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^{1}H NMR, UV and Circular Dichroism Study of Inclusion Complex Formation between the 5-Lipoxygenase Inhibitor Zileuton and β- and γ-Cyclodextrins

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Abstract. The interactions between the chiral 5-lipoxygenase inhibitor zileuton and β- and γ-cyclodextrins were characterised through solubility measurements and ^{1}H NMR, UV and circular dichroism spectroscopic studies. γ-Cyclodextrin was found to increase the solubility of racemic zileuton while the complexes formed with β-cyclodextrin were almost insoluble. The chiral recognition mechanism which comes into play between the cyclodextrins and the zileuton isomers was studied by ^{1}H NMR, UV, and CD spectroscopies. The apparent association constants for the inclusion complexes were obtained from the changes in chemical shifts, UV absorbance and ellipticity, respectively. It was found that zileuton interacted more strongly with β-cyclodextrin, while γ-cyclodextrin was a better chiral selector. The geometries of the different complexes were also postulated on the basis of 2D NMR ROESY experiments.

Key words: cyclodextrin inclusion complexes; zileuton; chiral recognition; ^{1}H NMR, UV, circular dichroism spectroscopic study.

1. Introduction

Zileuton (± *N*-(benzo[b]thien-2-ylethyl) *N*-hydroxyurea, Figure 1) is a powerful 5-lipoxygenase inhibitor belonging to the hydroxyurea class of compounds which is presently approved in the USA for the treatment of asthma. Being a specific and efficient inhibitor of leukotriene formation, zileuton could also find potential therapeutic use in the treatment of a variety of inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease [1].

Zileuton has a chiral centre (the carbon bearing the methyl group) and therefore two isomers can be isolated.

Zileuton exhibits a very limited water solubility [2] and its interaction with the cyclodextrins can lead, through the formation of inclusion complexes, to alterations of the physico-chemical and biological properties of the guest molecule which

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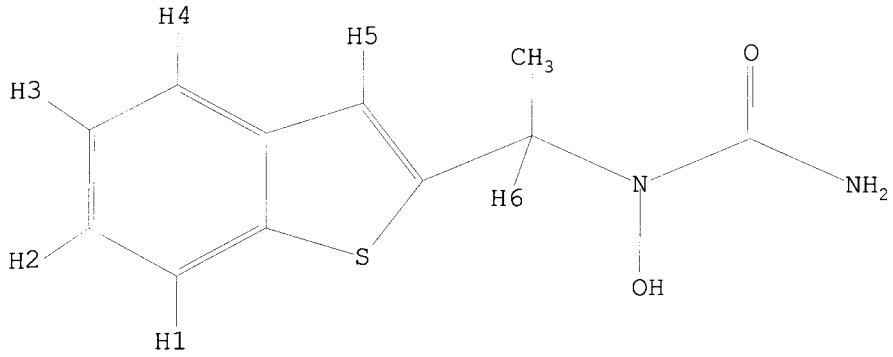

Figure 1. Structural formula and atom numbering scheme of zileuton.

might eventually have relevant pharmaceutical potential. Moreover enantioselective inclusion complex formation between zileuton and the cyclodextrins could eventually be exploited in the study of the differential metabolism of the zileuton enantiomers.

2. Experimental

2.1. MATERIALS

Zileuton was a generous gift of Abbot; since a preliminary HPLC analysis showed a purity of about 99%, any further purification was deemed unnecessary.

β- and γ-Cyclodextrins were purchased from Fluka; their purity was $>$99% and $>$98%, respectively. The water content of the cyclodextrins was determined by thermogravimetric analysis with a Perkin-Elmer TGA 7 instrument and the appropriate corrections were made to the concentrations of the relative solutions. D_2O (99.95% isotopic purity) was obtained from Merck.

2.2. SOLUBILITY MEASUREMENTS

Excess racemic zileuton ($\sim$5 mg) was placed in a screw cap vial and mixed with 3 mL of an aqueous solution containing different concentrations of β- (5.0×10^{-4}, 1.0×10^{-3}, 2.0×10^{-3}, 5.0×10^{-3}, 1.0×10^{-2} M) and γ- (1.0×10^{-3}, 2.0×10^{-3}, 5.0×10^{-3}, 1.0×10^{-2}, 2.5×10^{-2} M) cyclodextrins. The samples were placed in a thermostatic bath at 25 °C and subjected to magnetic stirring for 72 hours. The samples were then filtered through a 0.22 μm MILLEX-GS filter (Millipore) and diluted 1:10 with doubly distilled, deionized water. The zileuton content was ascertained by measuring the intensity of the UV absorption band at 229 nm, whose molar absorptivity has been previously determined [3].

2.3. CHIRAL SEPARATION OF ZILEUTON ENANTIOMERS BY HPLC

The enantioselective separation was performed with a CHIRALPAK AD (250 × 10 mm i.d. – Daicel Chemical Industries) semipreparative column and a mobile phase containing 90 : 10 v/v *n*-hexane/2-propanol. The flow rate was set at 2 mL/min and the column effluent was monitored at 229 nm with a multi wavelength diode array detector (Waters 991). The sign of the optical rotation for each enantiomer was determined with a Perkin Elmer 241 polarimeter at the sodium D line. The (+) and (−) isomers eluted at 28.42 and 45.35 minutes, respectively. A representative chromatogram is shown in Figure 2. The separated enantiomers were collected and dried under nitrogen flux. Both isomers were found to be ∼99.5% enantiomerically pure.

2.4. ^{1}H NMR STUDY

^{1}H NMR spectroscopy was performed on a Bruker AMX 400 spectrometer operating at 400.13 MHz. The probe temperature was set at 25 ± 1 °C using a Haake control system. Chemical shifts were measured relative to external sodium 4,4 diethyl-4-silapentane sulphonate (DSS) at 0 ppm. 1D spectra were collected by co-addition of 256 scans using a 45° observe pulse. 2D homonuclear shift correlation experiments with a delay period to optimise for long range coupling [4] were performed to help identifying the zileuton resonances; the relative assignments were (see Figure 1 for atom numbering): H-1, 7.838 ppm (broad doublet); H-2 and H-3, 7.442–7.385 ppm (multiplet); H-4, 7.917 ppm (broad doublet); H-5 ppm, 7.358 ppm (singlet); H-6, 5.655 ppm (quartet); H-(CH_3), 1.670 ppm (doublet).

2D ROESY (intermolecular nuclear Overhauser enhancement in the rotating frame) pulse sequences [5] were used to study the geometries of the inclusion complexes. The mixing time for ROESY was set at 400 ms. Processing and plotting of the data were performed on a Bruker AMX data station.

2.5. UV AND CIRCULAR DICHROISM (CD) SPECTROSCOPIC INVESTIGATION

UV absorption spectra were recorded at 25 ± 0.5 °C on a Perkin Elmer Lambda 16 spectrophotometer using 1 cm matched quartz cuvettes.

CD measurements were carried out on a Jasco J-710 spectropolarimeter (Jasco Corporation). A 1 cm quartz cell was used to hold the samples. The CD spectra were recorded at 25 ± 0.5 °C in the 200–350 nm range with a scan speed of 100 nm/min, a response time of 1 s and a band width of 10 nm. The recording of each spectrum was repeated four times and the averaged spectra were obtained on the data processor.

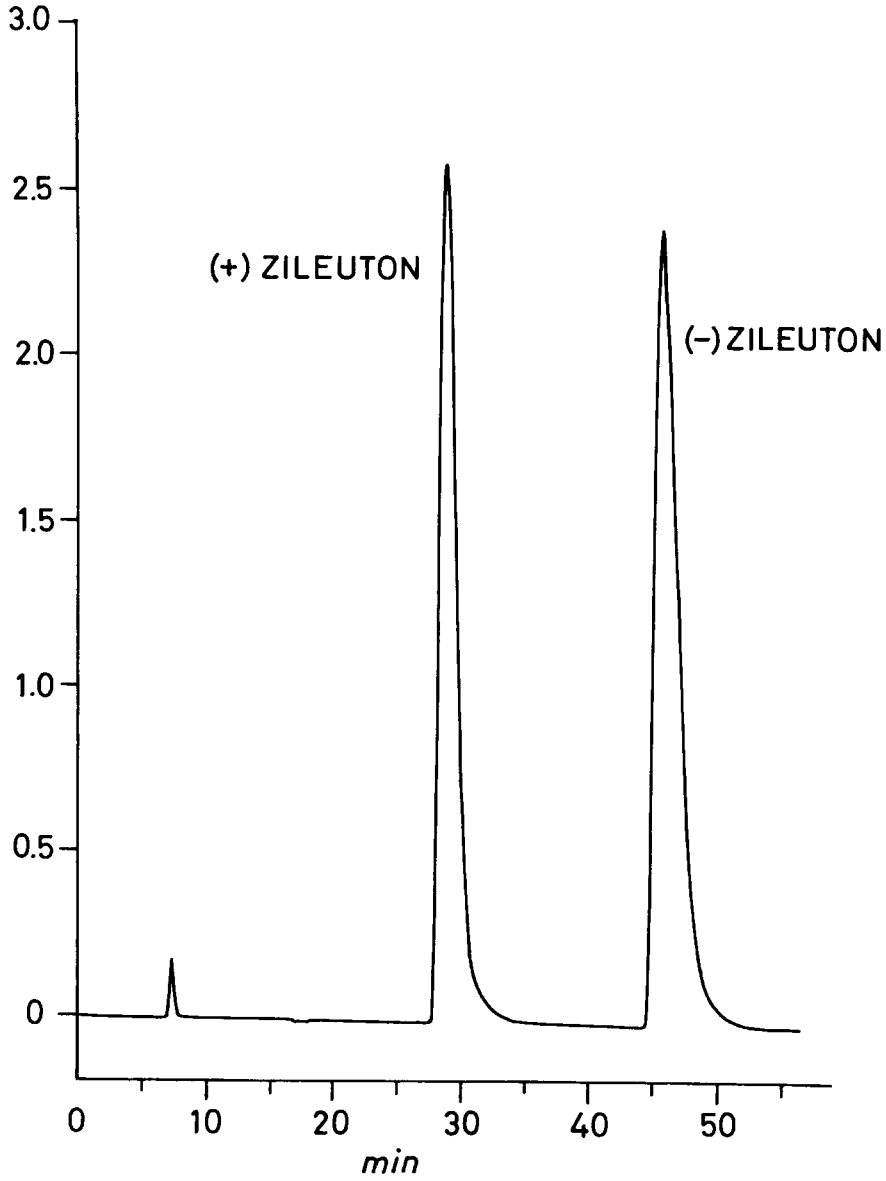

Figure 2. Chiral HPLC chromatogram of the zileuton enantiomers. Resolution factor R = 8.9; capacity factors K = 3.01 (for the (+) isomer) and 5.43 (for the (−) isomer); enantio selectivity factor α = 1.8.

3. Results and Discussion

3.1. SOLUBILITY MEASUREMENTS

A preliminary study was performed to ascertain the influence of β- and γ-cyclodextrins on the zileuton aqueous solubility.

The solubility isotherms are reported in Figure 3. As can be seen an almost insoluble complex was formed between zileuton and β-cyclodextrin (B_I type diagram), whereas a monotonous increase in the guest solubility was noted with γ-cyclodextrin (A_L type diagram). This increase of zileuton solubility (from 6×10^{-4} M in pure water up to 2.6×10^{-3} M in the presence of 2.5×10^{-2} M γ-cyclodextrin) could be exploited in order to improve the drug bioavalability.

3.2. ^{1}H NMR STUDY

NMR spectroscopy can afford the most direct evidence for true inclusion complex formation as H-3′ and H-5′ of the host point toward the interior of the cyclodextrin cavity and the shifts of their resonances are due to magnetic anisotropic effects exerted by the guest. The H-$6'_{a,b}$ atoms lie on the upper surface of the molecule, at the narrow rim of the torus, while all other hydrogens are located on the exterior of the cavity and are therefore affected to a lesser extent by the presence of the guest.

The guest resonances are also affected by the inclusion process, the chemical shift of the anisotropically shielded atoms being modified on the NMR spectrum. Moreover, due to the potential ability of the cyclodextrins to form diastereomeric complexes with chiral compounds, it is to be expected that the equivalent hydrogens of the zileuton enantiomers would give resonances which differ in chemical shift after inclusion. This was indeed the case with the zileuton γ-cyclodextrin complex (Figure 4) which showed an upfield shift for all the guest resonances and splitting of the signals, particularly remarkable in the case of H-5. The very small solubility of the complex formed with β-cyclodextrin (see Figure 3) made the detection of such splitting difficult.

To obtain more information on the chiral recognition mechanism that comes into play between β- and γ-cyclodextrins and zileuton, we decided to run NMR experiments making use of the isolated enantiomers which were previously separated through HPLC.

The (+) zileuton effect on the chemical shift of β-cyclodextrin H-3′, H-5′ and H-$6'_{a,b}$ is shown in Figure 5. The greatest upfield shift was observed for H-5′ followed by H-$6'_{a,b}$ and H-3′. The other host protons did only exhibit negligible shift changes. Similar observations were also made for the (−) zileuton-β-cyclodextrin complex.

Figure 6 reports the $\Delta\delta_s$ for γ-cyclodextrin H-3′, H-5′ and H-$6'_{a,b}$ upon interaction with (+) zileuton. As can be seen the H-5′ resonance was always the most affected but the changes for H-$6'_{a,b}$ and H-3′ were very similar, an observation which did also apply for the (−) zileuton-γ-cyclodextrin complex.

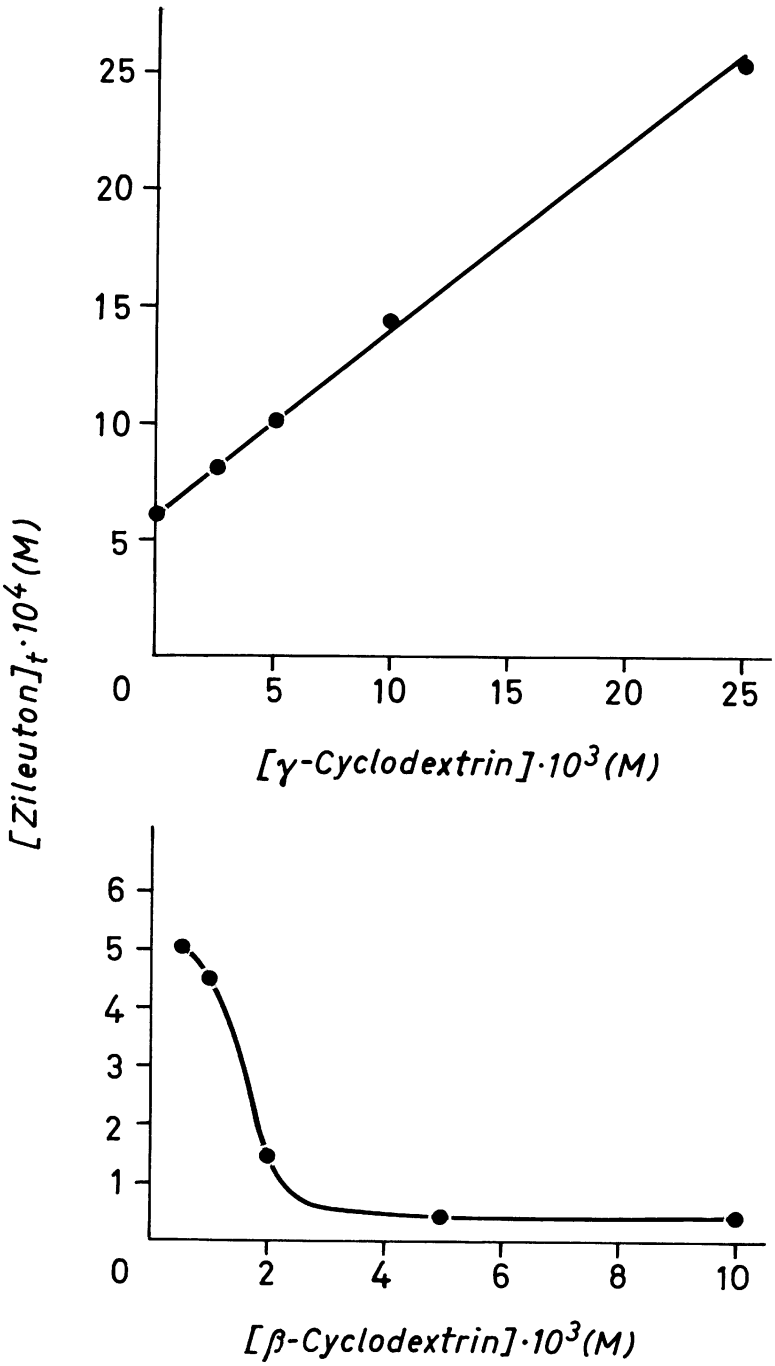

Figure 3. Solubility isotherm of racemic zileuton in aqueous solution in the presence of different concentrations of β-cyclodextrin (lower plot) and γ-cyclodextrin (upper plot).

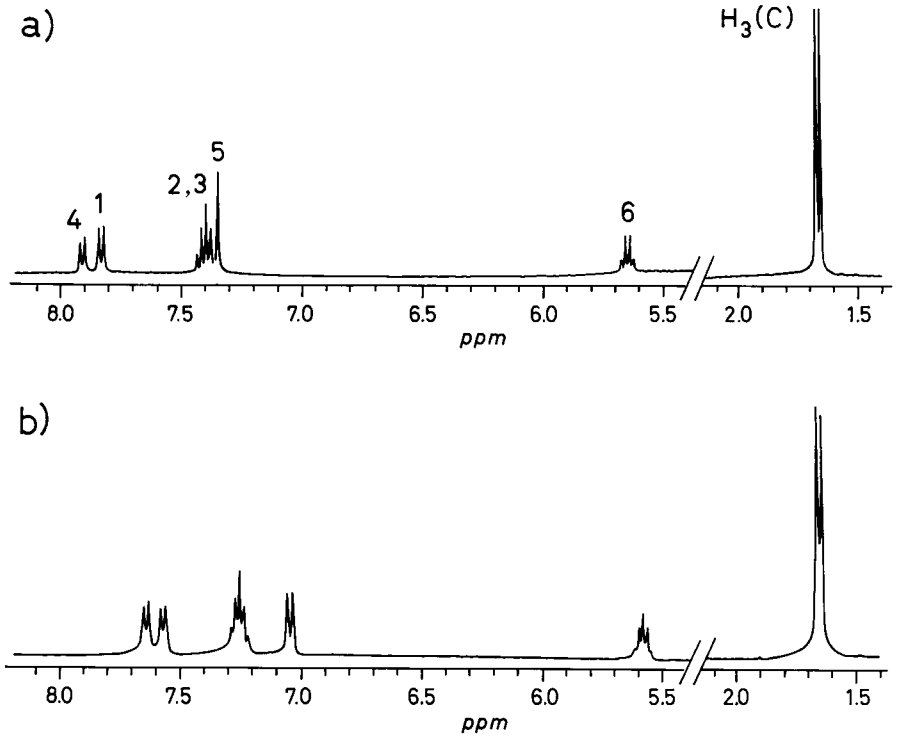

Figure 4. 400 MHz ^{1}H NMR spectra of racemic zileuton (1.2×10^{-3} M) in D_2O in the absence (a) and in the presence (b) of 6.5×10^{-3} M γ-cyclodextrin.

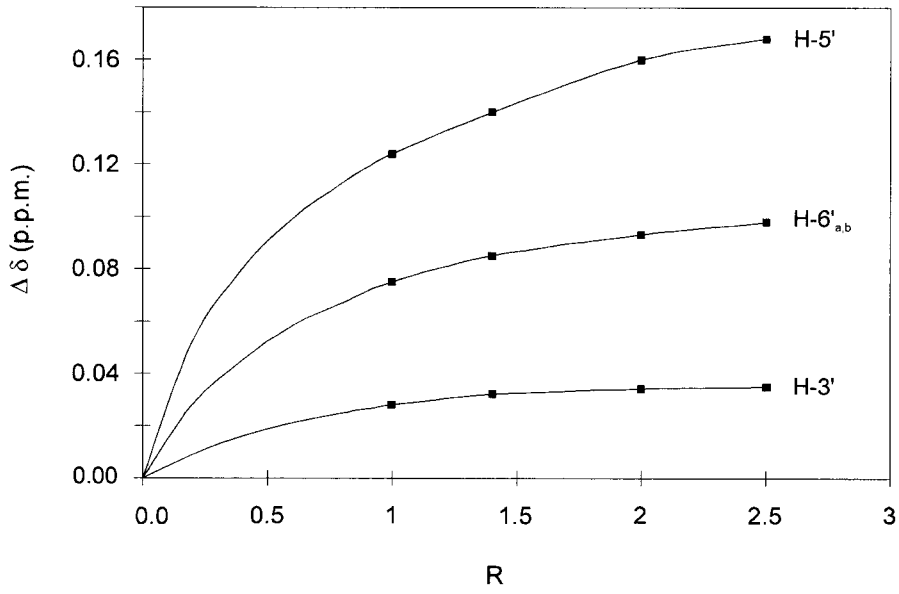

Figure 5. Plots of the chemical shift changes for β-cyclodextrin H-5′, H-6′$_{a,b}$ and H-3′ against the molar ratio of (+)zileuton to β-cyclodextrin.

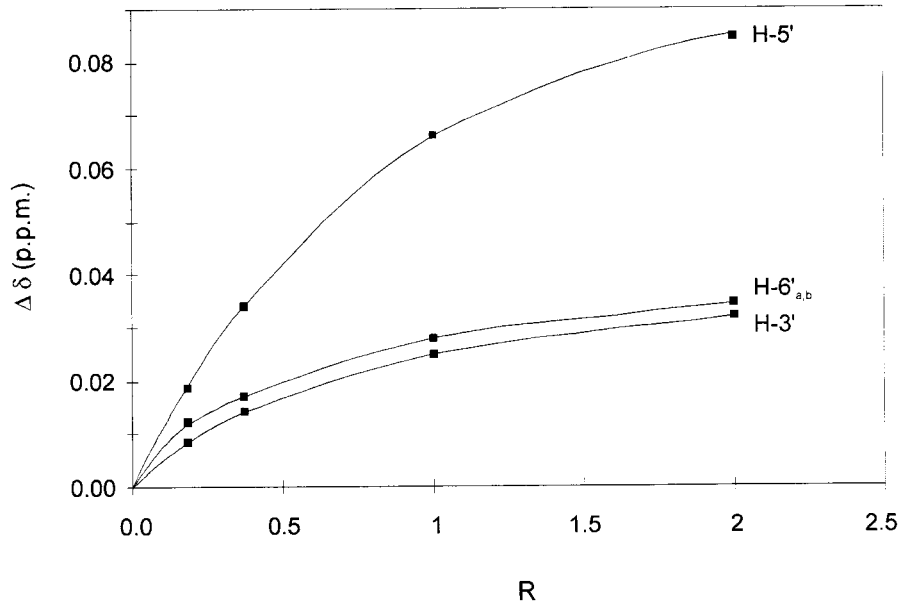

Figure 6. Plots of the chemical shift changes for β-cyclodextrin H-5′, H-6′$_{a,b}$ and H-3′ against the molar ratio of (+) zileuton to γ-cyclodextrin.

Chiral discrimination did show up clearly in the chemical shift changes of the guest resonances in the presence of γ-cyclodextrin and was particularly evident for zileuton H-5. Another remarkable feature was the different behaviour of the H-2,3 guest resonances which were not affected by inclusion complex formation with β-cyclodextrin but showed with γ-cyclodextrin a maximum upfield shift of 0.148 ppm under our experimental conditions (host concentration 6.50×10^{-3} M; guest concentration 1.2×10^{-3} M).

2D ROESY experiments run with the (+) zileuton-γ-cyclodextrin complex displayed small nuclear Overhauser effects between all the guest resonances and the H-3′, H-5′ and H-6′$_{a,b}$ of the host (Figure 7), pointing to a deep penetration of zileuton into the cyclodextrin cavity. Very similar features were also displayed by the (−) zileuton-γ-cyclodextrin complex while the very low solubility of the zileuton-β-cyclodextrin complexes did not allow observation of any NOE.

On the basis of the 1D and 2D NMR results, the structures of the zileuton inclusion complexes with β- and γ-cyclodextrins (in aqueous solution) were postulated in which the aromatic guest is inserted through the narrower opening of the host cavity while the hydroxy urea moiety mainly interacts with the wider rim (see Figure 8).

The chemical shift changes observed upon complex formation were used to determine the apparent association constants. A 1 : 1 complex stoichiometry was assumed throughout this study because zileuton presents only one interaction moiety (the benzothiophene ring system) and its molecular dimensions seem to preclude

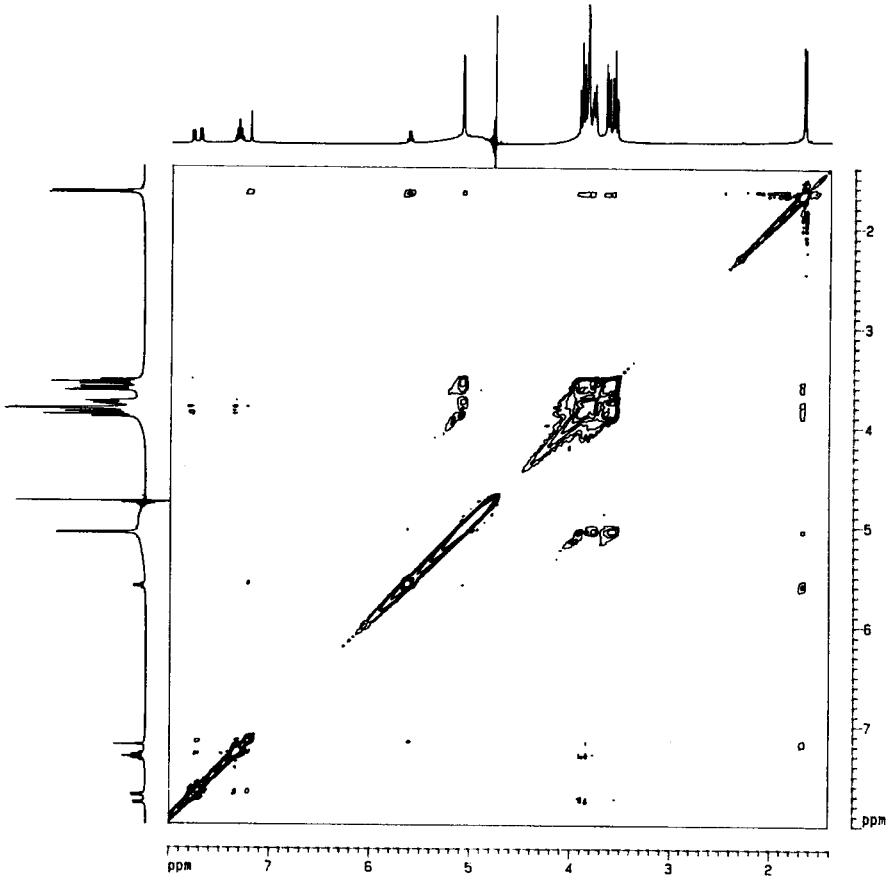

Figure 7. 400 MHz ROESY spectrum of γ-cyclodextrin (6.2×10^{-4} M) – (+) zileuton (1.2×10^{-3} M) solution in D_2O.

the inclusion of more than one guest entity. The following equation was used for each diastereomeric complex:

$$K_{ap} = \frac{[c]}{([cd]_t - [c]) \cdot ([z]_t - [c])} \tag{1}$$

where [c], $[cd]_t$ and $[z]_t$ indicate the complex, total cyclodextrin and total zileuton concentrations, respectively. The concentrations of the zileuton enantiomers were in the range of $1.0 \times 10^{-4} - 2.2 \times 10^{-4}$ M with β-cyclodextrin concentrations varying between 3.2×10^{-5} M and 1.8×10^{-4} M; concentrations of γ-cyclodextrin between 6.2×10^{-4} and 6.5×10^{-3} M were used with the guest isomers ranging between 1.2×10^{-3} and 1.38×10^{-3} M. The complex concentration [c] was related to the observed chemical shift through the relationship [c] =

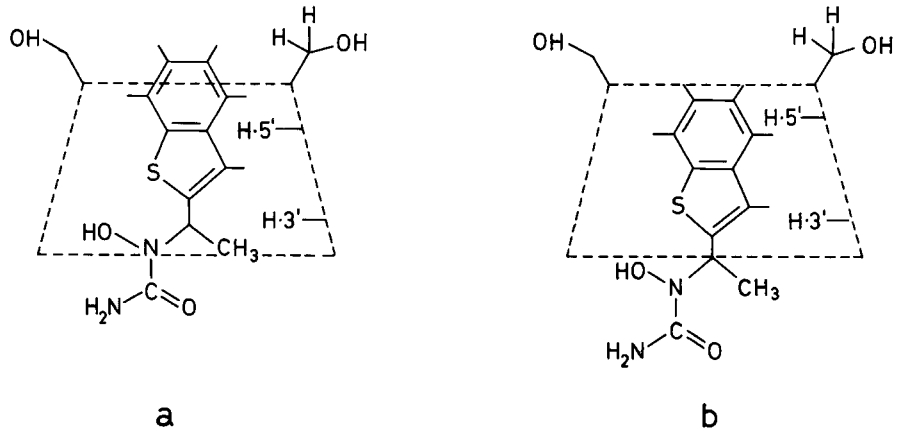

Figure 8. Suggested host-guest geometries for the inclusion complexes of zileuton with β-cyclodextrin (a) and γ-cyclodextrin (b).

Table I. Apparent association constants and chemical shift differences (H-5′ of the cyclodextrins) for complex formation between zileuton and β- and γ-cyclodextrins in aqueous solution at 25 °C

Complex	K_{ap} (M^{-1})	$\Delta\delta_c$ [a] (ppm)
(+) zileuton-β-cyclodextrin	5436 ± 735[b]	0.305 ± 0.005[b]
(−) zileuton-β-cyclodextrin	5752 ± 246	0.337 ± 0.005
(+) zileuton-γ-cyclodextrin	2544 ± 220	0.122 ± 0.005
(−) zileuton-γ-cyclodextrin	1844 ± 176	0.131 ± 0.005

[a] Defined as $\delta_0 - \delta_c$, where δ_0 and δ_c are the chemical shifts in the uncomplexed and fully complexed states, respectively.
[b] Standard deviation.

$\Delta\delta_{obs}/\Delta\delta_c \cdot [cd]_t$, where $\Delta\delta_c$ is the chemical shift difference for the pure complex. After some rearrangements we obtain:

$$1/K_{ap} = [z]_t \cdot \Delta\delta_c/\Delta\delta_{obs} - [z]_t - [cd]_t + \Delta\delta_{obs}/\Delta\delta_c \quad (2)$$

The signal position of cyclodextrin H-5′ was chosen to determine the apparent association constants as this signal appeared most sensitive to the inclusion process.

The values of K_{ap} and $\Delta\delta_c$ for the various complexes were obtained by non linear least squares fitting of Equation (2) using the BMDP software package [6] and are reported in Table I.

The differences found in the K_{ap} values for inclusion in β- and γ-cyclodextrins seemed to confirm that inclusion processes are very sensitive to the relative size of the cavity and the substrate: β-cyclodextrin (6.0–6.4 Å internal diameter) gave rise to a snug fit with the guest while the binding to γ-cyclodextrin with its larger

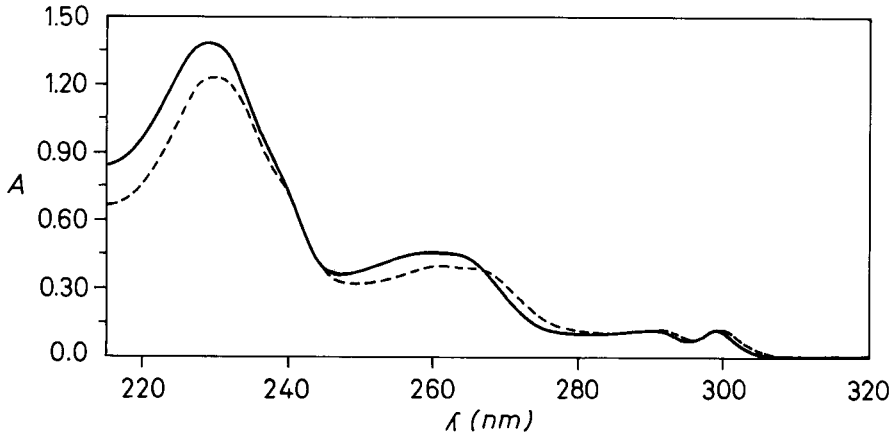

Figure 9. UV spectra of (+) zileuton (5.05×10^{-5} M) in aqueous solution in the absence (——) and in the presence (- - - -) of 4.5×10^{-3} M β-cyclodextrin.

cavity was poorer, in accord with previous observations [7]. Moreover the K_{ap} values as well as the $\Delta\delta_c$ values (for cyclodextrin H-5′) were practically indistinguishable for the diastereomeric complexes of zileuton with β-cyclodextrin, while γ-cyclodextrin exhibited a slight preference for complexation with the (+) isomer of the guest; this preference is responsible for the chiral discrimination observed in the NMR spectra.

3.3. UV AND CIRCULAR DICHROISM STUDIES

The UV spectra of (+) zileuton both in the absence and in the presence of β-cyclodextrin is shown in Figure 9.

The UV spectrum of zileuton in aqueous solution consists of three bands centred at 288–289 nm, 257–259 nm and 227–230 nm, respectively. All these absorption bands have been predicted to arise from $\pi \rightarrow \pi^*$ transitions [3]. In the presence of β-cyclodextrin a negligible effect was observed on the wavelength of the absorption maxima while there was a decrease in their intensity. Similar results were also obtained in the presence of γ-cyclodextrin.

The effect of the cyclodextrins on the UV spectra of zileuton was quantitatively investigated by holding the concentration of the guest constant (at 5.05×10^{-5} M and 6.8×10^{-5} M for the (+) and (−) isomers of zileuton, respectively) and by varying the host concentration between 2.5×10^{-4} and 4.5×10^{-3} M for β-cyclodextrin and between 2.5×10^{-4} and 5.0×10^{-3} M for γ-cyclodextrin. The K_{ap} values for the different complexes are reported in Table II. They were computed using the following equation on the assumption of a 1 : 1 complex stoichiometry (see NMR results):

Table II. UV and CD determined apparent association constants, molar absorptivity and molecular ellipticity differences for complex formation between zileuton and β- and γ-cyclodextrins in aqueous solution at 25 °C

Complex	UV		CD	
	K_{ap} (M^{-1})	$\Delta\epsilon$[a] ($M^{-1}\cdot cm^{-1}$)	K_{ap} (M^{-1})	$\Delta[\Theta]$[b] ($deg\cdot cm^2\cdot dmol^{-1}$)
(+) zileuton-β-cyclodextrin	5866 ± 738[c]	3211 ± 15[c]	5071 ± 182[c]	51200 ± 190[c]
(−) zileuton-β-cyclodextrin	5686 ± 216	3174 ± 48	4508 ± 218	-25500 ± 65
(+) zileuton-γ-cyclodextrin	1683 ± 184	1248 ± 17	1581 ± 303	8400 ± 455
(−) zileuton-γ-cyclodextrin	936 ± 120	1996 ± 50	720 ± 211	3725 ± 270

[a] Defined as $\epsilon_0 - \epsilon_c$, where ϵ_0 and ϵ_c are the molar absorptivities of the high frequency band in the uncomplexed and fully complexed states, respectively.
[b] Defined as $[\Theta]_c - [\Theta]_0$, where $[\Theta]_c$ and $[\Theta]_0$ are the molecular ellipticities of the high frequency band in the fully complexed and uncomplexed states, respectively.
[c] Standard deviation.

$$\frac{[cd]_t}{\Delta A} = \frac{1}{K_{ap}([z]_t \cdot \Delta\epsilon - \Delta A)} + \frac{1}{\Delta\epsilon} \tag{3}$$

where ΔA is the absorbance change at λ = 229 nm upon addition of cyclodextrin while $\Delta\epsilon$ is the difference in molar absorptivity between free and complexed zileuton; $[cd]_t$ and $[z]_t$ are the total cyclodextrin and total zileuton concentrations, respectively. Equation (3) was solved by non linear least squares fitting, using the BMDP software package.

Quite good agreement was observed between the K_{ap} values derived by NMR spectroscopy and those obtained by the UV measurements, confirming the stronger interaction of zileuton with β-cyclodextrin while a better chiral discrimination was exerted by γ-cyclodextrin.

These conclusions were also supported by the circular dichroism study. Figure 10 shows the CD spectra of (+) zileuton both in the absence and in the presence of β-cyclodextrin. The CD spectrum of (+) zileuton in aqueous solution consists of three absorption bands located at 233 nm (molecular ellipticity $[\Theta]$ = 12000 $deg\cdot cm^2\cdot dmol^{-1}$), ∼262 nm ($[\Theta]$ = 6800 $deg\cdot cm^2\cdot dmol^{-1}$) and ∼290 nm ($[\Theta]$ = -1950 $deg\cdot cm^2\cdot dmol^{-1}$). Upon addition of β- cyclodextrin there was a big increase in the high frequency band intensity, while the rest of the spectrum was only slightly affected. For (−) zileuton the absorption band at 233 nm became more negative and was red shifted by ∼7 nm while the one at 262 nm almost disappeared. The γ-cyclodextrin effect on the guest CD spectrum was much less pronounced, resulting in an intensity increase of the 233 nm band of (+) zileuton while the corresponding band of the (−) isomer became less negative.

In order to compute the K_{ap} values for the different complexes the concentration of the guest was held constant (at 5.05×10^{-5} M and 6.8×10^{-5} M for the (+)

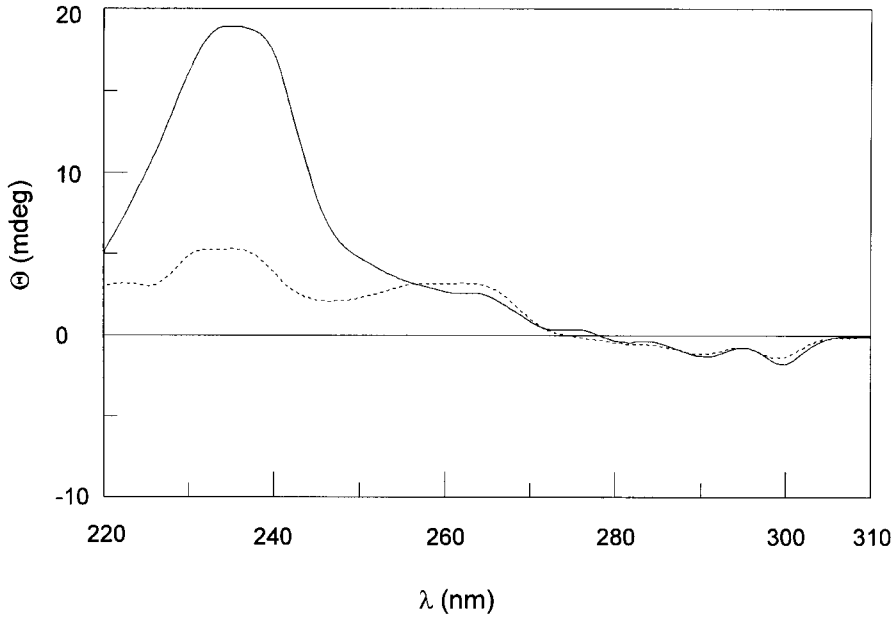

Figure 10. CD spectra of (+) zileuton (5.05×10^{-5} M) in aqueous solution in the absence (- - - -) and in the presence (——) of 2.5×10^{-4} M β-cyclodextrin.

and (−) isomers of zileuton, respectively) and the host concentration was varied between 2.5×10^{-4} and 4.5×10^{-3} M for β-cyclodextrin and between 2.5×10^{-4} and 5.0×10^{-3} M for γ-cyclodextrin. The experimental data were fitted to the following equation:

$$\frac{[\mathrm{cd}]_t}{\Delta\Theta} = \frac{1}{K_{ap}([z]_t \cdot \Delta[\Theta]' - \Delta\Theta)} + \frac{1}{\Delta[\Theta]'} \tag{4}$$

where $\Delta\Theta$ is the observed ellipticity change for the high frequency band of the guest molecule; $\Delta[\Theta]' = \Delta[\Theta] \times 10^{-2}$, $\Delta[\Theta]$ being the corresponding molecular ellipticity difference between complexed and free zileuton. Equation (4) was solved by non linear least squares fitting; the K_{ap} values are reported in Table II.

4. Conclusion

The requirements for chiral recognition by cyclodextrins have been postulated by Armstrong et al. [8]: (a) an inclusion complex must be formed and (b) there must be a relatively tight fit between the guest and the host and the chiral centre or one of its substituents must be near and interact with the mouth of the cyclodextrin cavity. In this regard the unidirectional 2′- and 3′-hydroxyl groups of the host are considered to play a relevant role. All these requirements are satisfied for the complexes

formed between zileuton and β-and γ-cyclodextrins. The K_{ap} values for these complexes are characteristic of a quite tight interaction which was stronger with β-cyclodextrin, possibly because of a better host-guest complementarity. However the K_{ap} values only showed a very small difference, if any, for the β-cyclodextrin complexes with (+) and (−) zileuton enantiomers. This lack of enantioselectivity could possibly be accounted for by the conformational flexibility of the hydroxyurea moiety of the guest [3] which made both enantiomers to fit equally well in the host cavity.

γ-Cyclodextrin was found to be a slightly better chiral selector but the experimental results did not allow us to postulate a sound mechanism for chiral recognition.

Nevertheless the increased solubility of zileuton in the presence of γ-cyclodextrin together with the preferential inclusion of the (+) isomer of the guest could have a relevant importance from a pharmaceutical point of view, also taking into account that a stereoselective glucoronidation of the zileuton isomers by human hepatic microsomes has been recently demonstrated [9].

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