J Incl Phenom Macrocycl Chem (2007) 57:185–190
DOI 10.1007/s10847-006-9198-7

ORIGINAL ARTICLE

Inclusion complexes of fusidic acid and three structurally related compounds with cyclodextrins

Kim Lambertsen Larsen · Stig Bruse Andersen · Anne Louise Mørkbak · Reinhard Wimmer

Received: 15 May 2006 / Accepted: 20 October 2006 / Published online: 17 January 2007

Abstract The inclusion complexes between fusidate, 3-keto fusidate, 11-keto fusidate and 11-deoxy fusidate and α-, β-, and γ-cyclodextrin (CD) were studied using capillary electrophoresis. By monitoring the changes in mobility of the negatively charged compounds in the presence of varying amount of CD the stability constants of the complexes formed could be obtained. In the case of α- and β-CD the obtained results could be modelled to a simple model assuming 1:1 stoichiometry, revealing, not surprisingly, that β-CD formed a stronger complex compared to α-CD. A model assuming 1:2 (fusidate:CD) stoichiometry could be fitted to the data obtained with γ-CD. The results showed that the different fusidanes formed very strong 1:1 complexes with γ-CD as well as a quite weak 1:2 complex. 3-keto-, 11-keto- and 11-deoxy-fusidate formed stronger complexes compared to fusidate, probably due to an decrease in hydrophilicity caused by the reduced number of hydroxyl groups. The complex between γ-CD and fusidate was studied by use of 2D-NMR spectroscopy. The results showed that most of the hydrogen atoms of fusidate show interactions with the hydrogen atoms in the cavity of γ-CD. The interaction pattern suggests that fusidate may be fully embedded in the cavity of γ-CD. No interactions between fusidate and the hydrogen atoms situated at the outside of the CD were found.

Keywords Cyclodextrin · Fusidic acid · Capillary electrophoresis · Complex formation · 2D-ROESY NMR

Abbreviations

CD	Cyclodextrin
COSY	Correlation spectroscopy
DQF	Double Quantum Filtered
ROESY	Rotating Overhauser Effect Spectroscopy

Introduction

Fusidic acid is a steroid-like antibiotic compound belonging to the group of fusidanes produced by fermentation by the fungus *Fusidium coccineum* (Fig. 1). Fusidic acid is widely used in dermatology [1] and is marketed in a wide range of formulations for oral, topical and intravenous use [2].

Cyclodextrins, cyclic α-1,4 linked glucose oligosaccharides, are well known to be able to form complexes with steroid and steroid like molecules, resulting in complexes with often higher solubility, dissolution rate and bioavailability, compared to the drug alone [3–6]. This has spurred a numbers of studies on the structure-complex stability relationship between steroids and cyclodextrins [7–11], as well as detailed structural studies of the complexes formed between cyclodextrins and steroids and steroid like molecules [11–19].

K. L. Larsen (✉) · S. B. Andersen · R. Wimmer
Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University, Sohngaardsholmsvej 49/57, 9000 Aalborg, Denmark
e-mail: kll@bio.aau.dk

A. L. Mørkbak
Department of Clinical Biochemistry, AS, Aarhus University Hospital, Aarhus, Denmark

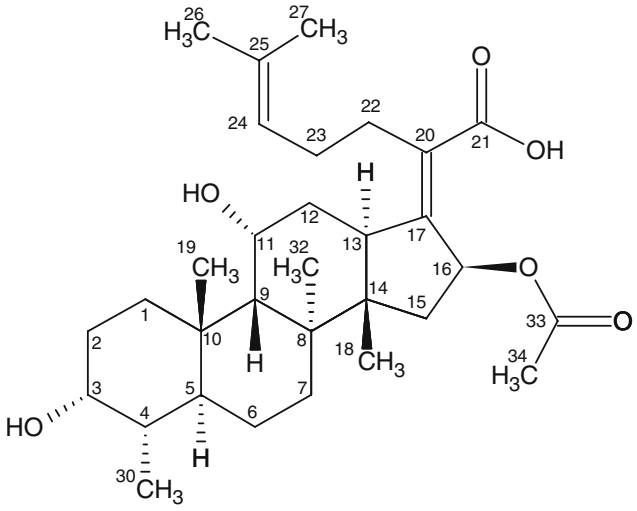

Fig. 1 Chemical structure of fusidic acid

A detailed study of the complex between β- and γ-CD and fusidate based on various NMR techniques has been published previously [17, 18]. They show that γ-CD forms 1:1 complexes with fusidate, where the fusidate is fully accommodated in the cavity of the CD. β-CD on the other hand was found to form 1:2 (fusidate:CD) complexes with one β-CD covering each end of the molecule.

In this work we present a study of the complexes formed by fusidate and three related compounds with α- β- and γ-CD by use of affinity capillary electrophoresis. Furthermore, the complex between fusidate and γ-CD was investigated by 2D-NMR.

Experimental

Chemicals

Pharmaceutical grade cyclodextrins were obtained from Wacker-Chemie, Vallensbæk, Denmark. Fusidic acid, 11-deoxy fusidic acid, 3-ketofusidic acid and 11-keto fusidic acid was provided by Løvens Kemiske Fabrik, Ballerup, Denmark.

Capillary electrophoresis

Capillary electrophoresis was performed on a Beckman P/ACE MDQ (Beckman-Coulter, Fullerton, CA) equipped with a photodiode array detector. Fused silica capillaries with an inner diameter of 50 μm were obtained from Composite Metal Services LTD., Worcester, UK. CE analyses were performed in capillaries of a total length of 50 cm with 40.2 cm to the detector. Prior to first usage the capillaries were cleaned with 0.1 M HCl for 5 min at 40 psi, 0.1 M NaOH for 5 min at 40 psi and water for 2 min at 40 psi. Prior to analysis the capillaries were cleaned using 0.1 M NaOH for 30 s at 40 psi and water for 6 s at 20 psi. The capillaries were filled with buffer containing 50 mM phosphate buffer pH 8.0 and varying concentrations of cyclodextrins. Samples containing 2 mM solutions of fusidic acid and related compounds in 50 mM phosphate buffer, pH 8.0 containing 35 mM γ-CD were injected by applying vacuum for 5 s at 0.5 psi at the anionic side of the capillary. Before separation was commenced a small amount of the buffer solution was injected by applying a pressure of 0.5 psi for 10 s to the anionic side of the capillary. Separation was carried out using two identical buffer reservoirs applying 15 kV for 15 min. Temperature was kept constant at 25 °C and absorbance was monitored from 190 to 300 nm. About 2 mM 4-iodo benzoate and 1 mM benzyl alcohol was used as intern standard and EOF marker respectively.

NMR

NMR spectra were recorded on a BRUKER DRX600 spectrometer equipped with a 5 mm triple axis gradient TXI(H/C/N) probe. The NMR samples consisted of 25 mM fusidic acid and 25 mM γ-CD in D_2O. pD was adjusted to 9.5 (uncorrected meter readings) by addition of NaOD. All the spectra were recorded at 298 K. ^{1}H 2D DQF-COSY and ROESY with a 250 ms continuous wave spin-lock of 1.4 kHz was applied to obtain the assignment. The NMR data were processed with the BRUKER XwinNMR Ver. 2.5 software and the spectral analysis was done with XEASY Ver. 1.3.13 [20].

Results and discussion

Capillary electrophoresis

The stability of the inclusion complexes formed between four fusidates and α-, β- and γ-CD was studied by use of capillary electrophoresis using the direct absorbency detection methodology as described by Larsen and Zimmerman, 1999 [21] and Lee and Lin, 1996 [22]. Complex formation between the CD and the charged guest molecules will result in a reduction in the effective electrophoretic mobility of the guest molecule. By monitoring the change in electrophoretic mobility of the fusidates as function of the concentration

of CD the stability constants can be derived by fitting the data to appropriate equations. Assuming the formation of 1:1 complexes between the guest molecule and CD, the stability constant ($K_{1:1}$) and the mobility of the complex (μ_{ACD-}) can be obtained by non-linear regression analysis based on eq. 1.

$$\Delta\mu = \mu_{eff} - \mu_{A^-} = \frac{\mu_{A^-} + K_{1:1} \times [CD]_0 \times \mu_{ACD^-}}{1 + K_{1:1} \times [CD]_0} - \mu_{A^-} \quad (1)$$

Where μ_{eff} is the effective mobility of the guest, μ_{A-} is the mobility of the guest in absence of cyclodextrins, $[CD]_0$ is the concentration of CD in the buffer solutions. The mobility change $\Delta\mu$ can be derived by subtracting the mobility of the guest in pure buffer solution μ_{A-} from the effective mobility μ_{eff}. μ_{A-} was measured in 50 mM phosphate buffer, pH 8.0 to -1.27×10^{-4}, -1.30×10^{-4}, -1.25×10^{-4} and -0.76×10^{-4} $cm^2V^{-1}s^{-1}$ for fusidate, 3-keto fusidate, 11-keto fusidate and 11-deoxy fusidate, respectively.

In the case of α- and β-CD it was possible to fit the data to an equation assuming a 1:1 complex stoichiometry (Figs. 2, 3). This was not the case for the data obtained with γ-CD. Therefore an equation assuming 1:2 [guest:CD] complex stoichiometry was developed, Eq. 2

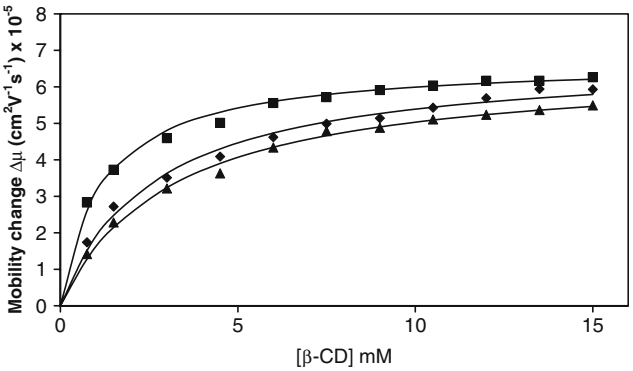

Fig. 3 Electrophoretic mobility change of fusidates as function of β-cyclodextrin concentration in 50 mM phosphate buffer pH 8.0 at 25°C. ♦, fusidate; •, 11-keto fusidate; ■, 3-keto fusidate. Solid curves were generated from Eq. 1 assuming a 1:1 complex stoichiometry using the parameters obtained by non-linear regression analysis

where $K_{1:2}$ and $\mu_{ACD_2^-}$ denotes the stability constant and mobility of the 1:2 complex, respectively. Using this equation it was possible to fit the data obtained for the complexes of the fusidates with γ-CD (Fig. 4). The stability constants obtained (Table 1) reveal that all three CD's form complexes with the fusidates in the strength order for α-CD < β-CD < γ-CD. α-CD formed relatively weak complexes which were also expected, since the fusidates or parts of the molecules are too

$$\Delta\mu = \mu_{eff} - \mu_{A^-} = \frac{\mu_{A^-} + K_{1:1} \times [CD]_0 \times \mu_{ACD^-} + K_{1:1} \times K_{1:2} \times [CD]_0^2 \times \mu_{ACD_2^-}}{1 + K_{1:1} \times [CD]_0 + K_{1:1} \times K_{1:2} \times [CD]_0^2} - \mu_{A^-} \quad (2)$$

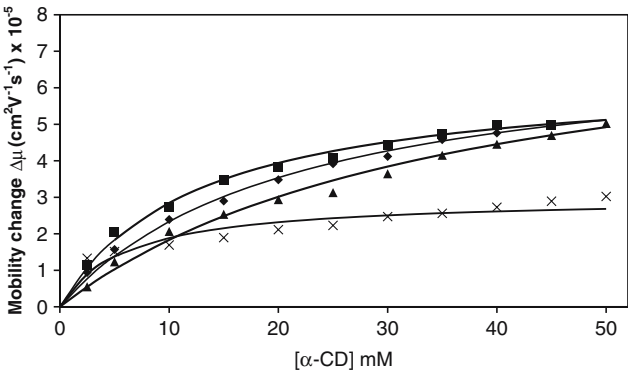

Fig. 2 Electrophoretic mobility change of fusidates as function of α-cyclodextrin concentration in 50 mM phosphate buffer pH 8.0 at 25°C. ♦, fusidate; •, 11-keto fusidate; ■, 3-keto fusidate; ×, 11-deoxy fusidate. Solid curves were generated from Eq. 1 assuming a 1:1 complex stoichiometry using the parameters obtained by non-linear regression analysis

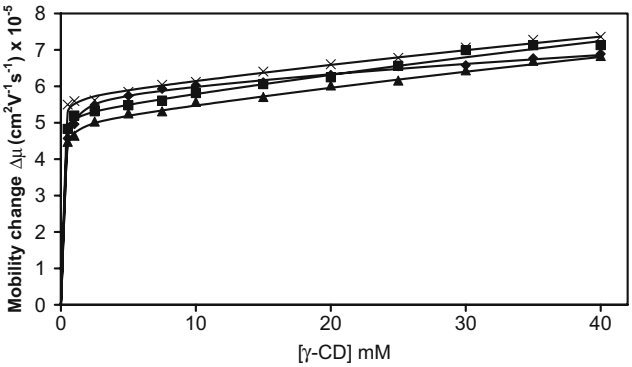

Fig. 4 Electrophoretic mobility change of fusidates as function of γ-cyclodextrin concentration in 50 mM phosphate buffer pH 8.0 at 25°C. ♦, fusidate; •, 11-keto fusidate; ■, 3-keto fusidate; ×, 11-deoxy fusidate. Solid curves were generated from Eq. 2 assuming a 1:2 (γ-CD:fusidate) complex stoichiometry using the parameters obtained by non-linear regression analysis

Table 1 Inclusion complex stability constants for fusidate and related compounds with α-, β-, and γ-CD in 50 mM phosphate buffer pH 8.0 at 25°C

	α-CD		β-CD		γ-CD			
	Log $K_{1:1}$	STD	Log $K_{1:1}$	STD	Log $K_{1:1}$	STD	Log $K_{1:2}$	STD
Fusidate	1.67	0.03	2.58	0.04	3.87	0.02	0.89	0.01
3-keto fusidate	1.90	0.03	2.93	0.03	4.37	0.12	0.63	0.02
11-keto fusidate	1.44	0.06	2.51	0.03	4.15	0.06	0.63	0.02
11-deoxy fusidate	2.23	0.11	2.62	0.18	4.37	0.09	0.63	0.02

bulky to fit into the narrow cavity of α-CD. Our data suggest that β-CD form a relative strong 1:1 complex with the fusidates. This contrasts the results presented by Al-Soufi and co-workers [18] who by use of 1D-NMR and 2D-NMR techniques show that β-CD can form 1:1 and 1:2 complexes of similar strength with log $K_{1:1}$ = 3.9 and log $K_{1:2}$ = 3.3, in the case of sodium fusidate. Furthermore, under our experimental conditions the stability constant with fusidate was found to be considerable lower, log $K_{1:1}$ = 2.58. The strongest complexes were found with γ-CD, but as mentioned above the data failed to be modelled by assuming a simple 1:1 complex stoichiometry. Only a more elaborate 1:2 model could sufficiently describe the data obtained. The data reveals that γ-CD formed a strong 1:1 complex, log $K_{1:1}$ = 3.9, and a very weak 1:2 complex, log $K_{1:2}$ = 0.9 with fusidate. Albeit that the complex strength is similar to that found for sodium fusidate, log $K_{1:1}$ = 4.8, Al-Soufi et al. [18], did not find any evidence for the presence of a 1:2 complex in the case of γ-CD. A 2D-NMR study of the complexes between sodium fusidate and β- and γ-CD, respectively, have been presented by Jover et al. [17]. They show that fusidate is fully embedded into the cavity of γ-CD, whereas β-CD only is capable in forming complexes with the A and B-rings as well as the side chains, supporting the probability that β-CD can form 1:2 complexes. Although it might look as if the results presented here and the results presented by Jover et al. [17], and Al-Soufi et al. [18] were conflicting, the differences found can be explained by the strengths and limitations of the different analysis techniques, as well as the difficulty to distinguish between higher order complexes. Analysis of CD complexes by affinity capillary electrophoresis has the strength to reveal very weak interactions (e.g. Larsen et al. (1998) [23] and Larsen and Zimmerman (1999) [21]), which can be seen here by the discovery of a very weak 1:2 complex formed between the fusidates and γ-CD. Stability constants of this strength are often too low to be measured for most experimental techniques, including NMR based methods. On the other hand the capillary electrophoresis technique was not able to discern between an interaction consisting of a 1:1 complex and one consisting of 1:1 and 1:2 complexes of similar strength which may exists in the case of α- and β-CD.

When comparing the stability constants obtained for the different complexes formed between the fusidates and the CD's, the stability constants of α- and β-CD are in general only minimally affected by the exchange of polar hydroxyl groups to less polar carbonyl groups. This correlates well with the assumption that these CD's primarily interact with the outer parts of the molecule. In contrast the complexes formed with γ-CD and the fusidates show a general tendency towards stronger complexes in agreement with the notion that γ-CD fully encircles the central part of fusidate (ring C and D) as shown by Jover et al. [17].

NMR

The complex between γ-CD and fusidate was studied by 2D-ROESY NMR (Fig. 5). It was only possible to fully distinguish the interaction between fusidate protons and C-5 protons from the cavity of γ-CD. Signals from C-3 and C-6 protons overlapped and could not be separated. No interaction with protons on the surface of the CD was detected. The interaction pattern obtained reveals that fusidate is embedded in the cavity of γ-CD with strong signals between the C-3/C-6 and C-5 protons to several protons distributed all over the molecule, with the strongest signals found on the B, C, and D-rings (Table 2). This shows that several complex geometries including different orientations of fusidate in the cavity are present simultaneously. This is partly consistent with the results presented by Jover et al. [17], who also find a strong interaction between the cavity protons of γ-CD and the C and D-ring of fusidate. In contrast to our results they did not find any signals to either the A of B-ring of the molecule. The differences observed between the NMR data of Jover et al. [17] and our own data can to a large extent be ascribed to the different concentrations used. We operated at higher sample concentrations and therefore a higher amount of complex in the sample tube, allowing us to also detect weaker interactions (stemming from less populated conformations). Most of the interactions that we observe in contrast to Jover et al. [17], are weak, whereas those interactions observed by both of us, tend to be strong interactions.

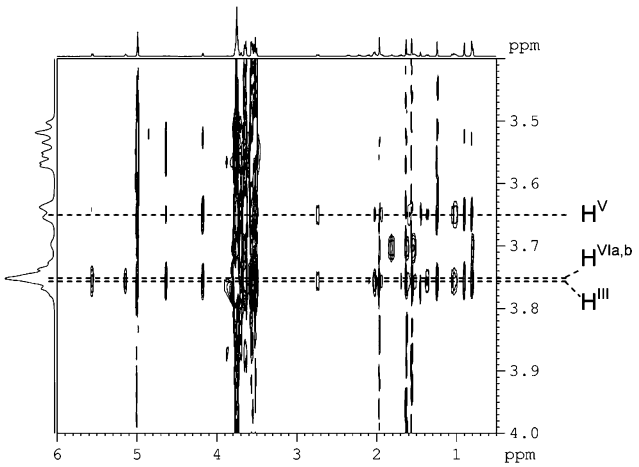

Fig. 5 Area of 2D-ROSEY spectrum of γ-cyclodextrin and fusidic acid

Table 2 Relative intensity of ROSEY intermolecular cross-peaks observed between protons of fusidate and γ-cyclodextrin

Location	C-atom	H-3/6	H-5
A ring	1α	++	+
	1β	++	+
	2α	–	–
	2β	++	
	3β	–	–
	4β		(+)
A/B-ring	5β		
	19	+++	+++
B-ring	6α	++	+
	6β	+	+
	7α	+	+
	7β	+	+
B/C-ring	9β	–	–
	32	+++	+++
C-ring	11β	+++	+++
	12α	+	+
	12β	++	+
C/D-ring	13α	++	++
	18	++	++
D-ring	15α	++	+
	15β	+	+
	16α	+++	++
Side chain	22	+	
	23		
	24	++	+
	26		
	27		
Ester	34		

Weak, +; m, ++; strong, +++; could not be resolved, –

Conclusion

Affinity capillary electrophoretic studied suggest that α- and β-CD form 1:1 complexes with all fusidanes studies. γ-CD was found to form 1:2 complexes, consisting of one relatively strong complex 1:1 complex and a very weak 1:2 complex. The stabilities of the inclusion complexes found for the fusidanes studied were, especially in the case of γ-CD, affected by exchange of polar substituents to less polar ones. 2D-NMR revealed that fusidate can be fully included into the cavity of γ-CD.

References

1. Wilkinson, J.D.: Fusidic acid in dermatology. Br. J. Dermatol. Suppl. **139**, 37–40 (1998)
2. Turnidge, J.: Fusidic acid pharmacology, pharmacokinetics and pharmacodynamics. Int. J. Antimicrob. Agents. **12**, S23 (1999)
3. Habon, I., Stadler-Szöke, A., Szejtli, J.: Improvement of the solubility of steroids by formation of cyclodextrin inclusion complex. Acta. Biochim. Biophys. Acad. Sci. Hung. **19**, 86 (1984)
4. Ahmed, S.M.: Improvement of solubility and dissolution of 19-norprogesterone via inclusion complexation. J. Inclusion Phenom. **30**, 111–125 (1998)
5. Uekama, K., Otagiri, M., Uemura, Y., Fujinaga, T., Arimori, K., Matsuo, N., Tasaki, K., Sugii, A.: Improvement of oral bioavailability of prednisolone by beta-cyclodextrin complexation in humans. J. Pharmacobiodyn **6**, 124–127 (1983)
6. Uekama, K., Sakai, A., Arimori, K., Otagiri, M., Saitô, H.: Different mode of prednisolone within alpha-cyclodextrins, beta-cyclodextrins and gamma-cyclodextrins in aqueous-solution and in solid state. Pharm. Acta. Helv. **60**, 117–121 (1985)
7. Uekama, K., Fujinaga, T., Otagiri, M., Yamasaki, M.: Inclusion complexations of steroid-hormones with cyclodextrins in water and solid-phase. Int. J. Pharm. **10**, 1–15 (1982)
8. Liu, F.Y., Kildsig, D.O., Mitra, A.K.: Beta-cyclodextrin steroid complexation – Effects of steroid structure on association equilibria. Pharm. Res. **7**, 869–873 (1990)
9. Djedaïni, F., Perly, B.: Nuclear-Magnetic-Resonance investigation of the stoichiometries in beta-cyclodextrin-steroid inclusion complexes. J. Pharm. Sci. **80**, 1157–1161 (1991)
10. Marzona, M., Carpignano, R., Quargliotto, P.: Quantitative structure-stability relationships in the inclusion complexes of steroids with cyclodextrins. Ann. Chim. **82**, 517–537 (1992)
11. Forgo, P., Vincze, I., Kover, K.E.: Inclusion complexes of ketosteroids with beta-cyclodextrin. Steroids **68**, 321–327 (2003)
12. Cabrer, P.R., Alvarez-Parrilla, E., Meijide, F., Seijas, J.A., Nunez, E.R., Tato, J.V.: Complexation of sodium cholate and sodium deoxycholate by beta-cyclodextrin and derivatives. Langmuir **15**, 5489–5495 (1999)
13. Pean, C., Creminon, C., Wijkhuisen, A., Perly, B., Djedaïni-Pilard, F.: Reliable NMR experiments for the study of beta-cyclodextrin/prostaglandin E-2 inclusion complex. J. Chim. Phys. Phys. Chim. Biol. **96**, 1486–1493 (1999)
14. Forgo, P., Göndös, G.: A study of beta-cyclodextrin inclusion complexes with progesterone and hydrocortisone using rotating frame Overhauser spectroscopy. Monatsh. Chem. **133**, 101–106 (2002)
15. Cameron, K.S., Fletcher, D., Fielding, L.: An NMR study of cyclodextrin complexes of the steroidal neuromuscular

blocker drug Rocuronium Bromide. Magn. Reson. Chem. **40**, 251–260 (2002)
16. Bednarek, E., Bocian, W., Poznanski, J., Sitkowski, J., Sadlej-Sosnowska, N., Kozerski, L.: Complexation of steroid hormones: prednisolone, ethinyloestradiol and estriol with beta-cyclodextrin. An aqueous H-1 NMR study. J. Chem. Soc. Perkin. Trans. 2, 999–1004 (2002)
17. Jover, A., Budal, R.M., Al-Soufi, W., Meijide, F., Tato, J.V., Yunes, R.A.: Spectra and structure of complexes formed by sodium fusidate and potassium helvolate with beta- and gamma-cyclodextrin. Steroids **68**, 55–64 (2003)
18. Al-Soufi, W., Cabrer, P.R., Jover, A., Budal, R.M., Tato, J.V.: Determination of second-order association constants by global analysis of H-1 and C-13 NMR chemical shifts. Application to the complexation of sodium fusidate and potassium helvolate by beta- and gamma-cyclodextrin. Steroids **68**, 43–53 (2003)
19. Larsen, K.L., Aachmann, F.L., Wimmer, R., Stella, V.J., Madsen Kjølner, U.: Phase solubility and structure of the inclusion complexes of prednisolone and 6 alpha-methyl prednisolone with various cyclodextrins. J. Pharm. Sci. **94**, 507–515 (2005)
20. Bartels, C., Xia, T.-H., Billeter, M., Günter, P., Wüthrich, K.: The program XEASY for computer-supported NMR spectral-analysis of biological macromolecules. J. Biomol. NMR **5**, 1–10 (1995)
21. Larsen, K.L., Zimmerman, W.: Analysis and characterisation of cyclodextrins and their complexes by affinity capillary electrophoresis. J. Chromatogr. A. **836**, 3–14 (1999)
22. Lee, Y., Lin, I.: Capillary electrophoretic analysis of cyclodextrins and determination of formation constants for inclusion complexes. Electrophoresis **17**, 333–340 (1996)
23. Larsen, K.L., Endo, T., Ueda, H., Zimmerman, W.: Inclusion complex formation constants of alpha-, beta-, gamma-, delta-, epsilon-, zeta-, eta-, and theta-cyclodextrins determined with capillary zone electrophoresis. Carbohydr. Res. **309**, 153–159 (1998)