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Utility of cyclodextrins in the formulation of genistein Part 1. Preparation and physicochemical properties of genistein complexes with native cyclodextrins

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ABSTRACT

Isoflavones are suitable guest molecules for inclusion complex formation with cyclodextrins (CDs). The molecular encapsulation with CDs results in a solid, molecularly dispersed form and in a significantly improved aqueous solubility of isoflavones. Genistein, a key isoflavone constituent of *Ononidis spinosae radix* was found to form a supramolecular, non-covalent inclusion complex with both β-cyclodextrin (β-CD) and γ-cyclodextrin (γ-CD), while it did not form a stable complex with α-CD. The guest genistein was found to spatially located in the less polar cavity of cyclodextrin. The isolated binary genistein/CD complexes appeared novel crystalline lattices. The *in vitro* dissolution of genistein entrapped into both β- and γ-CD, significantly surpassed that of the plain isoflavone.

1. Introduction

Isoflavonoids are chemically flavonoids, in which the ring B is attached to the C-3 position of ring C. They predominantly occur in their glycosidic form, as acetyl- or malonyl-glycosides. Best-known isoflavonoids are daidzin, genistin, glycytin; their aglycons are daidzein, genistein, glycytein and another one is biochanin A (Fig. 1).

Isoflavones are considerably widespread constituents of the medicinal plants. They can be found in *Iridaceae*, *Rosaceae*, *Moraceae* families as well but occur especially in legumes (*Fabaceae*) and are generated largely in root of these herbs (*Glycine max*, soy bean; *Phaseolus vulgaris*, bean; *Medicago sativa*, alfalfa; *Astragalus*, *Trifolium*, clovers; *Ononis*, restharrows).

Isoflavones such as genistein (5,7,4′-trihydroxyisoflavone) and daidzein (7,4′-dihydroxyisoflavone) are regarded to be phytoestrogens because of their estrogenic activity in certain animal models [1,2].

Many studies reported about antioxidant activity of isoflavones [3] and their use seems promising in the treatment of post-menopausal osteoporosis, breast cancer, prostate cancer and inhibition of platelet activation [4–7].

Isoflavones have rather poor water solubility. For nutraceutical and pharmaceutical purposes they need to be solubilized, to provide sufficient concentration of available monomeric forms of the plant material.

The molecular interaction of isoflavones and cyclodextrin (CD) derivatives has already been studied by UV, circular dichroism and FTIR spectroscopic methods by Crupi et al. [8] and Stancanelli et al. [9]. These studies focused on the molecular inclusion between isoflavones and highly soluble, non-aggregating, chemically modified β-cyclodextrins. Inclusion complex formation was proven by spectroscopic and solubility methods, and the existence of 1:1 molar stoichiometry between chemically modified CDs and isoflavones in water was found. The host–guest interactions have been evidenced by monitoring the FTIR-ATR spectra, the changes in some guest molecule's bands relative to those observed in the spectra of the 1:1 physical mixtures and complexes.

The molecular interaction of 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) with genistein and daidzein was studied in aqueous

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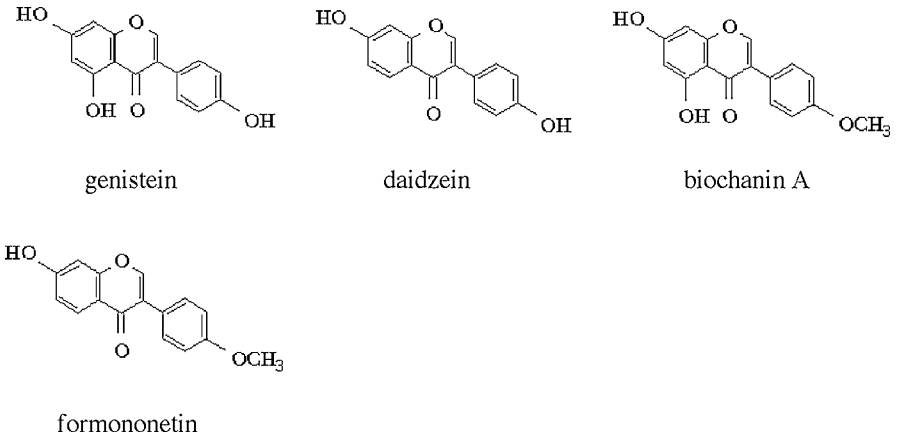

Fig. 1. Schematic representation of the structure of main isoflavone aglycons.

medium by UV–vis and CD techniques. The stoichiometric ratios of complexes formed and stability constants were determined by phase-solubility [10]. The reported stability constant values (around 10,000/M) indicated a very strong interaction between isoflavones and cyclodextrin derivatives.

So far, however, no data have been published in the literature on the inclusion complex formation between isoflavones and native, chemically non-modified α-, β- and γ-CDs.

European Patent Application disclosed the use of cyclodextrins (both native and chemically modified CDs), to mask the bitter taste of isoflavones when used as healthy food additives [11].

The purpose of this study was to enhance the aqueous solubility, the *in vitro* dissolution rate and the bioavailability of isoflavone genistein by encapsulation with different commonly used well approved native, parent CDs preparing a technically feasible isoflavone formulation for oral administration of the plant material.

2. Experimental

2.1. Materials

Genistein substance of analytical purity and D_2O (99.9%) were purchased from Sigma–Aldrich.

Cyclodextrins: the α-, β- and γ-CDs used for the present study were of pharmaceutical grade materials, manufactured by Wacker Chemie, Germany.

All other chemicals and reagents used in this study were of analytical grade.

2.2. Computer modeling studies for visualization of host–guest fitting

Previously published [12,13] X-ray structures of γ- and β-CD (CIWMIE10 and BCDEXD03) were downloaded from the database of Technische Universität Darmstad and were used as an input [14].

All calculations were run on Spartan for Windows '06 software version 1.1.1 [Wavefunction Inc. 18401 Von Karman Avenue, Suite 370 Irvine, CA 92612].

The input structure of ligand (genistein) was taken from the database of SPARTAN.

In case of both CDs, explicit water molecules were removed and missing hydrogen atoms were added. CD and ligand structures were pre-optimized separately using the PM3 semi-empirical method.

A centroid was defined for both CDs as the geometrical center of alpha-carbonic atoms their sugar units. The structures of input complexes were generated by the superposition and manual alignment of genistein's 4*H*-pyran-4-on ring to these centroids. Cyclodextrins and the inserted genistein were optimized together using the PM3 semi-empirical method. Optimizations were completed successfully, the calculations were converged, and there was no sign of major overlapping between Van der Waals surfaces of the CDs and the ligand in the output complexes (see Fig. 2).

2.3. Phase-solubility studies

The phase-solubility diagrams were registered in deionized water at 25 °C after a 10-h equilibration time according to Higuchi and Connors [15].

The phase-solubility studies were performed by equilibrating excess amounts of solid, crystalline genistein in aqueous cyclodextrin solutions that contained increasing CD concentrations in a way, that always excess, undissolved solid genistein remained in the solubility test systems providing the required equilibrium conditions.

After stirring for 10 h at 25 °C the undissolved genistein was filtered off on a membrane filter of 0.45 μm and the clear, equilibrated cyclodextrin solutions were assayed for dissolved genistein concentration by UV-spectrophotometry.

It was also proved that the presence of α-, β-, and γ-CDs had no effect on the spectrophotometric determination of genistein in water.

2.4. Preparation of the genistein cyclodextrin complexes

The genistein/CDs complex formulations were prepared by the wet kneading method [16,17] in ceramic mortars. Calculated amounts of CDs were wetted with deionized water and kneaded for 10 min at room temperature. The calculated amount of genistein substance was added into the wetted CD in one single portion in solid state without using any co-solvents or detergents.

The reaction mixtures were then kneaded intensively for 30 min yielding a nearly solid dense dough. The resulting material was allowed to dry at room temperature in air, to constant weight. The dry complexes were ground to fine powder and sieved.

2.5. Methods for characterization of the genistein/cyclodextrin complexes

The quantitative determination of genistein in the CD complexes was carried out by UV-spectrophotometry. Further evidences of host–guest interaction was proven by circular dichroism and NMR-spectroscopy.

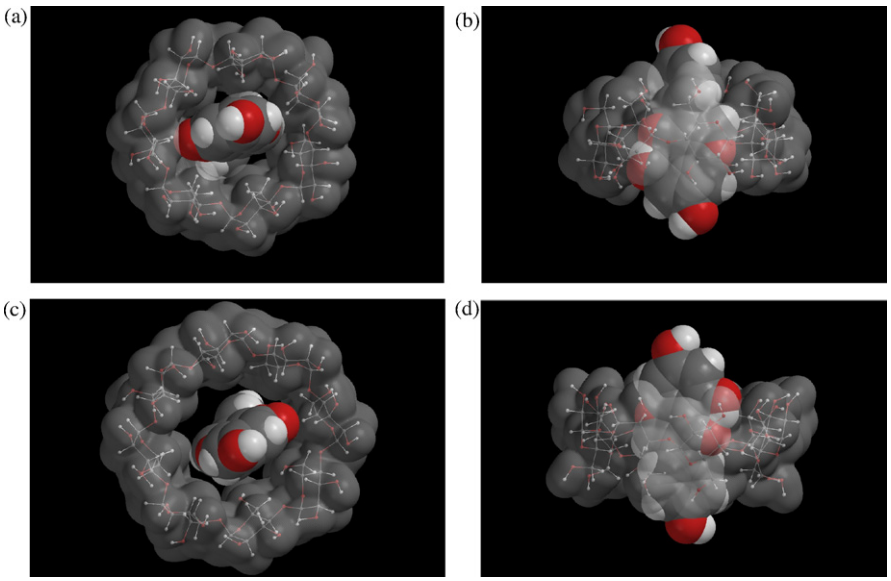

Fig. 2. Cyclodextrin/genistein tight fitting: a host–guest interaction between genistein and β-CD (a and b) or γ-CD (c and d).

The CDs do not absorb in the 200–800 nm wavelength range therefore they show no ellipticity (The native CDs could be investigated with ORD spectroscopy only.). Incorporation of an achiral molecule with measurable chromophore incorporates into the cavity of CD, the chiral molecule can induce intermolecularly circular dichroism (ICD) [18,19].

The spectra were recorded using a Jasco J720 Spectropolarimeter using a 10-mm cylindrical quartz cell was used for γ-CD and a 2-mm cell was used for β-CD. The spectra were accumulated six times with a bandwidth of 1.0 nm and a scanning step of 0.2 nm at a scan speed of 50 nm/min.

^{1}H NMR measurements were carried out on a Varian Inova spectrometer (600 MHz for ^{1}H) equipped with a dual 5-mm inverse-detection gradient (IDPFG) probehead. All spectra were recorded at 30.0 ± 0.1 °C and referenced to the residual HDO signal (4.72 ppm). The kneeded solid sample (containing β-CD or γ-CD and genistein) was dissolved in D_2O and shaked at room temperature for 48 h to achieve the equilibrium. The obtained suspension was centrifuged and a 0.7-ml supernatant saturated solution was introduced into the NMR tube.

The water content of the binary complexes was determined by loss on drying method; at 105 °C in vacuum, to constant weight in an open container over phosphorous-pentoxide.

The *in vitro* dissolution properties of the cyclodextrin-based genistein formulations were tested and compared under non-sink conditions in deionized water at 37.0 °C, by following the Uekama-proposed so-called dispersed amount method [20].

Samples were withdrawn from the dissolution medium at different time points (5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90 min) and the released genistein concentration was determined by UV-spectrophotometry.

3. Investigations and results

3.1. Structural considerations of the molecular encapsulation of genistein with cyclodextrins

The molecular dimensions of isoflavones are approximately in the 0.5 × 1.5 nm range; showing a good size-wise correlation with the geometry of the central cavity of CDs (0.47–0.83 nm). This is a good precondition for the formation of a tight-fit inclusion complex between genistein and parent CDs. Based on these structural considerations and on the previous encouraging results obtained with the molecular inclusion of ipriflavon (a semisynthetic 7-isopropoxy-isoflavone) by β-CD [21], a systematic work was initiated to improve some biopharmaceutical properties of genistein using CDs.

Optimizing the molecular fitting of genistein/β-CD and genistein/γ-CD spatially with semi-empirical PM3 method, it can be seen that geometrical matching of the two molecules is really possible (Fig. 2). On the other hand, it was also indicated by the computer modeling study that α-CD will hardly form a well-fitted host–guest type complex with genistein.

^{1}H NMR-spectroscopy is the most suitable method for quantification of non-covalent interactions at the molecular level and confirms the inclusion [22]. Previous ^{1}H NMR experiments on the structurally similar rutin revealed, that the single aromatic ring was inserted into the CD cavity based on chemical shift differences ($\Delta\delta$) [23]. In our study two-dimensional rotating frame nuclear Overhauser effect spectroscopy (ROESY) experiments were carried out to identify the interacting host–genistein moieties. The sufficient signal to noise ratio for a typical 2D ROESY experiments was only achieved in the genistein/β-CD system. A characteristic part of the 2D ROESY spectrum is presented in Fig. 3. The expansion of the spectrum contains intramolecular cross-peaks between the aromatic dubletts (7.06 ppm and 7.39 ppm) of the 4-hydroxyphenyl moiety. The protons at 7.39 ppm show a weak cross-peak between the singulet of the ring B (7.96 ppm) indicating that the protons at 7.39 ppm are in the meta-position the others at 7.09 ppm are in the ortho-position, respectively. From the viewpoint of encapsulation, intermolecular cross-peaks of the 4-hydroxyphenyl dublets with the inner CD protons H-5 (at 3.88 ppm) and H-3 (at 3.97 ppm) are important. The relative cross-peak intensities revealed that the whole aromatic ring is in the β-CD cavity and the mode of penetration occurred from the wider rim side, similar to our previous findings in case of imatinib [24]. A further evidence for this type of orientation of genistein is a weak cross-peak between the ring B singulet (7.96 ppm) and the H-5 proton of β-CD (3.88 ppm).

The encapsulation was also proved by circular dichroism spectroscopy. The ICD spectra of the investigated cyclodextrins are presented in Fig. 4. The induced band in circular dichroism spectrum indicates the inclusion of genistein into the cyclodextrin

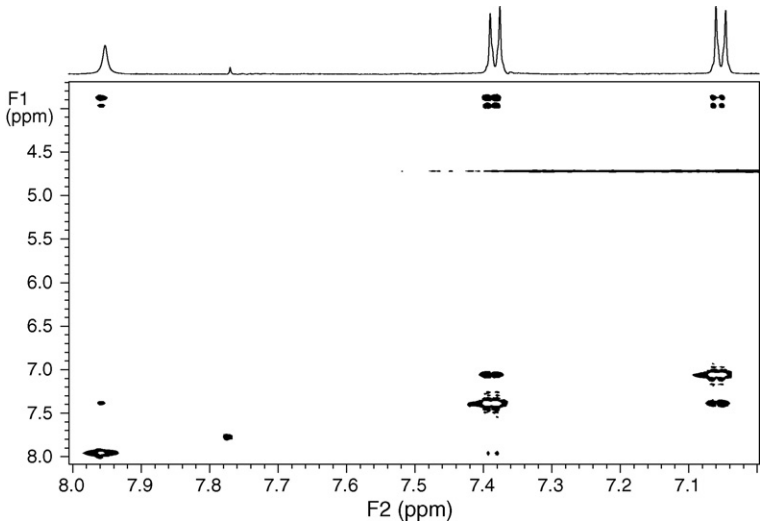

Fig. 3. Representative part of 2D ROESY spectrum showing the intermolecular proximities between genistein and β-CD (nt = 32, ni = 256, and mixing time = 300 ms).

cavity. The UV and CD spectra show identical maximum at 262.5 nm in case of both cyclodextrin, while the intensity of ICD spectrum of genistein/β-CD complex is higher than that of genistein/γ-CD, a more stable complex could be supposed in case of genistein/β-CD system.

3.2. Results of the phase-solubility study

The results of the solubility isotherms indicated that among the studied parent CDs β-CD forms the most stable inclusion complex with genistein in water. The slope of the solubility isotherms registered with β-CD indicated the formation of an inclusion complex with considerable stability. The solubility isotherm also showed the typical saturation type or Higuchi-B_S shaped curve, which indicates the formation of inclusion complexes with different molar stoichiometries (co-existence of 1:2, 2:3 mol/mol species) as well as their higher aggregates [20].

The solubility enhancement of the isoflavone in water at ambient temperature was the most pronounced by using β-CD complexing host. This about 9-fold solubility improvement achieved by β-CD was found reliably reproducible and of practical significance.

The complex forming potency of γ-CD under identical conditions appeared lower than that of the β-CD (see Fig. 5, the α-CD, β-CD and γ-CD solubility isotherm graphics). The circular dichroism spectra of genistein/β-CD and genistein/γ-CD complexes (Fig. 4) further confirmed the UV-spectrophotometric results and also were in a good agreement with the results reported in Stancanelli's work [10], despite the fact that those results were derived from the hydroxypropyl-β-CD-complexes of genistein.

The genistein complexation with α-CD was found, however, insignificant, and this experimental observation was in a good agreement with the results of the molecular modeling trials.

3.3. Characterization of the composition of binary host guest complexes of genistein and cyclodextrins

The UV-spectrophotometric determination of the genistein complexes indicated the existence of formulations with around 1:2 mol/mol stoichiometry.

The theoretical 1:2 guest/host complex refers to a genistein content of about 10% by weight.

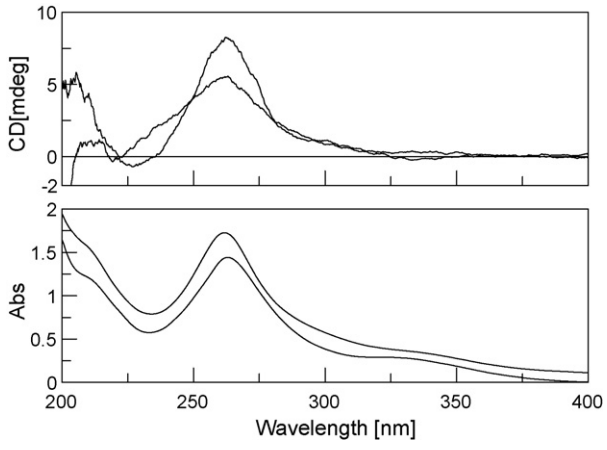

Fig. 4. Induced circular dichroism and UV spectra of genistein/β-CD (- - -) and genistein/γ-CD (––) complexes.

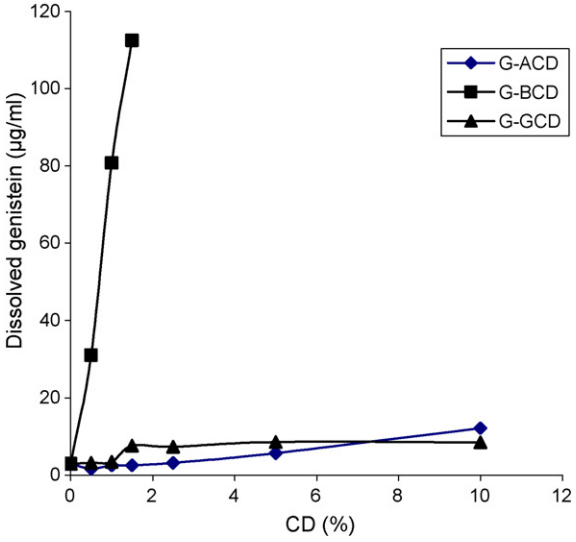

Fig. 5. Solubility isotherms of genistein with α-CD, β-CD and γ-CD in water.

Table 1
Composition of the inclusion complexes of genistein with β-CD and γ-CD in a 2:1 host:guest molar stoichiometry, determined by UV-spectrophotometry

Type of genistein complex	Genistein content (%)	Residual water content (%)
β-CD/kneaded	9.9 ± 0.04	7.5 ± 0.16
γ-CD kneaded	10.0 ± 0.10	5.5 ± 0.11

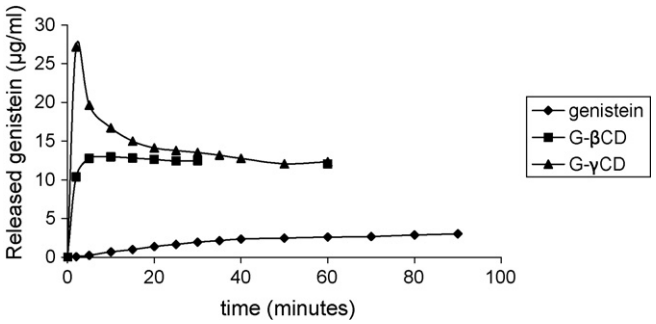

Fig. 6. *In vitro* dissolution profiles of genistein alone and its 1:2 mol/mol complexes with β-CD and γ-CD in water.

The compositions of genistein inclusion complexes prepared by wet kneading in the 1:2 molar ratios are listed in Table 1.

3.4. In vitro dissolution profile of free- and cyclodextrin-encapsulated genistein

The *in vitro* dissolution profiles of plain genistein and genistein entrapped in β- and γ-CD can be seen in Fig. 6.

It is clearly shown that the genistein/γ-CD complex provides the best dissolution rate with a peak concentration of 27 μg/ml, in contrast to the plain genistein alone (around 3 μg/ml) and the genistein/β-CD complex, which is just about 13 μg/ml).

The observed difference between the release properties of β-CD and γ-CD complexed genistein can be explained with the much better wettability characteristics of the γ-CD-based genistein bulk formulation in water.

4. Discussion

Based on the molecular modeling investigations and on the results of the phase-solubility studies, a correlation between the CD cavity size and the inclusion of isoflavone genistein was found. The interaction between genistein and the smallest cavity size α-CD was found to be negligible. It was further supported by the data of phase-solubility study.

However, both native β- and γ-CD were found to form an inclusion complex with the isoflavone, the real encapsulation was demonstrated with independent analytical techniques. The ICD spectrum confirmed the suggested inclusion, and ROESY experiment gave a clear explanation about the interacting moieties. In case of genistein, the 4-hydroxyphenyl ring immers from the narrower rim into the β-CD cavity.

The formation of an inclusion complex in aqueous solution has been proved by circular dichroism spectroscopy. The molecular entrapment of genistein with β- and γ-CDs was found to lead to an improved aqueous solubility and enhanced wettability and dissolution rate of the otherwise poorly soluble and hardly wettable substance.

The rates of dissolution of genistein β- and γ-CD complexes differ only in the initial, oversaturated dissolved genistein, observed in the first 1–10 min of the dissolution test. This oversaturated genistein concentration will drop after 15–20 min to the level achieved by β-CD complexed genistein, and will establish an around 15 μg/ml dissolved genistein in the dissolution medium. It was assumed that the bulk powder wetting properties of the two complexes are different. In order to find out the reason behind this dissolution difference, the wettability of bulk genistein/β-CD and genistein/γ-CD complex powders were compared. It was found that the contact angle of the γ-CD-based formulation was 18.8–20.5°, while the contact angle value for the corresponding β-CD genistein complex was between 45.0° and 47.8°.

Based on previous analogies and published results it is expected that these *in vitro* dissolution observations will result in enhanced genistein transport and absorption *in vivo*.

The *in vitro*/*in vivo* correlation studies are in progress and will soon be reported as the second part of the present study.

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References

[1] V. Beck, Steroid Biochem. Mol. Biol. 94 (2005) 499–518.
[2] C. Atkinson, Exp. Biol. Med. (Maywood) 230 (2005) 155–170.
[3] D.R. Doerge, H.C. Chang, J. Chromatogr. B 777 (2002) 269–279.
[4] T.L. Jia, H.Z. Wang, L.P. Xie, X.Y. Wang, R.Q. Zhang, Biochem. Pharmacol. 65 (2003) 709–715.
[5] H. Adlercreutz, H. Honjo, A. Higashi, T. Fotsis, E. Hämäläinen, T. Hasegawa, H. Okada, Am. J. Clin. Nutr. 54 (1991) 1093–1100.
[6] R.L. Schleicher, C.A. Lamartiniere, M. Zheng, M. Zhang, Cancer Lett. 136 (1998) 195–201.
[7] N.W. Scoene, C.A. Guidry, J. Nutr. Biochem. 10 (1999) 421–426.
[8] V. Crupi, R. Ficarra, M. Guardo, D. Majolino, R. Stancanelli, V. Venuti, J. Pharm. Biomed. Anal. 44 (2007) 110–117.
[9] R. Stancanelli, A. Mazzaglia, S. Tommasini, M.L. Calabró, M. Guardo, V. Villari, P. Ficarra, R. Ficarra, Proceedings of 13th International Cyclodextrin Symposium, Torino, May 14–17, 2006.
[10] R. Stancanelli, A. Mazzaglia, S. Tommasini, M.L. Calabró, M. Guardo, V. Villari, P. Ficarra, R. Ficarra, J. Pharm. Biomed. Anal. 44 (2007) 980–984.
[11] S. Kanaoka, et al., European Patent Application, EP 796624 (1997).
[12] K. Harata, Bull. Chem. Soc. Jpn. 60 (1987) 2763–2767.
[13] T. Steiner, G. Koelner, J. Am. Chem. Soc. 116 (1994) 5122–5128.
[14] http://csi.chemie.tu-darmstadt.de/ak/immel/structures/cyclodextrins/index.html.
[15] W.T. Higuchi, K.A. Connors, Adv. Anal. Methods Instrum. (1965) 123–134.
[16] J. Szejtli, in: J. Szejtli, T. Osa (Eds.), Comprehensive Supramolecular Chemistry, vol. 3, Elsevier Science Ltd., Oxford, 1996, pp. 14–26.
[17] V. Weiszfeiler, A. Stadler-Szoke, J. Szejtli, in: O. Huber, J. Szejtli (Eds.), Proceedings of the International Symposium on Cyclodextrins, 4th ed., Kluwer, Dordrecht, Netherlands, 1988, pp. 329–335.
[18] M. Suzuki, M. Kajtar, J. Szejtli, M. Vikmon, E. Fenyvesi, L. Szente, Carbohydr. Res. 214 (1991) 25–33.
[19] A. Buvari-Barcza, J. Kajtar, L. Szente, L. Barcza, J. Chem. Soc., Perkin Trans. 2 (1996) 489–491.
[20] K. Uekama, Y. Uemura, F. Hirayama, M. Otagiri, Chem. Pharm. Bull. 31 (1983) 3284.
[21] T. Loftsson, D. Hreinsdottir, M. Masson, J. Incl. Phenom. Macrocycl. Chem. 57 (2007) 545–552.
[22] H.-J. Schneider, F. Hacket, V. Rüdiger, H. Ikeda, Chem. Rev. 98 (1998) 1755–1785.
[23] M.L. Calabró, S. Tommasini, P. Donato, R. Stancanelli, D. Raneri, S. Catania, C. Costa, V. Villari, P. Ficarra, R. Ficarra, J. Pharm. Biomed. Anal. 36 (2005) 1019–1027.
[24] S. Béni, Z. Szakács, O. Csernák, L. Barcza, B. Noszál, Eur. J. Pharm. Sci. 30 (2007) 167–174.