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Determination of apparent association constants of steroid–cyclodextrin inclusion complexes using a modification of the Hummel–Dreyer method

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Abstract

A modified Hummel–Dreyer method was used to calculate the apparent association constants of steroid–cyclodextrin inclusion complexes. An external calibration technique was employed, using the *y*-intercept from a plot of peak area versus concentration to correct for sample solvent effects. Mobile phase temperature and sample diluent organic content were found to be critical factors affecting the accuracy and reproducibility of the results. For four of six sets of data, the modified Hummel–Dreyer method yielded statistically equivalent results to another HPLC method for determining apparent association constants. Limitations of the modified Hummel–Dreyer method are discussed. In particular, the accuracy of the method is poor when measuring small apparent association constants.

Keywords: Association constants; Complexation; Hummel–Dreyer method; Steroids; Cyclodextrin; Beclomethasone; Betamethasones; Dexamethasone

1. Introduction

Cyclodextrins are cyclic oligosaccharides consisting of six or more α-1,4-linked D-glucopyranose units. They exhibit the ability to form highly selective inclusion complexes with a variety of guest molecules [1,2]. This characteristic has led to the development of commercial stationary phases for both liquid and gas chromatography. A wide variety of native and derivatized cyclodextrins are available for use as mobile phase additives in HPLC and as run buffer additives for capillary electrophoresis (CE). While the majority of the published research using cyclodextrins in chromatography focuses on the separation of enantiomers, cyclodextrins demonstrate exceptional utility for separating closely related compounds including geometrical structural isomers [3–6].

Hummel and Dreyer developed a method for determining association constants from chromatographic data in 1962 [7]. They used an internal calibration method to calculate the amount of 2′-cytidylic acid bound by pancreatic RNAase. Solutions containing a constant amount of RNAase and varying amounts of 2′-cytidylic acid were placed on a column of cross-linked dextran and then eluted with a mobile phase containing 2′-cytidylic acid. A negative peak corresponding to the amount of ligand

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bound to the RNAase substrate was observed. The magnitude of this negative peak was found to decrease as the concentration of the ligand in the solution being analyzed increased. A plot of the concentration of the ligand versus the peak area showed the concentration of ligand that would yield a peak area of zero (the *x*-intercept). This concentration represents the amount of ligand to which the substrate present in the mobile phase is bound. Once this value is known, an association constant can be determined.

The internal calibration method used by Hummel and Dreyer to calculate the binding of 2′-cytidylic acid by pancreatic RNAase using gel filtration has been extended to analyze other systems. Sebille et al. determined the binding parameters of warfarin and furosemide with human serum albumin (HSA) using a size-exclusion HPLC column [8]. Similar work has been done to study the binding of propanolol to human α_1-acid glycoprotein [9]. Sun and Hsiao studied the competitive binding of five drugs and a detergent to bovine serum albumen [10].

None of the researchers above calculated the amount of the ligand represented by the negative peak directly. Data regarding the complex is extracted from concentration plots or Scatchard plots using an internal calibration Hummel–Dreyer method. Variations of the Hummel–Dreyer method that use the amount of the bound compound derived from the chromatographic peak to calculate K_f (the apparent association constant) are referred to as external calibration methods. Such methods have been used to quantify the amount of the negative peak without varying the concentration of the ligand in either the injection solution or the mobile phase. Sun and Hsiao studied the binding of L-tryptophan to bovine serum albumin using only one concentration of substrate and corrected the peak areas for all solutions against a blank injection [11]. Pinkerton and Koeplinger used a modification of the external standard method to analyze the binding of warfarin to HSA [12].

Sadleg-Sosnowska used the area of the positive peak that elutes at to to study the inclusion of several steroids with cyclodextrin [13,14]. The peak areas were corrected for the shift in molar absorptivity observed when cyclodextrin was added to solutions containing the steroids of interest. A molar absorptivity shift of 12 to 20% was reported for the steroids studied. Different results are obtained using the peak areas of the first and second peaks observed to calculate K_f for cyclodextrin complexes if this correction is not made [15]. However, this approach adds an additional source of error to the calculation of K_f, since the determination of the molar absorptivity change is made off-line. This error is illustrated by the result obtained for estriol, which was more than double the value measured using Fujimura et al.'s method. Fujimura et al.'s method is another way to measure association constants using HPLC [16]. It is a simplified version of the method originally published by Uekema et al. [17]. Fujimura et al.'s method involves analyzing a solution containing guest compounds using mobile phases containing various concentrations of cyclodextrin. The change in retention factors of each compound are monitored as the concentration of cyclodextrin changes. Association constants can be determined from these data, assuming that the complex has no retention on the stationary phase. The difference in values between the Hummel–Dreyer method and Fujimura et al.'s method observed by Sadleg-Sosnowska may be due to limitations in the method described by Fujimura et al. However, the potential for experimental bias introduced by using the area of the positive peak to calculate association constants makes it difficult to draw any meaningful conclusions from the data.

The goal of the research reported here was to determine K_f the formation of various steroid–cyclodextrin complexes using a modification of the Hummel–Dreyer method, and compare these K_f values to those obtained using Fujimura et al.'s method. No statistical analysis comparing results obtained by the Hummel–Dreyer method with results obtained using Fujimura et al.'s method has been reported in the literature. However, before this work could be performed, it was desirable to develop a modification of the Hummel–Dreyer method that would be easy to use and yield accurate, reproducible results. A modification of an external calibration method was developed, but difficulties were encountered that needed to be overcome prior to the generation of useful data.

It should be noted that the K_f values calculated using HPLC are biased low due to pressure. This

phenomenon was reported by Ringo and Evans [18]. However, since the method being evaluated is being compared to another HPLC method, statistical analysis is unaffected by this pressure effect.

The guest compounds studied in this report are synthetic adrenocorticosteroids used in pharmaceutical products for their anti-inflammatory, antipruritic, and vasoconstrictive actions. Betamethasone, beclomethasone, and dexamethasone were investigated. $\Delta^{9,11}$-Betamethasone was also investigated. This compound is a potential synthetic impurity of betamethasone that contains a double bond between C_9 and C_{11}, displacing the 9-fluoro and 11-hydroxyl groups of betamethasone. Numerous researchers have investigated the interactions of betamethasone and other steroids with various cyclodextrins. Cyclodextrins have been shown to improve bioavailability of steroids in topical formulations [19] and in oral formulations [20], to enhance the solubility [21,22] and dissolution rates [23,24] of steroids, and to increase the stability of formulations for some steroids [25–28]. Cyclodextrins have also been used as mobile phase modifiers in HPLC applications for the analysis of steroids [29–32]. Highly selective β-cylcodextrin-based molecular imprinted polymers have been synthesized using steroids as a template [33,34]. A modified cyclodextrin has been used to control the hydroxylation of an androstanediol derivative through inclusion [35].

2. Experimental

2.1. Apparatus

The HPLC systems used were Hewlett-Packard 1050 Series (Agilent Technologies, Palo Alto, CA, USA) with in-line degassers. One system was equipped with a variable wavelength detector while the other had a diode array detector. Symmetry C_{18} columns, 150 mm×4.6 mm I.D., 5 μm particle size (Waters, Milford, MA, USA) were used. Refrigerated circulating baths (Neslab Instruments, Portsmouth, NH, USA) were used to control column temperature. A flow-rate of 1.5 ml/min was used to maintain reasonable run times. Operating pressure ranged from 1200 to 2000 p.s.i. (1 p.s.i. = 6894.76 Pa). The steroids studied possess a UV absortion maximum at a wavelength of 240 nm, so this wavelength was used for detection. Turbochrom 4.0 (Perkin-Elmer, Norwalk, CT, USA) was used to acquire and process the chromatographic data.

2.2. Chemicals

HPLC-grade acetonitrile, methanol, and water (Fisher Scientific, Springfield, NJ, USA) were used without further purification: γ-Cyclodextrin (γ-CD) was purchased from Cerestar USA. (Hammond, IN, USA). Hydroxypropyl-γ-cyclodextrin (HP-γ-CD; average degree of substitution = 4.6) and dexamethasone were purchased from Fluka (Milwaukee, WI, USA) The Schering-Plough Research Institute (Kenilworth, NJ, USA) generously provided betamethasone, beclomethasone, and the $\Delta^{9,11}$-Betamethasone. $\Delta^{9,11}$-Betamethasone has the same structure as betamethasone except that the 9-fluoro and 11-hydroxyl groups have been lost to form a double bond between C_9 and C_{11}.

2.3. Mobile phase preparation

Mobile phases were prepared by pipetting 2.0 ml of a stock solution of each steroid into a 1 L volumetric flask and diluting to volume with acetonitrile–water (35:65 or 20:80, v/v). The resulting solution was then degassed using a sonic bath (Fisher Scientific). The steroid stock solutions were prepared by weighing approximately 300 mg of each steroid into a 50 ml volumetric flask and diluting to volume with methanol. Mobile phases prepared in this way contained approximately 0.03 m*M* of the steroid.

3. Results and discussion

The value of K_f for the steroid–cyclodextrin inclusion complex can be determined from the following equation:

$$K_f \frac{Q_{G\text{-}CD}}{[(G)_m](Q_{CD}\text{-}Q_{G\text{-}CD}} \quad (1)$$

where Q_{CD} is the amount of cyclodextrin injected, $Q_{G\text{-}CD}$ is the amount of the inclusion complex that is

formed, and $(G)_m$ is the concentration of the guest compound in the mobile phase [15]. Q_{CD} can be calculated since the concentration of the cyclodextrin solution and the injection volume are both known. $[(G)_m]$ is also known. It is the amount of complex that forms, $Q_{G\text{-}CD}$, which needs to be determined. Two values are needed to quantify $Q_{G\text{-}CD}$ from the negative peak: (1) a response factor for the steroid and (2) the amount of this peak that is due to the injection of a solution which does not contain the steroid into the HPLC system. The first piece of data is readily obtained and shall be discussed first. The accurate determination of peak area due only to the introduction of cyclodextrin will then be discussed.

3.1. Response factor determination

Solutions containing between 0.01 m*M* and 0.3 m*M* of the steroid being studied were injected into an HPLC system with mobile phases containing 0.03 m*M* of the steroid of interest. An overlay plot of typical chromatograms obtained for a set of steroid solutions is shown in Fig. 1. A calibration curve can be constructed by plotting peak area as a function of the amount of steroid introduced onto the column. Correlation coefficients greater than 0.99 were achieved for all calibration curves used for this work. A series of standard solutions were injected on each day that the Hummel–Dreyer method was performed.

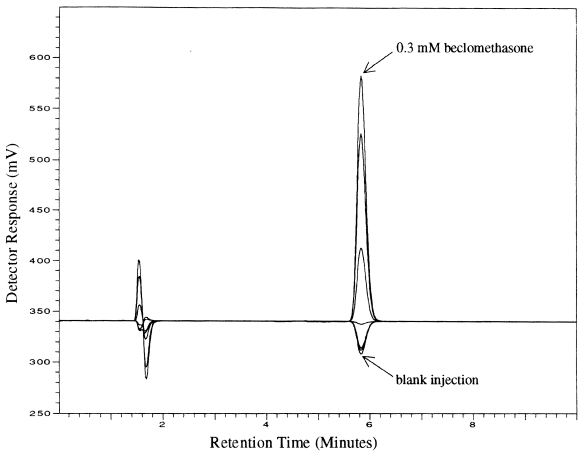

Fig. 1. Overlaid chromatograms of a blank injection and solutions containing between 0.01 m*M* and 0.3 m*M* beclomethasone. Mobile phase acetonitrile–water (35:65, v/v) containing 0.025 m*M* beclomethasone.

3.2. Determination of $Q_{(G\text{-}CD)}$

The calibration curves can be used to calculate the deficiency of steroid that results from injecting a solution containing cyclodextrin. However, calculating the amount of the steroid–cyclodextrin complex ($Q_{(G\text{-}CD)}$) formed is more complicated. The introduction of a plug of solvent that contains no steroid will disturb the equilibrium established between the steroid present in the mobile phase and the steroid interacting with the stationary phase. A deficiency is observed in the system when a solution is injected (unless that solution contains exactly the same amount of steroid as the mobile phase) regardless of whether cyclodextrin is present or not. Initial studies showed a discrepancy between the area of a blank injection and the *y*-intercept of the calibration curve. This should not be the case since the *y*-intercept of the calibration curve represents the peak area of a solution with a steroid concentration of zero. Two sets of standards were analyzed in duplicate on two separate days. Blank injections were made before each set. The *y*-intercept value and the average area of two blank injections were 109 200 and 103 400 on day 1, and 128 600 and 106 600 on day 2. A difference of 5% and 17% was observed between the *y*-intercept values and the average blank peak areas.

This work was performed without controlling the temperature of the mobile phase. While column temperature was controlled with a water bath, the temperature of the mobile phase entering the column was unregulated. Two problems can occur if the mobile phase is not at the proper temperature when it reaches the column. First, the formation of the inclusion complex may occur at an unknown temperature. It has been shown that a change of 1°C yields a change of approximately 2 to 4% in K_f for the steroids being studied [36]. Second, the equilibrium of the steroid between the stationary phase and mobile phase will be disrupted since this equilibrium is temperature dependent. When a solution is injected, the deficiency of steroid in the HPLC column is initiated at the column head. it is the temperature there that determines the quantity of steroid molecules that are going to be removed from the station-

ary phase. If the mobile phase is at a different temperature than the column then there will be a temperature gradient formed along the head of the column. The result will be irreproducible peak areas observed when solutions are injected. A small length of steel tubing (20 cm×0.010 in. I.D.; 1 in.=2.54 cm) was added just before the column in the water jacket. This allowed the mobile phase to reach the proper temperature immediately before entering the column. Fig. 2 illustrates the significant effect this had on baseline stability. The chromatograms were generated by injecting increasing concentrations of γCD into an HPLC system with a mobile phase of acetonitrile–water (20:80, v/v) containing dexamethasone. The set of chromatograms at the bottom of the figure was obtained in the absence of a length of steel tubing added to the head of the column. The top set of chromatograms was obtained after the steel tubing was added to the system. The relative standard deviations calculated for the analysis of the dexamethasone–γ-CD inclusion complex dropped from 9.6 to 3.5%. It is noteworthy that these experiments were performed during the winter, when laboratory temperatures can fluctuate drastically. This may have exacerbated the baseline fluctuation observed during these experiments.

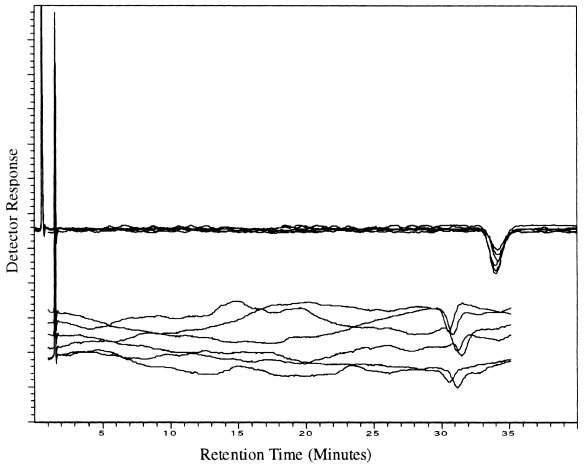

Fig. 2. Effect of mobile phase temperature on baseline stability using the Hummel–Dreyer method. Solutions containing 25, 50, 75, 100, and 150 mg of γ-CD in 50 ml of acetonitrile–water (20:80, v/v) were analyzed in the presence of a steel coil before the head of the column (top) and in the absence of the steel column (bottom). Mobile phase acetonitrile–water (20:80, v/v) containing 0.025 m*M* dexamethasone.

A set of standards containing five concentrations of beclomethasone ranging from 0.01 m*M* to 0.3 m*M* was analyzed with the mobile phase temperature controlled. The average y-intercept obtained from the resulting calibration curve was 139 900±3 800. Blank injections were made in duplicate before each set. The average area for eight blank injections was found to be 131 500±3 800. A difference of 6% is observed between these values. Even with mobile phase temperature controlled, it seems that the direct injection of blank may not yield the most accurate results. Using either of these values for the amount of steroid removed from the system by the sample diluent gave poor results for K_f. Table 1 summarizes the results for a set of γ-CD solutions ranging in concentration from 0.8 m*M* to 3.9 m*M*. K_f values generated using the peak area from blank injections and from the *y*-intercept of the calibration curve are shown. Either method of calculation results in a large standard deviation for the values of K_f calculated, with a trend towards increasing K_f values at higher concentrations of γ-CD. A second set of γ-CD solutions with concentrations ranging from 0.8 to 1.2 m*M* was prepared to verify these results. Both sets of solutions were analyzed. The same solution used to dilute the first set of solutions was used to prepare the second. Plots of the peak areas for both sets are shown in Fig. 3. This figure shows that the *y*-intercept for each set of solutions is different, while the slope is essentially the same. Since the amount of steroid removed from the column is a function of the composition of the sample diluent, the slight evapo-

Table 1
Comparison of results for beclomethasone, using the *y*-intercept of the standard curve and blank injections to correct the peak area of cyclodextrin injections

[γ-CD] (m*M*)	Area corrected using	
	y-intercept	Using blank inj. area
0.8	558	724
1.5	698	785
2.3	762	820
3.1	779	823
3.9	790	825
Average	717	795
SD	96.2	43.3

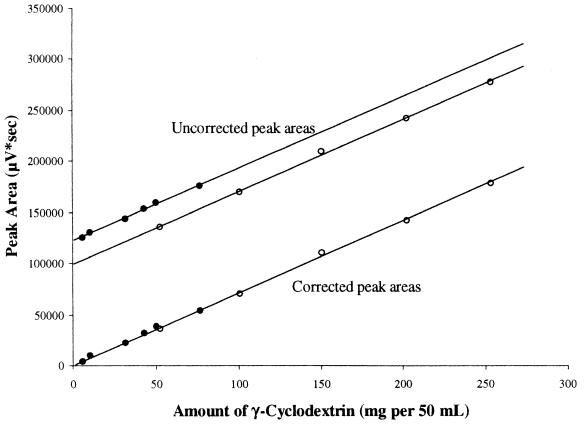

Fig. 3. Comparison of two sets of γ-CD solutions prepared on different days. See text for details.

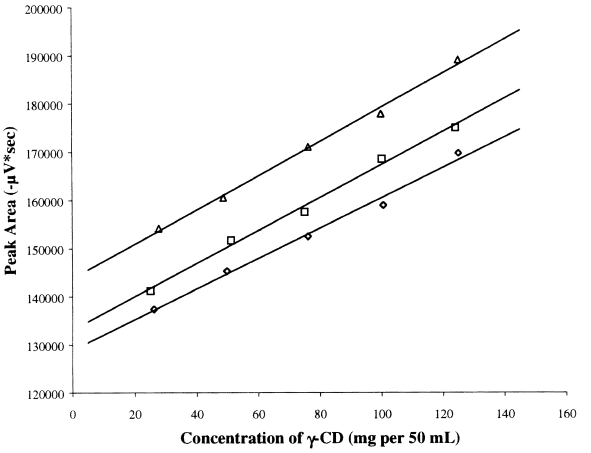

Fig. 4. Effect of sample diluent acetonitrile concentration on the peak areas of γ-CD solutions. Acetonitrile–water content (v/v) of sample diluent = 34:66 (◇), 35:65 (□), 36:64 (△). Mobile phase = acetonitrile–water (35:65, v/v) containing 0.025 m*M* betamethasone.

ration of acetonitrile from the sample diluent during a 24 h period resulted in a difference in the area of negative peak. When each set of data is corrected using the *y*-intercept for that set, the standard deviation for this data is reduced from 71 (RSD = 9.2%) to 30 (RSD = 3.9%).

In order to confirm that the concentration of acetonitrile in the sample diluent affects the negative peak area in the way described above, three sets of γ-CD solutions were prepared. The samples were diluted in acetonitrile–water (34:66, 35:65, or 36:64, v/v). Concentrations of γ-CD ranged from 0.4 m*M* to 1.9 m*M* γ-CD. The peak area versus concentration plots for all three sets of solutions are shown in Fig. 4. As expected, a decrease in organic content of the sample diluent results in a decrease in the steroid deficiency due to the injection of the diluent. Table 2 shows that similar K_f values are obtained from all three sets of data.

3.3. Determination of K_f

The modified Hummel–Dreyer method described above was used to analyze the inclusion complexes of beclomethasone with γ-CD and HP-γ-CD using mobile phases containing acetonitrile–water (20:80 and 35:65, v/v). These results were compared to previously reported data generated using Fujimura et al.'s method [36]. The results for all of the steroids analyzed using the Hummel–Dreyer method are summarized in Table 3. K_f values determined using Fujimura et al.'s method are also shown. A comparison of means was performed using the Student *t*-test to determine if the two data sets are statistically the same. The pooled standard deviation for the K_f of betamethasone and beclomethasone with γ-CD in acetonitrile–water (35:65, v/v) was calculated with eighteen degrees of freedom. Ten degrees of freedom were available for the calculations of the beclomethasone–HP-γ-CD complex. All other pooled standard deviations were calculated using eleven degrees of freedom.

The results show that both methods gave the same results in four of the six cases studied. The two cases

Table 2
Effect of sample diluent acetonitrile concentration on K_f after correcting peak areas for each concentration

[γ-CD] (m*M*)	Acetonitrile content (%)		
	34	35	36
0.4	159	159	185
0.75	165	183	174
1.2	155	164	180
1.5	150	178	172
1.9	164	170	183
Average	159	171	179
SD	6.2	9.9	5.7

Table 3
Summary of results obtained using the modified Hummel–Dreyer method and Fujimura et al.'s method

	K_f (M^{-1})		
	Hummel–Dreyer method	Fujimura et al.'s method	Comparison of means
Beclomethasone	713 (7.8%)	612 (3.0%)	fail
Beclomethasone[a]	480 (10.0%)	454 (*)	pass
Betamethasone	169 (6.5%)	212 (2.7%)	fail
Betamethasone[b]	818 (6.7%)	902 (*)	pass
Dexamethasone[b]	1026 (3.5%)	981 (*)	pass
$\Delta^{9,11}$-Betamethasone[b]	995 (6.0%)	827 (*)	pass

[a] Value for HP-y-CD complex.
[b] Value for γ-CD complex in acetonitrile water (20:80, v/v).
[*] Only one set of data was analyzed so the average standard deviation for γ-CD complexes at acetonitile–water composition of 35:65 (v/v) were used for *t*-test.

which failed the *t*-test are γ-CD inclusion complexes with betamethasone and in acetonitrile–water (35:65, v/v). One possible reason why these results are different could be that these complexes have some retention on the stationary phase. Fujimura et al.'s method assumes that the complex has no retention. It is this assumption that allows K_f to be determined from the dependency of $1/k$ on the concentration of cyclodextrin. If the complex did have some slight retention then k would be artificially higher and thus K_f would be lower when measured using Fujimura et al.'s method. This is the case for beclomethasone, but the K_f value obtained for betamethasone using Fujimura et al.'s method is higher than the value determined by the Hummel–Dreyer method. There are three reasons why it is unlikely that the beclomethasone complex is retained by the column. First, the peak for all of the complexes studied using the Hummel–Dreyer method was observed at the void time. The second observation that supports that the complex is unretained is drawn from the similarities between betamethasone and beclomethasone. It does not seem likely that the complex would be retained for one of these steroids and not the other. If the beclomethasone–γ-CD complex were retained by the column then one would expect that the beclomethasone–HP-γ-CD complex would be retained as well. However, this is not the case as the comparison of means for the HP-γ-CD data generated using both HPLC methods passes the Student *t*-test. Thirdly, if any of the steroid–cyclodextrin complexes studied were retained by the column then a greater difference in results between the two methods used would be expected at the *lower* concentration of acetonitrile studied. The data show that all of the steroids tested at the lower concentration of acetonitrile yielded equivalent results using both methods.

Clearly, there must be another reason which could explain the differences observed. When K_f is determined at acetonitrile–water (35:65, v/v) using the modified Hummel–Dreyer method, the amount of the negative peak area due to the steroid consumed by complexation with γ-CD ranged from 5 to 24% of the total area of the negative peak. The small percent of peak area due to complexation is a consequence of the low K_f for the steroid–γ-CD complex in 35% acetonitrile. Work done using Fujimura et al.'s method showed that K_f increases logarithmically as acetonitrile content is decreased [36]. The amount of the negative peak that related to complexation increased to between 25 and 75% when acetonitrile–water (20:80, v/v) was used. The use of peak areas to calculate values of K_f that are low is not as precise as the use of retention factors. This demonstrates the major weakness of the Hummel–Dreyer method for studying cyclodextrin complexation. However, the use of this method demonstrates the validity of the data generated using Fujimura et al.'s technique. It also serves to show how difficult it can be to compare K_f data obtained from different methods, even when these data are generated in the same laboratory under rigorously controlled experimental conditions.

4. Conclusions

A modified Hummel–Dreyer method was developed to evaluate the inclusion complexes of four steroids with γ-cyclodextrin and one steroid with HP-γ-CD. This method was found to be accurate when K_f is large. The use of a second HPLC technique to analyze the complexes of steroids with cyclodextrins confirmed the results obtained by Fujimura et al.'s method in four of six cases studied. A failure of the assumptions of Fujimura et al.'s method was not the cause of the deviation of the two differing results. The effects of temperature and sample diluent composition on the accuracy of the method were found to be significant when using the Hummel–Dreyer method to calculate K_f The modification of the Hummel–Dreyer method used in this report was found to yield imprecise results when measuring low K_f values, due to the small percentage of the negative peak that is due to complexation. However, the Hummel–Dreyer method is still the technique of choice when limited amounts of host compound are available.

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