagram where the solubility of drug/CD complex is limited in the aqueous media. The distinguish between the two A-types was done by comparing the correlation coefficient values (r^2) where the solubility curves with r^2 values greater than 0.99 is regarded as A_L type and less than 0.99 is regarded as A_P [31]. The stability constants $K_{1:1}$ and $K_{1:2}$ were calculated using equations (7) and (9), respectively.

Only αCD and HPαCD showed A_P-type diagrams with r^2 value less than 0.99 while all the other CDs except γCD showed A_L-type solubility curves with r^2 value greater than 0.99. The phase solubility profile of γCD with difluprednate showed a B_s–type diagram where the drug/CD complex has limited solubility in an aqueous medium. The solubility of difluprednate increases with an increase in γCD concentration until 5% (w/v) and started decreasing after that, hence the linear part of the curve was used to calculate the slope and r^2 values for γCD.

The analysis of the 1:3 and 1:4 (guest: host) inclusion models gave negative values for the stability constants suggesting that difluprednate predominantly forms 1:1 and 1:2 complexes with the CDs tested under mentioned conditions. The stability constants and the complexation efficiency (CE) of these CDs are listed in Table 3. These values are obtained from phase-solubility curves at room temperature where aqueous CD solutions are saturated with the drug.

γCD had the highest stability constant among the natural CDs signifying that the γCD cavity was of an appropriate size but the solubility decreases gradually at γCD concentration above 5%(w/v). This can be explained by the aggregation properties of the γCD where the γCD molecules and drug/γCD complexes self-assemble to form aggregates that precipitate from the solution [38].

The hydroxypropyl derivatives of βCD and γCD have inferior stability constant compared to the corresponding natural CDs. The reduced ability of these hydroxypropyl derivatives might be due to the steric hindrance of the substituent groups at the CD cavity. RMβCD has comparatively high stability constant. This can be explained by the increased hydrophobic cavity of the CD upon methylation of the OH-groups [39]. All the βCD derivatives give A_L-phase-solubility diagrams like the natural parent βCD. Finally, the difluprednate/SBEγCD complex has the highest stability constant among all the CDs tested.

Since the determination of stability constant values is strongly affected by the accuracy of the intercept (S_{int}) and intrinsic solubility (S_o) obtained from the phase-solubility plots (theoretically S_{int} should be equal to S_o) the use of complexation efficiency (CE) to compare the solubilizing potential of different CD can be a better approach [29,31,33]. In this study, the CE values are higher for γCD and its derivatives compared to other CDs with the highest value obtained for SBEγCD; meaning that this CD has the greatest solubilizing effects on difluprednate. This in turn relates to the stability constant values we have obtained in the study.

On the contrary, it should be mentioned that although sulfobutylated βCD and sulfobutylated γCD can be better solubilizers on a molar basis they have much higher molecular weight than the other CD derivatives tested and, thus, the difference will be much less on a weight basis. Furthermore, these CDs have numerous anionic moieties (i.e., sulfate groups) therefore will have a significantly greater effect on the osmolality than the unionized CD derivatives. 10% SBEβCD in water is about 286 mOsm and at concentrations above this will be hypertonic [40]. As a result, SBEγCD was not regarded for additional studies even though it has higher solubilizing power.

From the stability studies, it was evident that HPγCD was the best stabilizer under acidic conditions and the drug showed maximum stability at pH 5. Also, HPγCD was able to solubilize the drug better leading to the solubility of 3.5 mM at 15% (w/v) HPγCD. Hence, further studies were conducted with difluprednate and HPγCD.

Table 3
Stability Constants ($K_{1:1}$ and $K_{1:2}$) and complexation efficiency(CE) of difluprednate/CD complexes in pure water at room temperature (pH about 5).

System	$K_{1:1}$ (M^{-1})[a]	$K_{1:2}$ (M^{-1})[a]	CE	Molar ratio
αCD[b]	172	30	0.016	1:63
βCD	2010	–	0.011	1:87
γCD[b]	9040	–	0.052	1:20
HPαCD[b]	464	19	0.002	1:480
HPβCD	1430	–	0.008	1:122
HPγCD	6610	–	0.038	1:27
RMβCD	6550	–	0.038	1:27
SBEβCD	2020	–	0.011	1:86
SBEγCD	13400	–	0.075	1:14

[a] $K_{1:1}$: Stability constant of 1:1 complex, $K_{1:2}$: Stability constant of 1:2 complex.
[b] $K_{1:1}$ calculated from the linear part of the phase solubility diagram.

3.3. Effect of different polymers on difluprednate stability in HPγCD solution

Stability is one of the important factors that need to be considered during the drug development phase. In our case, HPγCD was able to stabilize the drug but the estimated shelf-life of the drug in aqueous HPγCD solution is less than required for the commercially available dosage forms. The desired shelf-life for any drug product to be considered in a pharmaceutical product should be ≥ 2 years. Consequently, further stabilization of difluprednate in aqueous solution was attempted through the addition of different polymers namely, poloxamer 407, tween 80, and tyloxapol, along with HPγCD. The commercially available dose of difluprednate in eye drops is 0.05% whereas we could solubilize 0.1% in aqueous 15% (w/v) HPγCD solution.

Hence, aqueous solution containing 15% HPγCD (w/v) and the polymers (from 0 to 4% w/v) were prepared to solubilize 0.1% difluprednate and these solutions were subjected to one cycle of autoclaving. Then the remaining drug concentration was measured by using the UHPLC method (section 2.2.1) (Fig. 5). This way, the stabilizing effect of both the components alone and their combination was determined. Unfortunately, tween 80 interfered with the HPγCD complexation of difluprednate, thus the difluprednate concentration was reduced to 0.03%.

Fig. 5 shows that the drug degradation decreases with increasing poloxamer concentration where the degradation was the least at 4% poloxamer – 15% HPγCD combination (less than 1% drug degradation). The solution with only poloxamer showed higher degradation compared to the poloxamer-HPγCD combination or only HPγCD. This result showed that the drug is better stabilized in the combination compared to only CD or poloxamer and that HPγCD is a better stabilizer than poloxamer when compared individually.

In the case of tween 80-HPγCD combination, the drug degradation decreases when the concentration of tween 80 is increased from 0.5 to 1% but again increases from 2% onwards. The combination did not in all cases provide for better stabilization compared to the individual components. 1% tween 80 in 15% HPγCD solution exhibited no drug degradation but the solubility of the drug was greatly reduced in presence of tween 80.

The tyloxapol-HPγCD combinations followed the same trend as with tween 80. The drug degradation decreases up to 1% tyloxapol in 15% HPγCD solution and then increases again from 2%. The drug degradation was the least at 1% tyloxapol when the combination was used and at 2% when only tyloxapol was used.

Overall, the minimum degradation was observed with the combination of 4% poloxamer and 15% HPγCD as well as 1% tween and 15% HPγCD. The combination of HPγCD and tween 80 was not regarded

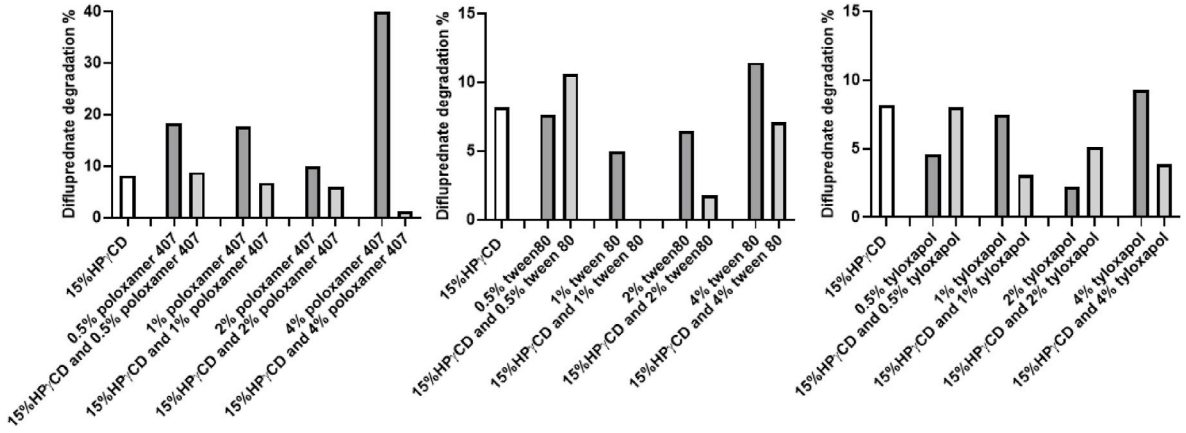

Fig. 5. Drug degradation % after one cycle of autoclaving with 15%(w/v) HPγCD and various % (w/v) of polymers: (a) poloxamer 407, (b) tween 80, and (c) tyloxapol.

advantageous as the difluprednate solubility was greatly reduced or only 0.37 mg/mL which is way less than what pure HPγCD could solubilize. This might be because the addition of surfactant/polymer to the complexation media results in competition with drug molecules for the CD cavity and consequent displacement of the drug molecules from the CD cavity resulting to lower solubility [41].

While in the case of poloxamer 407, stability could be achieved without compromising the target solubility. CD stabilizes the drug by complexation process while polymers like poloxamer by micelle formation and protection of the hydrophobic drug within the micelles [39,41]. Often combination of CD complexation and micellar solubilization can lead to a synergistic effect resulting in enhanced drug stability [32,42,43]. The formation of micellar-type CD aggregates enables solubilization of very poorly water-soluble drugs [42,44,45]. In addition, poloxamer normally acts as a stabilizer of dispersed systems [46]. Also, increased stability with an increased % of poloxamer could indicate the formation of micellar CD aggregates. Similar results have previously been observed with combinations of CDs and poloxamer [45,47]. Hence, 4% (w/v) poloxamer and 15% (w/v) HPγCD was the best combination in terms of stability without compromising the drug solubility.

This was followed by the determination of the particle size of the difluprednate/HPγCD/poloxamer aggregates by using NanoSight Wave which is given in Table 4.

The aggregate size in aqueous 15% HPγCD solution containing 0.1% (w/v) difluprednate is approx. 126 nm. The aggregate size of the samples increased when the combination of poloxamer 407 and HPγCD was used and increased with an increasing percentage of poloxamer 407.

Studies have shown that CDs interact with the poloxamer unimer, preferably by the inclusion of the hydrophobic propylene oxide segment into the CD cavity [48]. The ethylene oxide unit of poloxamer has relatively high hydrophilicity and hence has less tendency to penetrate into the cavities [49,50]. When poloxamer is introduced to the CD media, reorganization of the whole system will occur [51–53]. Similar changes were observed when poloxamer was added to an aqueous medium containing budesonide and HPβCD [54].

The morphology and size of these samples were further confirmed by transmission electron microscopy (TEM) as shown in Fig. 6. Aggregates with larger sizes (from 100 nm) do not have a spherical shape like the ones below 100 nm. Instead, they look like clusters of smaller spherically shaped aggregates [55]. As expected, the size of the dry aggregates observed from the TEM is somewhat smaller than the hydrodynamic diameter of the aggregates determined by NanoSight.

Table 4
Aggregate size of difluprednate/HPγCD/poloxamer aggregates in solutions containing different concentrations (% w/v) of poloxamer 407.

Poloxamer 407 concentration (% w/v)	Aggregate size (nm)
0.0%	125.9
0.5%	160.7
1.0%	194.1
2.0%	217.1
4.0%	234.1

3.4. Characterization of the difluprednate/HPγCD (binary) and difluprednate/HPγCD/poloxamer (ternary) complexes

The characterization of the binary and ternary complexes was done using various techniques to confirm the complexation between the drug and the cyclodextrin molecules.

3.4.1. Fourier Transform Infra-red (FTIR) spectroscopy

FTIR was used to elucidate the interaction between difluprednate and HPγCD, as shifts of the vibrational wavelengths of the components due to the presence of inclusion complexes could be expected [56].

HPγCD was characterized by bands at 2930 cm^{-1} due to the C–H stretching vibrations, bands at 3370 cm^{-1} related to the symmetric and antisymmetric O–H stretching mode, and other bands at lower frequencies. Concerning difluprednate, it had characteristic bands at 1660.44 cm^{-1} which is representative of the unsaturated ketone or secondary carbonyl and other bands from 1720.41 cm^{-1} to 1750.53 cm^{-1} related to the acyclic ketone and esters.

The FTIR of the difluprednate/HPγCD complex showed that the characteristic peaks of difluprednate have disappeared. This may indicate that there is formation of new solid phase suggesting that HPγCD formed a complex with difluprednate. Similarly, the characteristic peaks of difluprednate also disappeared in the complex with poloxamer. These changes indicated that HPγCD formed a complex with the drug that included the polymer (Fig. 7).

3.4.2. Differential Scanning Calorimetry (DSC)

DSC was applied to evaluate the solid-state characterization of pure HPγCD, pure difluprednate, their complex, and the complex in the presence of poloxamer 407. The DCS thermograms are shown in Fig. 8.

The disappearance or shifting of the individual endothermic peak to other temperatures is observed when a complex is formed between drug and CD molecules [57]. This indicates a change in the crystal lattice, melting, boiling, or sublimation points [58]. The DSC results showed the typical thermal curves from crystalline difluprednate with a well-defined sharp endothermic peak at 191 °C corresponding to the difluprednate melting point with the decomposition of the drug. The CDs themselves do not display any melting peak but decompose at a temperature above 300 °C [59–61]. The temperature range in this study

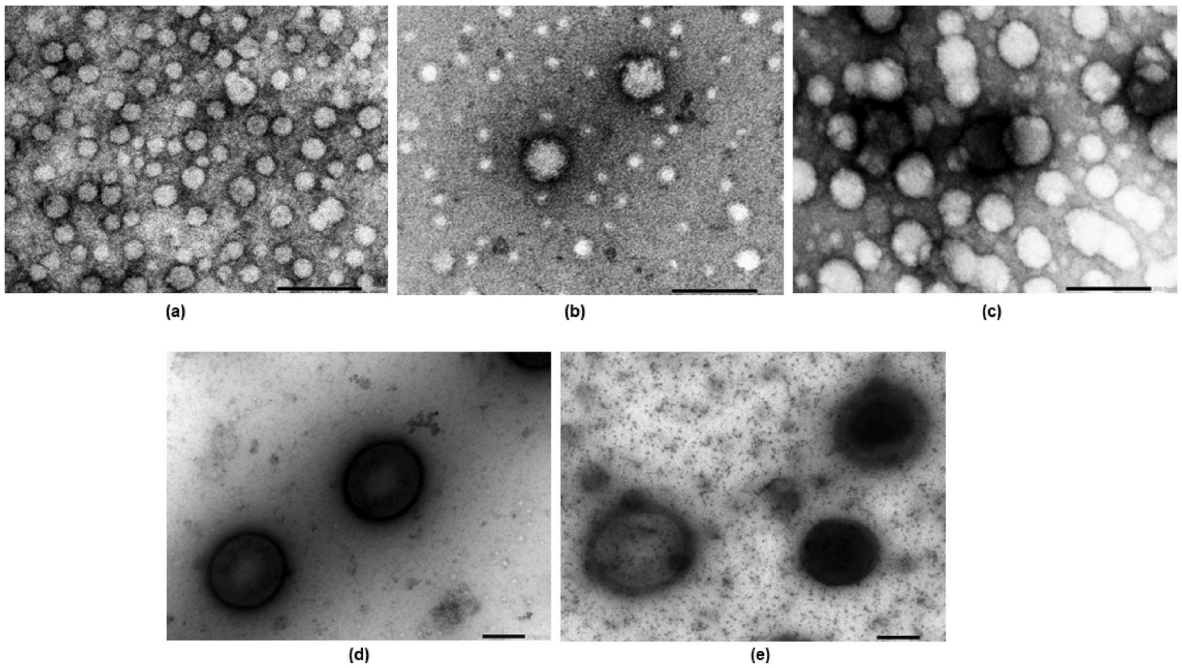

Fig. 6. Transmission electron microscopic images of (a) difluprednate/HPγCD and difluprednate/HPγCD/poloxamer complexes with different %(w/v) of poloxamer 407 (b) 0.5% poloxamer 407 (c) 1% poloxamer 407(d) 2% poloxamer 407 and (e) 4% poloxamer 407, at magnitude of 60 K.

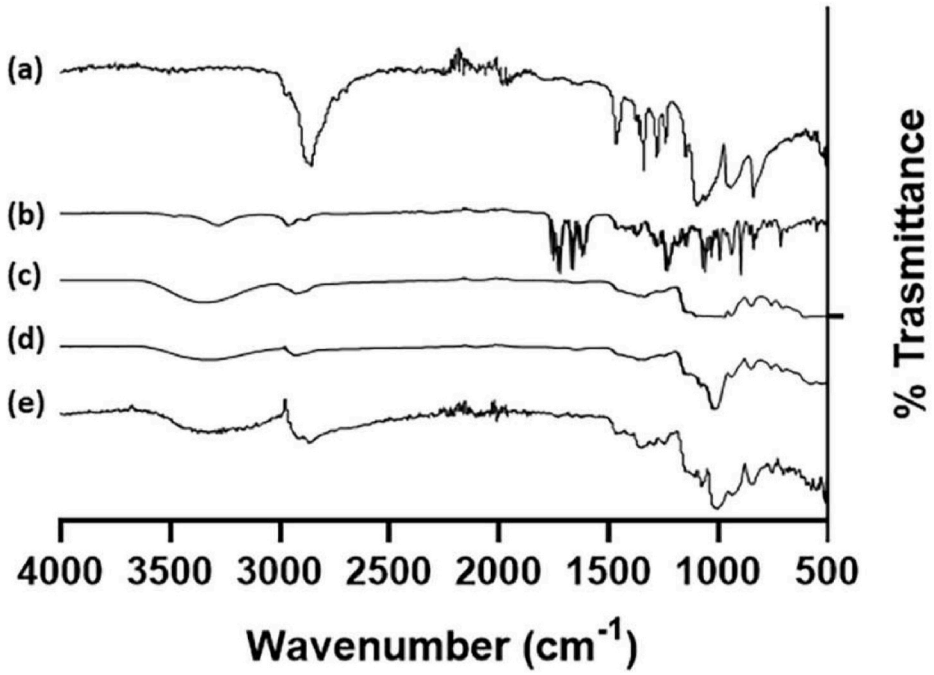

Fig. 7. FTIR spectra of (a) poloxamer 407 (b) difluprednate (c) HPγCD (d) difluprednate/HPγCD complex, and (e) difluprednate/HPγCD/poloxamer complex.

was 0–250 °C. However, HPγCD displayed broad endothermic peaks between 60 and 130 °C which is indicative of dehydration or the loss of water molecules from the CD cavity, upon heating. The difluprednate peak has completely disappeared in the complex solid-state with HPγCD and the one that has HPγCD and poloxamer 407 suggesting the inclusion complex formation and the existence of a new solid phase in both cases.

3.4.3 1. ^{1}H NMR spectroscopy. ^{1}H NMR spectroscopy has become one of the most important methods for structural elucidation of organic compounds in the solution state [62]. These studies not just provide information on the characteristics of guest/CD inclusion complexes but also the orientation of the guest molecule inside the hydrophobic CD cavity [63,64].

The inclusion complexes' formation leads to chemical shifts (Δδ*) in the ^{1}H NMR spectra of the guest and the CD molecule. Here, the difference in the chemical shifts of the difluprednate/HPγCD and difluprednate/HPγCD/poloxamer was observed in comparison to free HPγCD and Δδ* calculated using the following equation:

$$\Delta\delta^* = \delta_{complex} - \delta_{free} \quad (15)$$

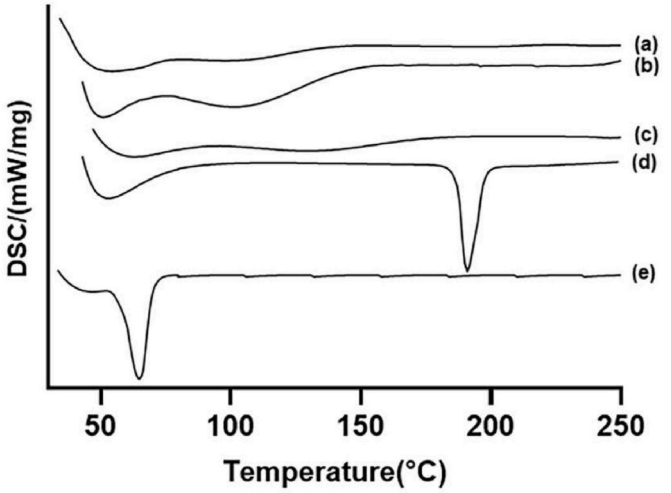

Fig. 8. DSC of (a)difluprednate/HPγCD/poloxamer complex (b) difluprednate/HPγCD complex (c)HPγCD (d) difluprednate and (e) poloxamer 407.

where complex and free are chemical shifts between free and bound CD molecules, respectively.

The CD inclusion complexes of various hydrophobic drugs have been intensively studied by ^{1}H NMR spectroscopy [65,66] and shown that the observed chemical shift variations of a specific host or guest can provide evidence for the formation of inclusion complexes in solution as major changes in the microenvironment are known to occur between the free and bound states [67].

The proton positions of HPγCD were assigned to its chemical structure. Based on the molecular shape of CDs, hydrogen atoms attached to carbon 3 and 5 of glucopyranose units (i.e., H3 and H5) are sited in the cavity interior while H1, H2, and H4 are located on the exterior surface. When inclusion complexes are formed, NMR studies show chemical shifts of the H3 and H5 signals and the intensity of the shifts can give information on the inclusion complex geometry [68].

All these protons displayed significant resonance alternation in the presence of guest molecules (difluprednate and poloxamer 407) and the ^{1}HNMR chemical shifts corresponding to HPγCD in free-state, binary, and ternary complexes are shown in ppm and listed in Table 5.

When the apolar region of a guest molecule enters into the hydrophobic CD cavity, it induces a shielding effect on the inner protons of the glucose units, namely H3 and H5, whereas the proton on the exterior of the torus (H1, H2, and H4) are relatively unaffected [69]. The shifts of H5 represent a 'deep' inclusion complex since its position in the CD cavity is deeper than H3 while the shifts of H3 indicate a 'shallow' complex or partial inclusion [68,69].

The changes in ^{1}H-chemical shifts ($\Delta\delta^*$) of the H1, H3, and H5 protons were −0.0025, −0.0043 and −0.0028, respectively, displaying upfield shifts. This can be explained by the fact that water is replaced by the hydrophobic aromatic ring(s) of the difluprednate molecule inside the cavity as these effects are an indication of reduced hydration due to steric hindrance or hydrogen bonding [70]. The upfield shift of the H3 proton, which is located on the inner surface at the secondary hydroxyl group had higher change ($\Delta\delta^* = -0.0043$ ppm) compared to H5 ($\Delta\delta^* = -0.0028$ ppm) which is situated at the inner surface of the cavity of primary hydroxyl side. As described earlier, the higher shielding effect on H3 with respect to H5 suggests that difluprednate forms a shallow complex with HPγCD [68,69]. Djedanini et al. explained that the upfield effects experienced by the host molecules are most probably due to ring-current and magnetic anisotropy effects created by the aromatic drug [71].

The downfield shift ($\Delta\delta^* > 0$) was observed for H2 and H4 when difluprednate formed a binary complex with HPγCD. This can be probably due to de-shielding effects of the van der Waals interaction between HPγCD and difluprednate molecules or due to variation of local polarity upon complex formation [67,69,72,73].

In the case of HPγCD in difluprednate/HPγCD/poloxamer ternary complex, resonance protons (H1, H3, and H5) underwent upfield and protons (H2 and H4) showed downfield shifts as similar to the difluprednate/HPγCD binary complex. The addition of the polymer increased the shielding effect of the characteristic protons in the outer cavity (H2 and H4) shown by greater $\Delta\delta^*$ values except for H1 where the shielding effect is decreased. The shielding effect on the protons H3 and H5 are not significantly affected by the addition of poloxamer. Hence, the binary and ternary complexes showed the binding behaviour with HPγCD which includes both inner cavity of CD and the exterior rim.

Table 5
The ^{1}H-chemical shifts of HPγCD alone and the variation in chemical shifts of HPγCD the presence of difluprednate and poloxamer 407.

Protons	HPγCD	difluprednate/HPγCD ($\Delta\ \delta^*$)	difluprednate/HPγCD/poloxamer 407 ($\Delta\ \delta^*$)
H1	5.151	−0.0025	−0.0014
H2	3.6571	0.0005	0.0014
H3	4.0548	−0.0043	−0.0043
H4	3.5032	0.0010	0.0034
H5	3.8895	−0.0028	−0.0021

4. Conclusions

The stability of difluprednate in an aqueous CD solution was determined as a function of the medium acidity and difluprednate was shown to be most stable at a pH of about 5. Based on the described solubility and stability studies, it can be concluded that HPγCD is the best CD tested for the preparation of aqueous difluprednate formulations. The stability and solubility were improved when the combination of HPγCD and polymers was used, particularly with HPγCD and poloxamer 407, with increasing micelle aggregate size as shown by NanoSight and TEM. Furthermore, characterization studies of the difluprednate/HPγCD complex in both solid-state and solutions using different techniques like DSC, NMR and FTIR verified the formation of a complex. It was possible to solubilize 0.1% difluprednate in aqueous HPγCD solution, which is twice as much as the commercially available eye drops, and stabilize in an aqueous solution using a combination of CD and polymer. However, in order to formulate into eye drops, there are other parameters like viscosity, tonicity, etc. that need to be considered, and further studies with different excipients are needed to achieve that goal.

Author contributions

Manisha Prajapati: Investigation, Data curation, Writing - original draft and editing. Thorsteinn Loftsson: Funding acquisition, Supervision, Writing – reviewing and editing.

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Declaration of competing interest

The authors declare that there is no conflict of interest. The work was performed by Manisha Prajapati and will be part of her PhD thesis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jddst.2022.103106.

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