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A Preliminary Investigation of the Solution Complexation of 4-Sulphonic calix[n]arenes with Testosterone

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Abstract

The complexation between water soluble calixarenes and testosterone has been studied. Stability constants of the host guest complexes of 4-sulphonic calix[n]arenes (n = 4, 6 and 8) with testosterone in water and buffers (pH 5.8, 7.3 and 10.0) were determined from phase solubility curves. These solubility curves indicated that the complexes were all of the A_L type. The constants were in the range 26–341 M^{-1}, dependent on the size of the calixarene and the pH of the solutions.

Introduction

Over the last fifty years the chemistry of macrocycles of both natural and synthetic origin has developed dramatically. Examples of this class of compounds include the crown ethers, cyclodextrins, cryptands and calixarenes. The chemical properties of these compounds have been employed in a wide variety of both academic and commercial applications. The ability of these compounds to form complexes is one property that has been utilised extensively. The crown ethers have found wide application in a range of synthetic chemical procedures especially in the area of phase transfer catalysis [1]. In the case of cyclodextrins their complexation properties have been utilised in a range of chromatographic and electrophoretic techniques [2, 3]. These compounds have been employed as mobile phase additives or as stationary phases in TLC, GLC, HPLC and CE. Both achiral and especially chiral chromatographic separations have benefited tremendously from this use of cyclodextrins. In the case of calixarenes their ion sequestering capabilities have been described in a number of patents. The selectivity of calixarenes in terms of ion complexation has been employed in the construction of ion selective electrodes [4].

The formation of inclusion complexes and the nature of the complexes has also been widely investigated by the pharmaceutical industry [5]. Over the last thirty years there have been many investigations into the use of cyclodextrins in drug formulation and delivery. The range of investigations has covered aspects such as enhanced solubility, stability improvement and dissolution/bioavailability modification. Many of the early investigations involved, naturally enough, native cyclodextrins and there are numerous examples of beneficial complexations in the literature. Subsequently it was discovered that there were toxicity problems associated with these natural cyclodextrins that limited their potential use in human and animal pharmaceutical products. Despite this setback investigations into the utilisation of cyclodextrins continues with modified cyclodextrins that appear to have improved toxicity profiles. It is reported that the FDA is at present considering a number of Drug Master Files of products containing cyclodextrins [5].

There are few publications in the literature that describe the formation of complexes between drug substances and calixarenes and there are no reports of possible uses of calixarenes as pharmaceutical enabling agents. Parini et. al. [6] have described the solid state interaction of steroids with calixarenes. The calixarenes used in this study were non water soluble compounds [4-tert-butylcalix[4]arene, 4-tert-butylcalix[6]arene, 4-tert-butylcalix[8]arene]. These authors suggest that co-grinding or co-precipitation cause steroids and calixarenes to interact via complex formation. Information presented to substantiate these claims include FTIR and DSC evidence. There is a suggestion in the paper that in solution the complexes formed between the calixarenes and the steroids are of low stability, however, no detailed study of the solution complexation was undertaken. Higler *et al.* [7] have described the synthesis of a series of steroid receptor molecules based on the combination of calix[4]arenes with a tetrahydroxy cavitand. These molecules were shown to complex several structurally related corticosteroids. The present paper describes investigations into the solution complexation of water soluble calixarenes with testosterone.

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Experimental

Chemicals

Testosterone was obtained from Sigma. The 4-sulphonic calix[4]arene, 4-sulphonic calix[6]arene and 4-sulphonic calix[8]arene were obtained from Acros Organics, Belgium. HPLC grade acetonitrile was purchased from Lab Scan Analytical Services, Dublin. Potassium dihydrogen orthophosphate, orthophosphoric acid and disodium hydrogen orthophosphate were obtained from BDH (Poole, England). All water was processed using a Millipore-Q Reagent System (Waters, England). Filtration of HPLC mobile phases was performed using Gelman FP-450 Filters (Pall Gelman Sciences, Northampton, England).

HPLC determination of testosterone

The chromatography system consisted of a Waters 712 WISP, a Waters 600E system controller and a Waters 990 Photodiode Array Detector. Data collection was by means of the Waters Powerline System. The HPLC column was a 250 × 4.6 mm Spherisorb 55 ODS 2 column [Supplied by AGB, N. Ireland]. The mobile phase was 25% water/75% acetonitrile. The flow rate was 1 mL/min and detection was at 240 nm. Standard solutions containing testosterone [1–10 μg/mL] and internal standard [methyltestosterone: 5 μg/mL] dissolved in the mobile phase were injected [20 μL] in triplicate onto the column and a calibration curve constructed using peak height ratios.

Testosterone solubility studies

Solubility measurements were carried out according to standard procedure. Testosterone (10 mg) was added to each sample vial and 10 mL of either water or buffer solutions containing various concentrations of 4-sulphonic calix[n]arene were added. The samples were equilibrated for 72 h at 25 °C using a shaking water bath. The samples were filtered and 1 mL of this solution was mixed with 200 μL of internal standard solution (methyltestosterone; 250 μg/mL) and diluted to 10 mL. The concentration of testosterone was then determined using the HPLC method described above. Each determination was conducted in triplicate.

Testosterone solubility curves

Testosterone solubility curves were constructed by plotting testosterone concentration versus calix[n]arene concentration. From these plots stability constants for the complexation of testosterone with calix[n]arenes were determined.

Results and discussion

Following initial investigations a reverse phase HPLC method suitable for the resolution of testosterone, methyltestosterone (internal standard) and 4-sulphonic calix[n]arenes was developed. This method involved the use of a conventional ODS column and a mobile phase consisting of 25% water/75% acetonitrile. The retention times for testosterone and the internal standard (methyltestosterone) were 7.3 and 8.5 min. respectively and these compounds were baseline resolved. For solutions containing the 4-sulphonic calix[n]arenes the retention times for testosterone and methyltestosterone were unchanged and the 4-sulphonic calix[n]arenes eluted at approximately 1.5 min. Calibration curves for testosterone were constructed using peak height ratios [typical equation $y = 0.22310x + 0.018431$; $r^2 = 1.000$] .

The solubility of testosterone in water and in buffer solutions at various pHs was determined in the absence and presence of the 4-sulphonic calix[n]arenes according to standard methods. In all cases the concentration of the 4-sulphonic calix[n]arenes was below the critical micelle concentration [8]. Phase solubility diagrams for testosterone were constructed from these data and Figure 2 illustrates these curves for solutions containing 4-sulphonic calix[4]arene, 4-sulphonic calix[6]arene and 4-sulphonic calix[8]arene in water. Corresponding phase diagrams were constructed for the experiments conducted with 4-sulphonic calix[6]arene in phosphate buffers at pH 5.8, 7.3 and 10.0 and Table 1 details the results of linear regression analysis of these data. All curves were linear with high r^2 values indicating A_L type behaviour [9] with the formation of soluble complexes. The slopes of the curves observed in the regression analysis would indicate that 1 : 1 complexation was occurring although the formation of complexes with other ratios is not precluded. Apparent stability constants K_c were calculated from the lines according to Equation (1).

$$K_c = \frac{\text{slope}}{\text{intercept(1-slope)}}. \qquad (1)$$

The calculated K_c values are recorded in Table 1. The calculated constants indicate that, in water, the K_c values increase with increasing numbers of phenyl moieties within the calixarene suggesting that the size of the cavity within the calix influences the complexation. Table 1 also details the K_c values for testosterone in the presence of the 4-sulphonic calix[n]arenes at pH 7.3 and also at 5.7 and 10 for 4-sulphonic calix[6]arene. These data suggest that the K_c values vary with pH. Uekama *et al.* [10] and Albers and Muller [11] have investigated the complexation of cyclodextrins and steroid hormones. Uekama *et al.* [10] demonstrated that with α-cyclodextrin the complexation was of either the A_L or A_P type whereas with β- or γ-cyclodextrins B_S type behaviour was predominant although a small number of the steroids displayed A_L or A_P type solubility curves. Similar observations were also made by Albers and Muller [11] when they studied complexations with β-cyclodextrin. The complexation between β-cyclodextrin and testosterone was shown to be of the B_S type and the solubility limit for the complex occurred at approximately 70 μg/mL of testosterone. These authors also investigated the solubility curves for a range of steroids with 2-hydroxypropyl β-cyclodextrin. This cyclodextrin is considerably more water soluble than the parent cyclodextrins.

Table 1. Phase solubility curve data (linear regression analysis) and apparent stability constants

Solution	x	y	r^2	$K_c \pm se$
4-Sulphonic calix[4]arene in water†	0.0031	7×10^{-5}	0.9227	44 ± 4
4-Sulphonic calix[6]arene in water†	0.0147	8×10^{-5}	0.9977	202 ± 5
4-Sulphonic calix[8]arene in water†	0.0330	1×10^{-4}	0.9959	355 ± 17
4-Sulphonic calix[6]arene in buffer pH 5.8*	0.0098	7×10^{-5}	0.9814	144 ± 9
4-Sulphonic calix[6]arene in buffer pH 7.3*	0.0232	7×10^{-5}	0.9734	346 ± 39
4-Sulphonic calix[4]arene in buffer pH 7.3*	0.0026	1×10^{-4}	0.9479	26 ± 22
4-Sulphonic calix[8]arene in buffer pH 7.3*	0.0159	1×10^{-4}	0.9850	156 ± 9
4-Sulphonic calix[6]arene in buffer pH 10.0*	0.0144	6×10^{-5}	0.9573	226 ± 27

†Solutions of 4-sulphonic calix[n]arene in water result in low pHs: for example a 1 mM solution of 4-sulphonic calix[6]arene has a pH of 2.3.
*Phosphate buffers prepared according to the British Pharmacopoeia 1998 Appendix 1D.

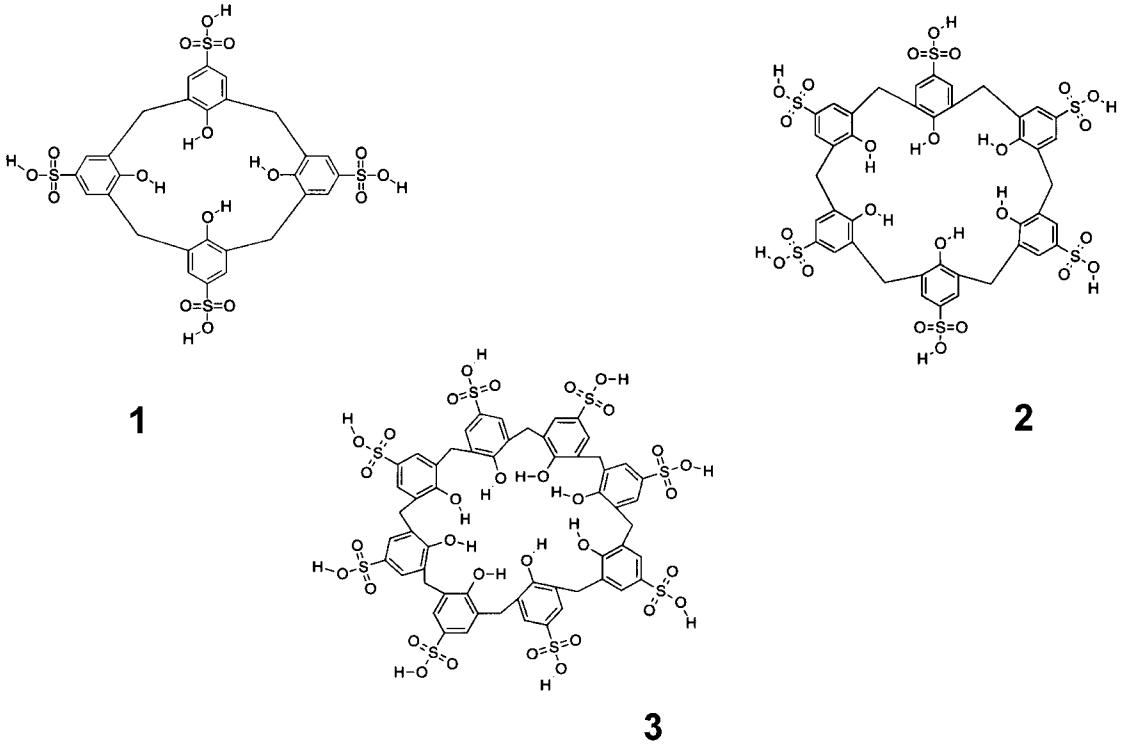

Figure 1. The structures of 4-sulphonic calix[4]arene (**1**), 4-sulphonic calix[6]arene (**2**) and 4-sulphonic calix[8]arene (**3**).

With this compound the series of steroids displayed A_L or A_P type solubility curves and in the case of testosterone solubilities of approximately 10 mg/mL were achieved in solutions containing 10% 2-hydroxypropyl β-cyclodextrin. Using data presented by Albers and Muller [11] it would appear that at a molar concentration of 2-hydroxypropyl β-cyclodextrin equivalent to the maximum molar concentration of calixarene used in the present study the solubility of testosterone was approximately eight times greater than the maximum observed in this investigation. Apparent stability constants for testosterone/cyclodextrin complexes were determined by Uekama *et al.* [10] as α-cyclodextrin (134) β-cyclodextrin (7540) and γ-cyclodextrin (16500). The values for β- and γ-cyclodextrin are considerably greater than those observed in the present study for the complexation between the calixarenes and testosterone.

The nature of the complexation between calixarenes and hosts is dependent on a number of factors. Figure 1 details the basic structures of the 4-sulphonic calix[n]arenes. The conformation of the calixarene structures in solution is governed by the size of the ring and also by the pH of the solution. It has been reported that the tetrameric calixarenes exist in the cone conformation, hexamers as the 'winged or hinged' conformations and the octamers as 'pleated loops' [12]. Arena *et. al.* [13] have described 4-sulphonic calix[4]arene as conformationally mobile whilst Atwood *et al.* [14] have proposed from X-ray studies that 4-sulphonic calix[6]arene exists in a double partial cone conformation (Figure 3). The pH of the solution influences the shape of the calixarene in terms of the dissociation of the phenolic OH groups and the extent of the hydrogen bonding of the hydroxyls. Alvarez *et al.* [15], following molecular modelling studies, have proposed that at neutral pH

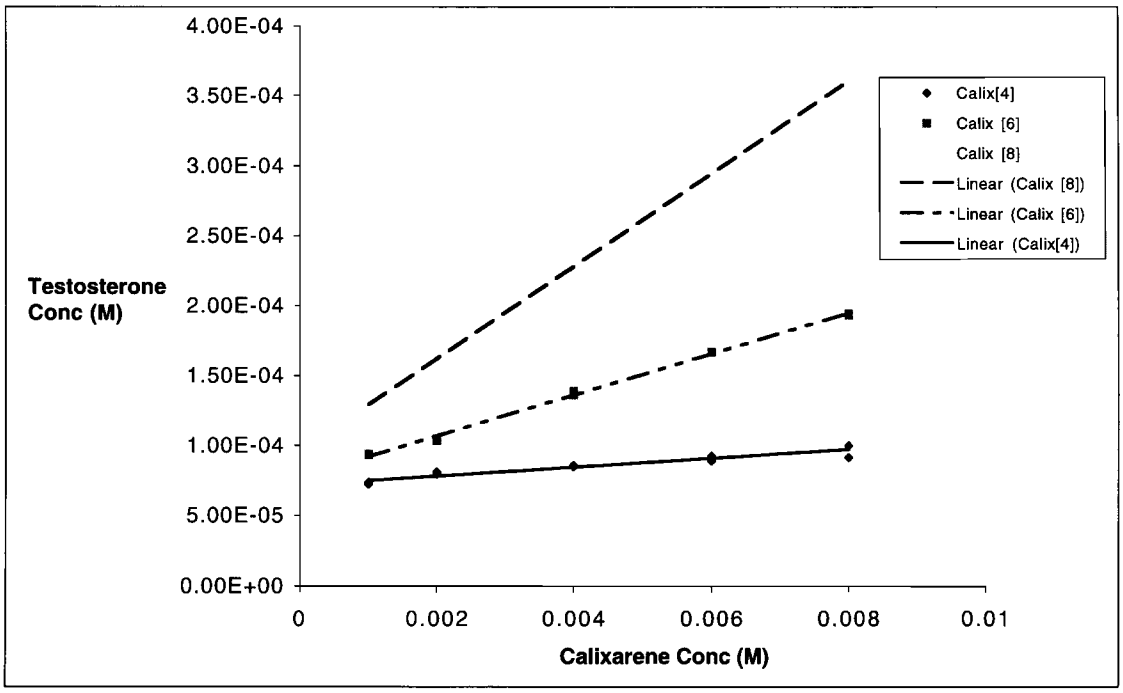

Figure 2. Phase solubility curves for testosterone in the presence of 4-sulphonic calix[4]arene, 4-sulphonic calix[6]arene and 4-sulphonic calix[8]arene.

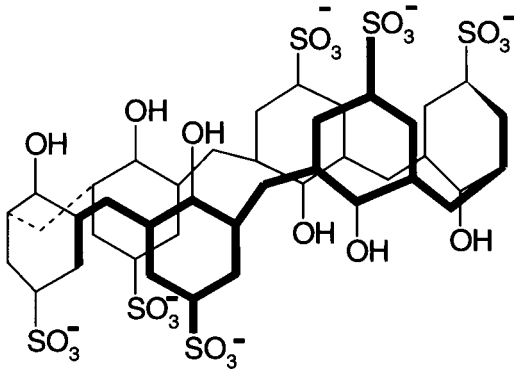

Figure 3. Structure of 4-sulphonic calix[6]arene (**2**) showing the double partial cone conformation (based on the proposed structure by Atwood *et al.* [13]).

4-sulphonic calix[6]arene exists as the octaanionic host and the most stable conformation is similar to the double partial cone proposed by Atwood *et al.* [14] whilst in the hexaanionic form, with the phenolic hydroxyls fully protonated, a flattened, puckered conformation is adopted. Shinkai *et al.* [16] studied the complexation of tetrasodium 4-sulphonic calix[4]arene with trimethylanilinium chloride in D_2O at pD 0.4 and pD 7.3. At the lower pD the calixarene selectively complexes the phenyl moiety whereas at pD 7.3 both the phenyl and the trimethylammonium moieties are included in the cavity in a nonspecific fashion.

The process of complexation in the case of the 4-sulphonic calix[*n*]arenes and testosterone, as studied in this work, is uncertain. Preliminary proton NMR studies were inconclusive due to the low solubility of testosterone in D_2O. UV spectroscopic studies were untenable due to the strong absorption of the calixarenes in the 200–300 nm region, λ_{max} for testosterone being 240 nm. The intrinsic solubility and the equilibrium solubility [as determined by the intercept of solubility curves] of testosterone were not influenced by pH. On the basis of the data obtained to date it would seem likely that the size and the shape of the calixarenes is influencing the complexation. The 4-sulphonic calix[4]arene shows very weak complexation (See Table 1) perhaps reflecting the fact that the cavity of this species is too small to fully accommodate the testosterone molecule. Shinkai *et al.* [17] have estimated that the diameter of the upper rim of calix[4]arenes as 3.8 Å, the calix[6]arene as 5.0 Å and reported that the calix[8]arene diameter to be difficult to estimate due to conformational fluctuation. The dissociation of the hydroxyl groups of 4-sulphonic calix[*n*]arenes will obviously influence the conformation of these compounds. The reported pK_a values for the phenolic OH groups in 4-sulphonic calix[*n*]arenes vary considerably and appear to be influenced by the nature of the electrolytes present [18–21]. UV spectrophotometric studies on 4-sulphonic calix[6]arene has demonstrated a red shift in the absorption spectrum over the pH range 2.0 to 13 [22]. The authors attribute this shift to the intramolecular hydrogen bonding of adjacent hydroxyl groups following dissociation. Similar observations for 4-sulphonic calix[*n*]arenes in general were reported [21]. It would therefore appear that, over the pH range studied in this investigation, there are changes in the hydrogen bonding of the calixarene hydroxyls which result in conformational changes in the calixarenes. These changes are reflected in the changing stability constants of the complexes with testosterone. The variability observed in the stability constants of the complexes of testosterone with 4-sulphonic calix[6]arene and 4-sulphonic calix[8]arene cannot yet be clearly defined. Present studies aimed at isolating the calixarene/testosterone complexes and further NMR studies using more water sol-

uble 4-ene-3-keto steroids (cortisone, hydrocortisone) will hopefully provide insight into the complexation process.

Conclusion

The complexation of 4-sulphonic calix[*n*]arenes and testosterone in aqueous solutions at various pHs has been demonstrated. The low values for the apparent stability constants for the complexation between 4-sulphonic calix[4]arene and testosterone suggest that the steroid does not enter the calix due to the size of the cavity. Moderate complexation is observed in the case of 4-sulphonic calix[6]arene and 4-sulphonic calix[8]arene. This complexation results in enhanced solubility of testosterone which, in the case of 4-sulphonic calix[6]arene and 4-sulphonic calix[8]arene, is superior to the enhancement observed with β-cyclodextrin and approaches that with 2-hydroxypropyl β-cyclodextrin. Concurrent investigations have demonstrated the (aqueous) complexation of 4-sulphonic calix[*n*]arenes with a range of other drugs suggesting that these water soluble calixarenes may be as versatile as the cyclodextrins in their complexation properties. It would appear that water soluble calixarenes may have potential as pharmaceutical enabling agents although the toxicity aspects of these compounds would need to be investigated.

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