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Pullulan–cyclodextrin microspheres A chromatographic approach for the evaluation of the drug–cyclodextrin interactions and the determination of the drug release profiles

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Abstract

Pullulan microspheres containing cyclodextrin (CyD) were obtained by chemical crosslinking with epichlorohydrin of an alkaline solution of pullulan (Pul) and α-, β- or γ-CyD. The amount of α-, β- and γ-CyD in microspheres was 120, 156, and 138 μmol/g, respectively, as determined from the percentage of iodine incorporated in the hydrophobic cavity of CyD's. Microspheres were packed in a glass column and the liquid chromatographic behaviour by isocratic elution of different drugs or typical organic compounds (TOC), taken as model drugs, was investigated. The increase of the retention volume (V_R) of each compound, depending on the interaction(s) between CyD's cavity and the considered molecule, is characterized by a broadening of the peaks. The interaction coefficient K, corresponding to the ratio between the V_R value of each tested molecule on Pul–α-, Pul–β- and Pul–γ-CyD active stationary phase and the V_R value of benzoic acid on St/maltodextrin neutral stationary phase, was determined. According to K values, the accurate prediction can be done on the potential drugs to be conditioned in suitable CyD cavity. Values of K allow to anticipate the release profiles of drugs considered.
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Keywords: Drug release profiles; Drug–cyclodextrin interactions; Pullulan–cyclodextrins

1. Introduction

Polymeric matrices containing conjugated cyclodextrins (CyD's) able to form inclusion complexes have found a variety of industrial, food and pharmaceutical applications [1]. In the pharmaceutical field, CyD's are used essentially for improving drug stability, dissolution rates and bioavailability [2–4]. CyD's can be polymerised by themselves using only a crosslinking agent, such as epichlorohydrin or diisocyanates [5–7] or by linking them to pre-existing polymers [8,9]. If the access to the CyD cavity is not obstructed, CyD–polymer conjugates can still form inclusion complexes. However, ligand affinity for CyD–polymer conjugates is usually lower than

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that observed for free CyD's [10,11]. The inclusion complex formation for parent CyD's can be easily monitored by a variety of methods [12–17]. Values of equilibrium constants for the binding of various guest molecules to α-, β-, or γ-CyD are largely present in the literature [18]. CyD–polymer gels were largely used as stationary phase in chromatographic separation and theoretical studies [19–21]. The chromatographic separation stems from the selectivity that the different compounds display towards a given CyD present in the stationary phase. Chemicals that interact strongly with CyD have large values of the association equilibrium constant (K_a) and exhibit relatively long retention times (or volumes). On the other hand, compounds that weakly bind CyD (low K_a values) are rapidly eluted. Accounting for the retention behaviour of compounds on a given CyD stationary phase, accurate predictions can be obtained concerning the stability of the CyD inclusion complexes formed (i.e. values of K_a may be predicted).

This study reports the preparation and characterization of CyD-containing pullulan microspheres by a single step procedure. The interaction of different drugs or model drug molecules with CyD's, linked in a swellable network of pullulan microspheres, by means of liquid chromatography, was also investigated. The suitable method here developed can predict appropriate drugs to be included in Pul–CyD microspheres as a potential biodegradable drug delivery system. Also, a correlation between the retention volume (V_R), the association equilibrium constants, the drug loading and the drug release profiles is examined.

2. Materials and methods

2.1. Materials

Pullulan (Pul), Mw=200 000 g/mol, was purchased from Hayashibara Laboratories LTD (Okayama, Japan). Maltodextrin (MD; DP=7), were kindly provided from Professor M. Rinaudo (CERMAV-CNRS, Grenoble, France).

α-, β-, and γ-CyD's were provided from Roquette Frères (Lestrem, France). Cellulose acetate butyrate (CAB) was purchased from Eastman Inc. (Kingsport, TN, USA).

Drugs and model drugs (i.e. TOC's), used for chromatographic studies, were provided from different suppliers. The chemical structure of drugs and TOC's are shown in Fig. 1. The molecular mass standards, deuterated water, D(+)-sucrose, and maltodextrins (with different degrees of polymerization, DP=3, 5, 7, 9, 13) were obtained from CERMAV (Grenoble, France). Dextrans with different molecular masses, namely 10.000, 17.500, 40.000, 70.000, 500.000, and 2.000.000 (Blue Dextran) g/mol, were provided from Pharmacia (Uppsala, Sweden). All chemicals were of the highest analytical grade.

2.2. Preparation of microspheres

Crosslinked Pul–CyD microspheres were obtained using a cylindrical glass reactor, provided with an anchor type glass stirrer, and a reflux condenser. The reactor was maintained at a constant temperature (50 °C) with a thermostatic water bath. In detail, 2 g of Pul and 2 g of CyD were dissolved in 20 ml NaOH solution (10% w/v) under stirring, in the presence of 50 mg of $NaBH_4$. After a complete removal of the air bubbles under vacuum, the solution was poured in 100 ml of dispersion medium (1,2-dichloroethane) containing 2.4 g of CAB (as dispersion agent). The obtained w/o emulsion was stirred for 1 h (stirring speed of 800 rev./min), then 4 ml of ECH were added, and the cross-linking reaction was carried out for 20 h at 50 °C. The cross-linked microspheres were recovered by filtration through a syntered glass filter, under vacuum. The removal of residuals was performed by washing the microspheres in the following order: 1,2-dichloroethane, acetone, water–acetic acid solution (30% v/v), water, and methanol. Then, microspheres were completely dried after overnight exposure to 60 °C, under vacuum. Microspheres from Pul–MD were prepared as reported above.

2.3. Cyclodextrin determination in microspheres

CyD determination in microspheres was carried out by the iodine and typical organic compounds (TOCs) approach as described in detail elsewhere [22].

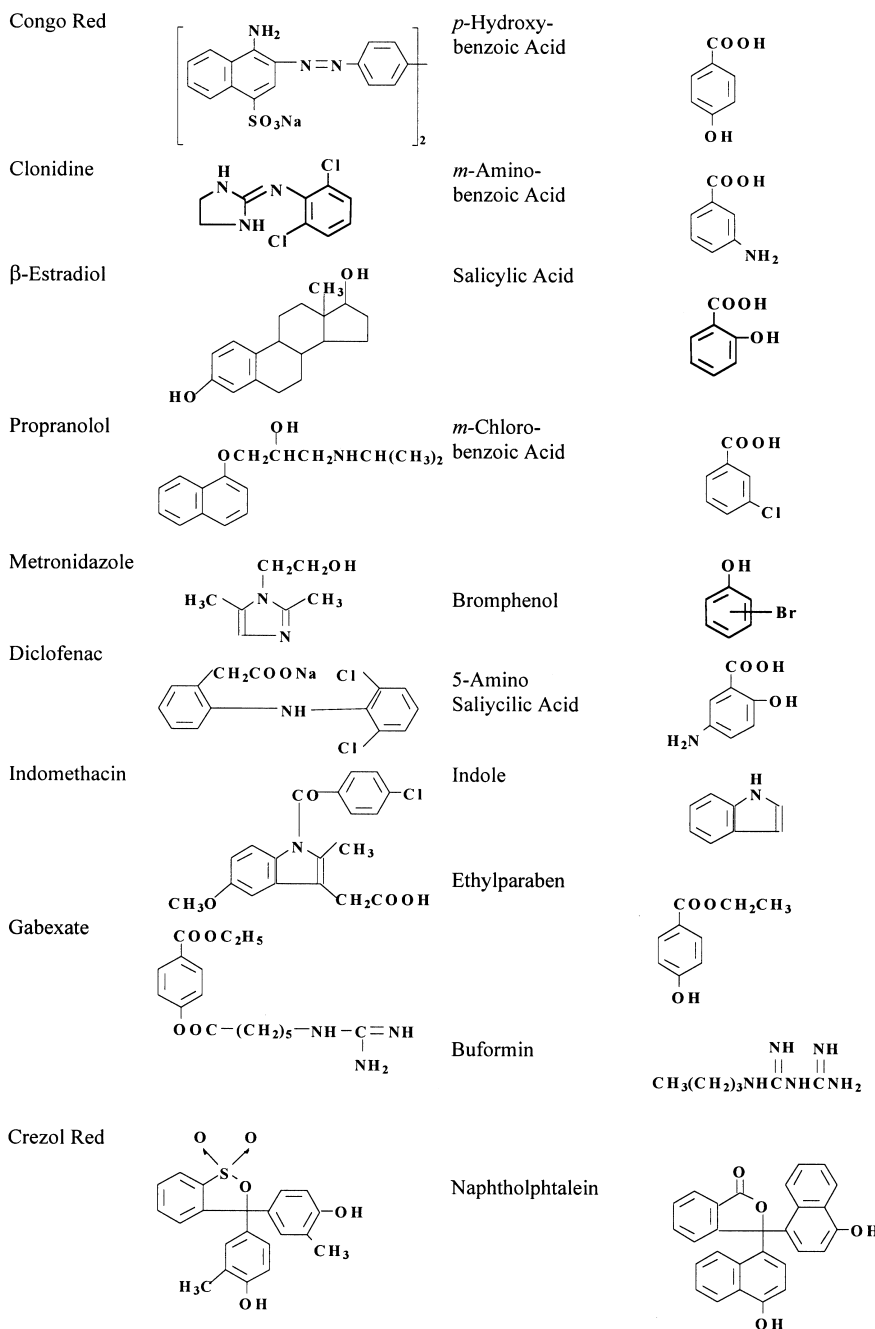

Fig. 1. Chemical structures of drugs or organic compounds used for chromatographic studies (TOCs).

2.3.1. Iodine approach

Subsequently, 100 mg microspheres were soaked in 10 ml of 0.1 N solution of iodine in 10% solution of potassium iodide, and kept for 48 h under gentle stirring. After 48 h the equilibrium was reached and 5 ml of iodine solution were taken up and assayed

for iodine content by titration with a 0.1 N $Na_2S_2O_3$, in the presence of starch (1%) as the indicator. As control, blank samples of Pul–MD microspheres were checked for iodine retention.

The amount of effective cyclodextrin in microspheres was determined as follows:

$$\text{CyD}(\%) = (I_1 - I_2) \times q_1 \quad (1)$$

where I_1 is % iodine retained by the Pul–CyD microspheres, I_2 is % iodine retained by Pul–MD microspheres, and

$$q_1 = \text{Mw}_{\text{CyD}}/\text{Mw}_{\text{Iodine}} \quad (2)$$

is the molecular mass ratio ($q_1 = 3.82$ for α-CyD, $q_1 = 4.46$ for β-CyD, and $q_1 = 5.10$ for γ-CyD).

2.3.2. Typical organic compounds approach

Cyclodextrin determination in microspheres was also carried out by the absorption of TOCs as follows: 100 mg of Pul–α-, Pul–β-, and Pul–γ-CyD microspheres were dispersed in 10 ml of an aqueous solution of indol (In) (1 mg/ml), 3-methylbenzoic acid (3-MBA) (0.5 mg/ml), and congo red (CR) (2.5 mg/ml), respectively, and kept for 48 h under gentle stirring. The total amount of TOC included in the microspheres was determined by UV–Vis spectrophotometrical analysis from the difference between the initial concentration of the TOC and the amount of the TOC in the supernatant after 48 h. As reference, a blank sample of Pul–MD microspheres was used in order to determine the amount of physically retained TOC. The amount of TOC retained by the CyD cavity was determined by difference between the total and physically retained organic compounds. The amount of effective CyD in microspheres was calculated using the same rationament as described above.

2.4. Microspheres recovery

After washing and drying, the prepared microspheres were weighed. The obtained weight was compared to the initial mass of Pul plus CyD and the recovery was calculated by using the following equation:

$$\text{Recovery}(\%) = W_{\text{Ms}}/(W_{\text{Pul}} + W_{\text{CyD}}) \times 100 \quad (3)$$

where W_{Ms}, W_{Pul} and W_{CyD}, represent the weight of the dried microspheres, Pul and CyD, respectively.

2.5. Morphological and dimensional analysis of microspheres

The morphology of the microspheres was evaluated by observation at optical and scanning electron microscopy (SEM). Microsphere size and size distribution were determined using a system of five sieves with the exclusion diameter of 50, 160, 250, 400 and 600 μm. The samples were sieved and each fraction was weighed and compared with the total amount of microspheres.

2.6. Wrack density

Wrack density was determined by weighting the volume of 1 ml of microspheres measured with a graduated cylinder (diameter = 12 mm).

2.7. Water retention in microspheres

Water retention tests have been performed following the Pepper's method [23].

2.8. Swelling degree of microspheres

The volume expansion of microspheres was determined at equilibrium, after having placed the microspheres in water. The volume of the swollen beads (V_s) compared with the dried volume (V_d), measured by placing the microspheres in a graduated cylinder (diameter = 12 mm), was defined as the swelling factor (q).

2.9. Determination of microsphere porosity

The porosity of Pul–CyD microspheres was determined by inverse size exclusion chromatography (ISEC) as described elsewhere [24]. ISEC was performed using a glass column (10 × 0.55 cm I.D.) packed under pressure with a suspension of Pul–CyD microspheres in the swollen state. The chromatographic equipment consisted of a peristaltic pump and a differential refractometer detector (model R 401, Waters).

2.10. Liquid chromatography procedure

The chromatographic analyses were carried out using a peristaltic pump provided with a injection valve (sample loop, 20 μl) connected to an UV–Vis variable wavelenghts detector (Merck, Germany) and a Spectra Physics Integrator. The columns (10×0.55 cm I.D.) were filled, under pressure of the peristaltic pump, with an aqueous suspension of Pul–CyD microspheres (50–160 μm). Experiments were carried out at room temperature. The flow-rate was 0.3 ml/min. The mobile phases used were high-performance liquid chromatography (HPLC) water and phosphate buffer (pH=7.4); they were filtered through a 0.2 μm membrane filter and degassed before use.

2.11. Drug loading

Drug-loaded Pul–CyD microspheres were prepared by soaking 100 mg dried microspheres in an aqueous solution of the drug (0.5–2.5 mg/ml), for 24 h at room temperature, under gentle stirring. Samples from the clear solution were taken up and assayed for drug content. Thereafter, microspheres were filtered, washed three times with 10 ml distilled water, and dried under vacuum. The amount of the retained drug per mg of dried microspheres was determined accounting for the amount of drug in the filtrate, obtained after microsphere isolation, by UV–Vis spectrophotometrical analysis, using a previously made calibration curve.

The efficiency of drug loading was calculated as follows:

$$\mathrm{Eff}\,(\%) = D_p / D_t \quad (4)$$

where D_p is the amount of the drug present in microspheres determined by the difference between the initial concentration of the drug and the drug concentration after separation and washing of the microspheres. D_t is the theoretical amount of the drug calculated as follows:

$$D_t\,(\%) = a \times \mathrm{Mw}_{\mathrm{Drug}} / \mathrm{Mw}_{\mathrm{CyD}} \quad (5)$$

where a is the amount of CyD in microspheres (w/w).

2.12. In vitro drug release kinetics

In vitro drug release studies were performed by soaking 50 to 100 mg of microspheres in 100 ml of phosphate buffer, pH=7.4, under gentle stirring. At regular time intervals, 10 ml of the released medium were withdrawn and the drug content was spectrophotometrically determined. The same volume of receiving medium was added to replace the volume of the extracted samples.

3. Results and discussions

3.1. Preparation and characterization of microspheres

Most of CyD-bounded stationary phase used for HPLC was prepared by grafting CyD onto silica beads [25] or by coating a polymer onto the surface of silica beads [26]. This stationary phase can then be modified by grafting appropriate ligands. In order to minimalize the number of the phases, as well as to obtain the maximum accessibility of the CyD cavity, we propose here the preparation of CyD-bounded polysaccharide microspheres as stationary phase obtained by a single step procedure.

Pullulan–cyclodextrin microspheres were successfully obtained by chemically crosslinking with epichlorohydrin a mixture of Pul–CyD 1:1 (w/w) (Table 1). The use of a Pul:CyD mixture allows the obtaining of a concentrated starting polymer solution with a relative low viscosity, and therefore spherical microparticles displaying a homogeneous porous internal structure (Fig. 2A). Moreover, the presence of pullulan, a member of the extracellular polysaccharide family often used in the last decade in pharmaceutics, confers to microspheres stability and biocompatibility. In addition, the association of a hydrophilic polymer to a CyD improves the swelling properties of the microspheres thus increasing the accessibility of a guest molecule into CyD's cavity.

As reported in Table 1, the recovery yield of Pul–Cyd microspheres shows a slight increase by increasing the molecular mass of the CyD used, namely α-, β-, and γ-CyD. A higher molecular mass of the CyD means, in fact, a higher number of the

Table 1
The main characteristics of Pul–CyD microspheres[a]

Pul–CyD Ms (type)	Yield (%)	Bulk density (g/ml)	Swelling degree (*q*)	Water regain (%, w/w)	Porous volume (ml/g)	Iodine retained on Ms (%, w/w)	Iodine retained by CyD (%, w/w)	TOC retained on Ms (%, w/w)	TOC retained by CyD (%, w/w)	CyD in Ms determined from (μmol/g)	
										Iodine approach	TOC approach
Pul–α-CyD	80.7±2.8	0.82±0.02	8.5±0.15	5.3±0.14	4.63±0.38	18.5±0.27	3.04±0.47	1.6±0.18	1.48±0.21	119±18.5	126±18.4
Pul–β-CyD	84.2±3.1	0.84±0.05	10.4±0.26	6.0±0.08	4.42±0.15	19.43±0.20	3.97±0.05	3.8±0.19	1.92±0.47	156±2.4	141±34.8
Pul–γ-CyD	88.5±3.4	0.88±0.05	9.5±0.23	5.5±0.15	4.25±0.28	22.48±0.62	7.02±0.43	18.5±0.60	7.5±2.1	138±8.6	107±30.1
Pul–MD	88.5±2.3	0.75±0.04	8.5±0.22	6.1±0.18	4.28±0.21	15.46±0.19	–	–	–	–	–

[a] Data are the mean of three independent experiments±SD.

hydroxyl groups and therefore a higher probability of linking of CyD in the polymer network.

Particularly, the linking of CyD in the polymer network could be realized either between two polymer chains (by intermediate of two molecules of ECH), or as pendant group (by intermediate of a single molecule of ECH). As a consequence, the crosslinking density will be the result of the bridges created by ECH with two polymer chains (Pul–ECH–Pul), and by additional crosslinking with CyD (Pul–ECH–CyD–ECH–Pul). The higher the amount of CyD linked as pendant group, the smaller the crosslinking degree.

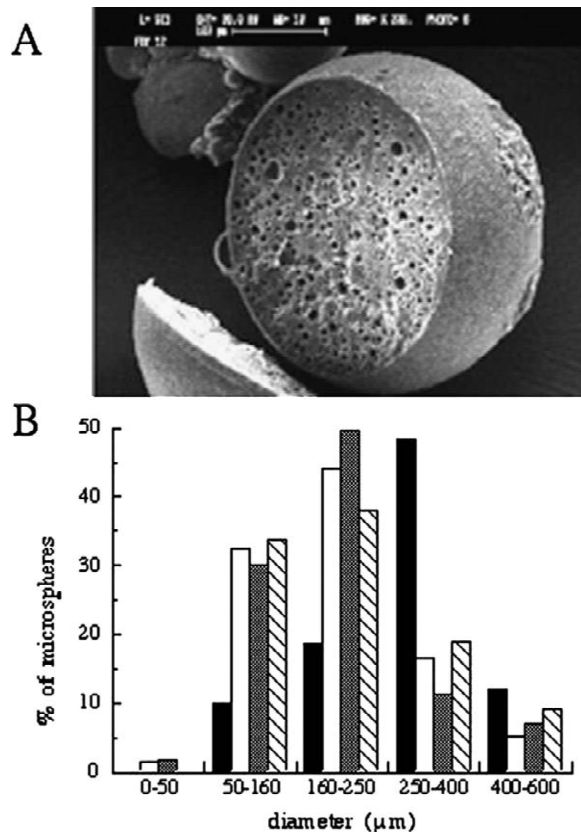

Fig. 2. SEM photograph of Pul–α-CyD microspheres (Panel A) and size distribution (Panel B) of Pul (white) Pul–α-CyD (black), Pul–β-CyD (gray) and Pul–γ-CyD (lines) microspheres.

It should be underlined that the swelling degree of microspheres as well as their water retention slowly increase by increasing the number of hydroxyl groups of the conjugated CyD, in the case of Pul–α- and Pul–β-CyD. Different behaviour has been noted for Pul–γ-CyD, since probably CyD is linked as additional crosslinker (Table 1). However, on the basis of SD values obtained for each sample, no significant difference in term of microspheres size distribution was obtained. All types of Pul–CyD microspheres are in fact characterized by a relatively large size distribution (Fig. 2B) being comprised between 50 and 250 μm. Particularly, the fraction characterized by dimensions comprised between 50 and 160 μm was further used for the experiments.

3.2. Determination of cyclodextrins in the stationary phase

Since Pul and CyD are characterized by the same structure of the repeating unit, the use of common analytical method (e.g. determination of the reducing sugars) [27,28] to determine the percentage of CyD in microspheres was not possible. In this view, the properties of iodine ion present in the KI solution to

form stable 1:1 or 1:2 inclusion complexes with α-, β-, and γ-CyD were exploited [22,29–31]. As it is well known, iodine displays a good affinity for polysaccharides, thus the effective amount of iodine retained in CyD cavity was calculated by difference between complexed and physically retained iodine according to Eqs. (1) and (2) (see the Experimental Section 2.3).

The results concerning to the amount of CyD associated to the microspheres, as determined by the iodine approach, are reported in Table 1. These results are in good agreement with those obtained by the alternative approach based on organic compounds which form very strong 1:1 inclusion complexes with CyD's, such as indol with α-CyD ($K_a = 6.0\times10^7$ M^{-1}), 3-methyl benzoic acid with β-CyD ($K_a = 1.3\times10^7$ M^{-1}) and Congo Red with γ-CyD ($K_a = 1.7\times10^4$ M^{-1}) [18]. Moreover, these results are directly proportional to the yield of CyD incorporation in Pul–Cyd microsphere preparation (i.e., γ-CyD > β-CyD > α-CyD).

In addition, these values are higher if compared to the amount of CyD immobilised on silica gel [8] and are related with the swellable properties of the matrix.

3.3. Determination of microsphere porosity

Since steric interactions between drug molecules and the microparticulate stationary phase could affect the intrinsic retention time, the porosity of Pul–CyD stationary phase was determined by ISEC method. In this case, the mobile phase is used to characterize the stationary phase thus providing data about the total pore volume (ml/g) and the maximum pore size of the microspheres.

As molecular mass standards, maltodextrins (DP = 3, 5, 7, 9, 13) were used for small pores, whilst dextrans with different molecular mass were used for large pores. Plotting the logarithm of the molecular mass of standard molecules against the elution volume (Fig. 3), the maximum radius of the pores was determined. Since no significant differences between the elution volume of the low molecular standards was detected for Pul–α-CyD, Pul–β-CyD and Pul–γ-CyD, we concluded that the dimensions of the smallest pores are enough large with respect to

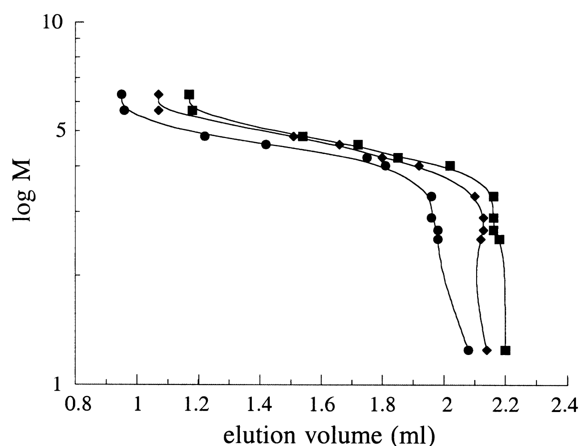

Fig. 3. Pore characteristics of Pul–α-CyD (●), Pul–β-CyD (■) and Pul–γ-CyD (♦) microparticulate stationary phase. Mobile phase, water; flow-rate, 0.3 ml/min; injected volume, 20 μl; solute concentration, 10 g/l (refractometric detection).

that of the largest tested molecules, and therefore steric interactions between drug molecules and the microparticulate stationary phase were discarded. Also, the porous volume of the microspheres was determined, showing no significant differences between Pul–α-CyD, Pul–β-CyD and Pul–γ-CyD (see Table 1).

3.4. Retention of Pul–CyD microspheres

In a first set of experiments the stationary phase was checked for the presence of carboxylic groups which generally appears in alkaline medium, during the preparation of polysaccharide microspheres. As expected, no carboxylic groups was detected due to the presence of a strong reduction agent, such as $NaBH_4$, in the system. Therefore, these microspheres were further mixed with neutral Pul–MD microspheres, in order to ensure the same number of moles of CyD per gram of microspheres for each CyD. Then, the interaction of Pul–CyD microspheres with drugs or TOCs was estimated from the retention volume (V_R) measured by liquid chromatography. The value of V_R of each tested molecule depends on the interaction with CyD's (Table 2).

In order to prove such correlation, TOCs with well-known values of the association equilibrium constant (K_a) were tested [18]. Therefore, benzoic acid (BA; $K_a = 10^3$ M^{-1}), 3-amino benzoic acid

Table 2
Retention volume and interaction constants values of different drugs or TOC eluted using different stationary phases

Drugs or TOC's[a]	Stationary phase							
	Pul–α-CyD		Pul–β-CyD		Pul–γ-CyD		Pul–MD	
	V_R (ml)	K_α	V_R (ml)	K_β	V_R (ml)	K_γ	V_R (ml)	K_{MD}
Benzoic acid (Na^+)	2.22	1.07	2.97	1.44	2.56	1.24	–[b]	–
Benzoic acid	3.95	1.91	3.31	1.60	3.03	1.47	2.06	1.00
3-Amino benzoic acid	9.65	4.68	–	–	–	–	4.42	2.14
4-Hydroxybenzoic acid	5.40	2.62	–	–	–	–	–	–
1-Cl-Benzoic acid	3.99	1.93	2.60	1.26	2.45	1.19	–	–
Salycilic acid (Na^+)	2.56	1.24	3.39	1.64	–	–	–	–
Salycilic acid	2.57	1.24	3.24	1.57	2.76	1.34	–	–
5-Aminosalycilic acid	2.98	1.44	3.30	1.60	3.51	1.70	–	–
Indol	9.45	4.58	∞	∞	6.25	3.03	–	–
p-Hydroxybenzoate ethyl ester	n.d.	–	2.25	1.09	9.39	4.55	4.70	2.28
Gabexate mesylate	n.d.	–	2.24	1.08	2.72	1.32	–	–
Buformin tosylate	2.68	1.30	2.70	1.31	2.89	1.40	–	–
Clonidine–HCl	2.31	1.12	2.16	1.04	3.11	1.51	2.06	1.00
Br-Cryptine mesylate	–	–	2.02	0.98	2.31	1.12	–	–
Diclofenac	4.82	2.34	5.96	2.89	2.60	1.26	2.15	1.04
β-Estradiol	n.d.	–	n.d.	–	n.d.	–	–	–
Indomethacin	2.59	1.25	3.85	1.86	4.78	2.32	–	–
Metronidazol	3.2	1.55	4.87	2.36	3.47	1.68	–	–
Propranolol	3.88	1.88	2.46	1.19	4.65	2.25	–	–
I_2 + KI	3.36	1.63	3.46	1.67	3.17	1.53	–	–
	∞	∞	∞	∞	12.13	5.88		
Blue Dextran	0.94	0.45	1.16	0.56	1.08	0.52	1.10	0.53
Congo Red	2.45	1.19	2.69	1.30	∞	∞	–	–
Br–Cresol Purple	–	–	–	–	2.16	1.04	–	–
Br-Phenol Blue	1.75	0.85	1.88	0.91	1.72	0.83	–	–
m-Cresol Red	–	–	–	–	1.87	0.90	–	–
Fluoresceine	–	–	–	–	1.87	0.90	–	–
Mixt Indicator	–	–	–	–	2.02	0.98	–	–
α-Naphtholphtaleine	–	–	1.88	0.91	∞	∞	–	–
Acetone	3.28	1.59	–	–	–	–	–	–

Data are the mean of three independent experiments.
[a] TOC's, typical organic compounds.
[b] –, experiment not done; ∞, never eluted; n.d., not determined.

(3-ABA; $K_a = 10^5$ M^{-1}), and indole (In; $K_a = 6.0 \times 10^5$ M^{-1}) were chosen as models to test the retention behaviour of α-CyD-containing stationary phase, while congo red (CR; $K_a = 1.7 \times 10^4$ M^{-1}) was used to check the retention behaviour of γ-CyD-containing stationary phase.

As evidenced in Table 2, V_R values for 3-ABA, In, and CR are higher with respect to other compounds (e.g. BA) while their K_a values are lower. Moreover, the V_R increase is characterized by a broadening of the peaks (Fig. 4A). In the absence of specific interaction between TOCs and Pul–MD neutral stationary phase, narrow peaks are obtained (Fig. 4B).

For an easier classification of the tested molecules, BA was taken as reference and the interaction parameters K_α, K_β and K_γ were defined as the ratio between the V_R value of each tested molecule on Pul–α-CyD, Pul–β-CyD, or Pul–γ-CyD, respectively, and the V_R value of BA obtained on the neutral Pul–MD stationary phase. A direct comparison between K values of the same molecule runned on the three types of the active stationary phase is reliable since the amount of each type of CyD

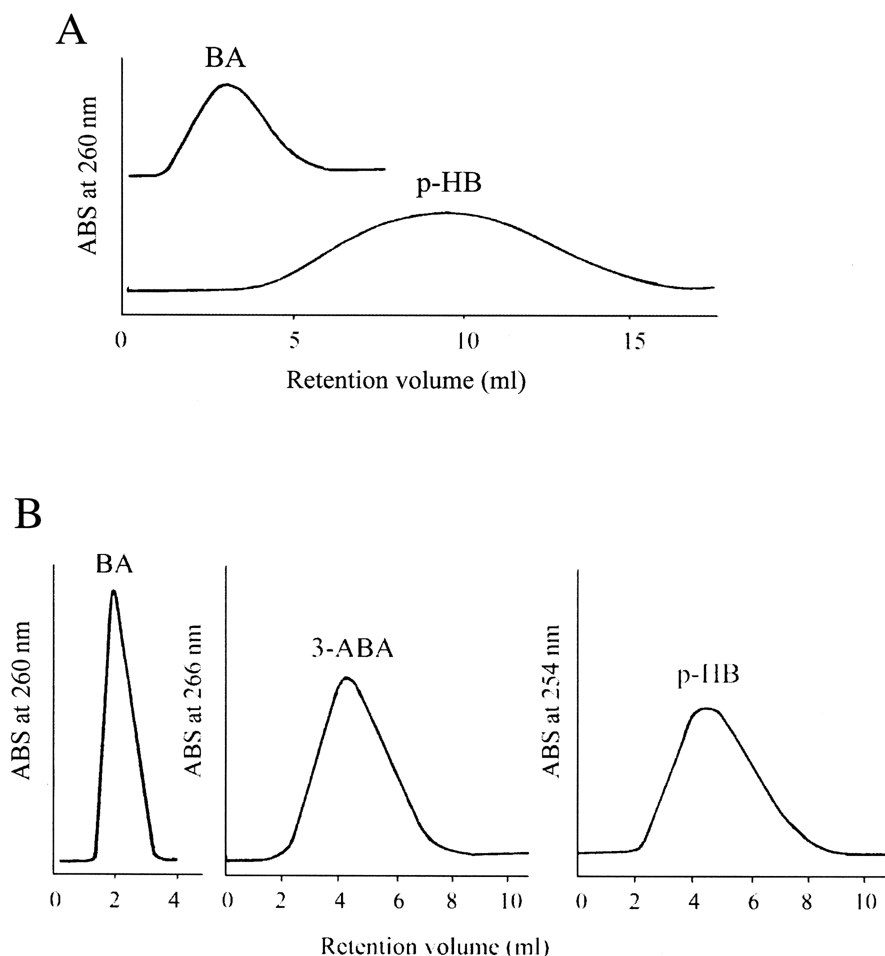

Fig. 4. Panel A: Comparison between liquid chromatographic profiles of BA and *p*-HB on Pul–β-CyD and Pul–γ-CyD active stationary phases, respectively. Panel B: Liquid chromatographic profiles of BA, 3-ABA, and *p*-HB on Pul–MD neutral stationary phase taken as references. Mobile phase, water; flow-rate, 0.3 ml/min; injected volume, 20 μl; solute concentration, BA 0.5 g/l; 3-ABA and *p*-HB 0.2 g/l.

present in microspheres (μ*M*/g) was adjusted to be the same. Fig. 5A shows the retention profiles of BA on Pul–α-CyD, Pul–β-CyD and Pul–γ-CyD stationary phase displaying the increase of the interaction coefficient (i.e. α-CyD>β-CyD>γ-CyD). These results are confirmed by literature data [18] obtained by alternative methods.

As shown in Table 2, all tested drugs and TOCs display a certain affinity for Pul–CyD microspheres (K_α, K_β, and $K_\gamma > 1$). This behaviour reflects the presence of one ore more benzene rings or indole-like structures in the drug molecules and TOCs considered; their size and geometry fit well the host CyD cavity. On the other hand, some compounds (i.e. CR, α-naphtolphtaleine, iodine, *p*-HB, gabexate mesylate, β-estradiol) can form very strong inclusion complexes, as CR and α-naphtolphtaleine with Pul–γ-CyD, iodine species with Pul–α-, and Pul–β-CyD otherwise they are spectroscopically silent, such as p-HB and gabexate mesylate runned on Pul–α-CyD stationary phase or β-estradiol runned on Pul–α- β- or γ-CyD stationary phase. The V_R values of all these chemicals are reported in Table 2 with the symbol ∞ (never eluted) and n.d. (not detected), respectively.

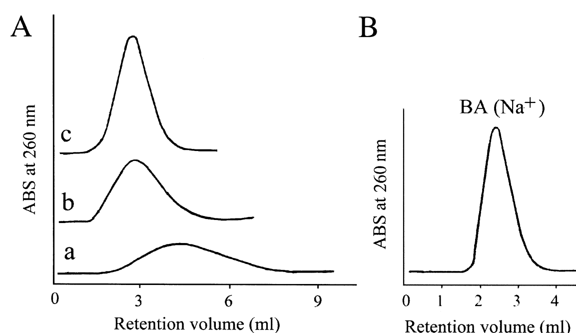

Fig. 5. Panel A: Liquid chromatographic profiles of BA on Pul–α-CyD (line a), Pul–β-CyD (line b) and Pul–γ-CyD (line c) stationary phase. Panel B: Liquid chromatographic profiles of BA(Na^+) on Pul–α-CyD stationary phase. Chromatographic conditions are the same as reported in Fig. 4.

Even if the relative size and geometry are the most important factors that rend thermodynamically favourable the formation of a given inclusion complex, other determinants could affect complex stability. The interaction coefficients K, with respect to the neutral Pul–MD stationary phase, for 3-ABA and p-hydroxybenzoate are 2.14 and 2.28, respectively, indicating that cooperative binding occurs (acid–base interactions, hydrogen bonds).

The stability of CyD inclusion complexes has been demonstrated to be dependent upon the charge of the host and guest molecule [32]. Hence, the pH of the release medium may affect experimental data; all guest molecules tested possess ionizable acidic or basic functional groups. In almost all cases, the binding ability of the formally charged species (either anionic or cationic) is smaller than that of the corresponding neutral species [BA, BA(Na^+), SA, SA(Na^+)] (see Fig. 5B and Table 2). This behaviour may reflect the diminished hydrophobic interactions between the charged guest molecules and the nonpolar CyD cavity.

At this point, it should be underlined that for checking the drug retention within Pul–Cyd microsphere stationary phase, water does not represent the best eluent, but it was chosen on the basis of the following considerations:

(a) organic eluents or their mixtures with water, reduce or inhibit the swelling of hydrophilic microspheres often causing microparticle collapse and therefore not permitting the access to CyD.
(b) buffers often present a high inorganic ions concentration that could influence the CyD–drug interactions [29,33]
(c) it is well established that the stability of CyD inclusion complexes depends upon the charge of the host molecule. Almost all charged molecules (more hydrophilic) have association constants much smaller than their protonated form (less hydrophilic). Using buffers as eluents, the pH will be the same along the column, but the degree of ionization could be different. In water, the dilution leads to decreasing of pH and to increasing of the degree of ionization, and therefore the pH declining could be partially compensated. In this view, some, chromatographic experiments were performed also in phosphate buffer (pH = 7.4).

The chromatographic profiles obtained with phosphate buffer (pH = 7.4) as the eluent instead of distilled water, gave obviously different results. For instance, values of the interaction coefficient K_α increase from 1.07 to 3.45 for BA(Na^+) ($pK_a = 4.2$), from 1.24 to 3.45 for SA ($pK_{a1} = 3.0$ and $pK_{a2} = 13.4$), from 4.68 to 7.06 for 3-ABA ($pK_{a1} = 2.4$ and $pK_{a2} = 4.9$), and from 4.58 to 7.20 for In, but it is almost unchanged in the case of 4-HBA (from 2.62 to 2.91). The increased values of K_α in phosphate buffer are not related to molecule ionization. Ionized compounds are less hydrophobic and therefore the K_α values should be low.

Fig. 6 shows the liquid chromatogram of an aqueous solution of free (line a) and mixed p-HB with γ-CyD (1:1 molar ratio) (line b) on the Pul–γ-CyD stationary phase. The interaction between p-HB and γ-CyD is very strong, proved by the high value of the interaction coefficient ($K_\gamma = 4.55$). When the mixture of p-HB and γ-CyD is injected onto the column, two separated peaks appear, showing that a complete baseline separation of the complex from free p-HB may occur on the Pul–γ-CyD stationary phase. On the other hand, when the same mixture was injected onto the Pul–α-CyD stationary phase it was never eluted. p-HB was firstly released from its

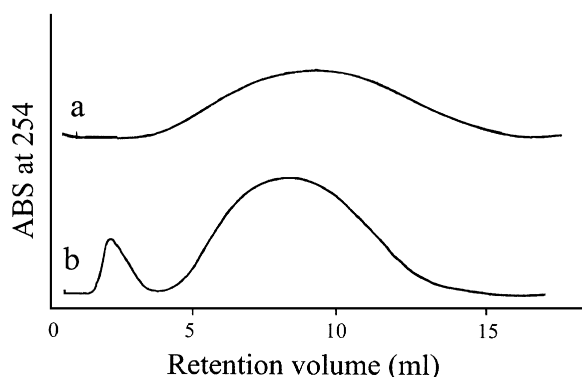

Fig. 6. Liquid chromatographic profiles of *p*-HB (line a) and of the *p*-HB–γ-CyD mixture (1:1 molar ratio; line b) on the Pul–γ-CyD stationary phase. Chromatographic conditions are the same as reported in Fig. 4 apart from solute concentration of experiments reported in panel A (1 g/l).

complex with γ-CyD and then retained by the Pul–α-CyD stationary phase, forming strong inclusion complexes.

Finally, it is worthy to be discussed the retention behaviour of the iodine species present in the iodine–iodide (I_2–KI) solution on the Pul–α-CyD, β-CyD, or γ-CyD stationary phase (see Fig. 7). The first peak was tentatively assigned to the I_3^- species retained by pullulan chains present in the stationary phase. On the other hand, the second peak was attributed to the I_3^- species retained by the γ-CyD cavity (line c). The absence of the second peak when the iodine–iodide (I_2–KI) solution was eluted from the Pul–α-CyD and Pul–β-CyD stationary phases (see line a and b) may be related to the very strong interactions between the I_3^- species and α-CyD and β-CyD; I_3^- was never eluted. In addition, the Pul–α-CyD and Pul–β-CyD stationary phases remain always brown coloured after retention tests due I_3^- complexation, whilst the colour of Pul–γ-CyD microspheres disappeared after the resolution of the second peak. According to literature, α-CyD and β-CyD form strong 1:1 inclusion complexes with the I_3^- species present in iodine–iodide solutions, while γ-CyD forms weaker 1:2 inclusion complexes [22,29,30].

3.5. Drug loading

The microsphere inclusion of the drugs was performed in aqueous solution. The use of an aqueous solution allows to obtain good results in terms of drug content, since a good hydration (swelling) of microspheres and therefore an easier diffusion of the drug towards the cavity of CyD's can be obtained. Table 3 shows the entrapment efficiency of drugs or TOC's. Compounds characterized by high values of the interaction coefficient K (i.e. In, 3-ABA, CR) display the highest loading efficiency without depending on the number of washing steps. On the contrary, compounds characterized by low *K* values display a low entrapment efficiency being directly related to the number of washing steps.

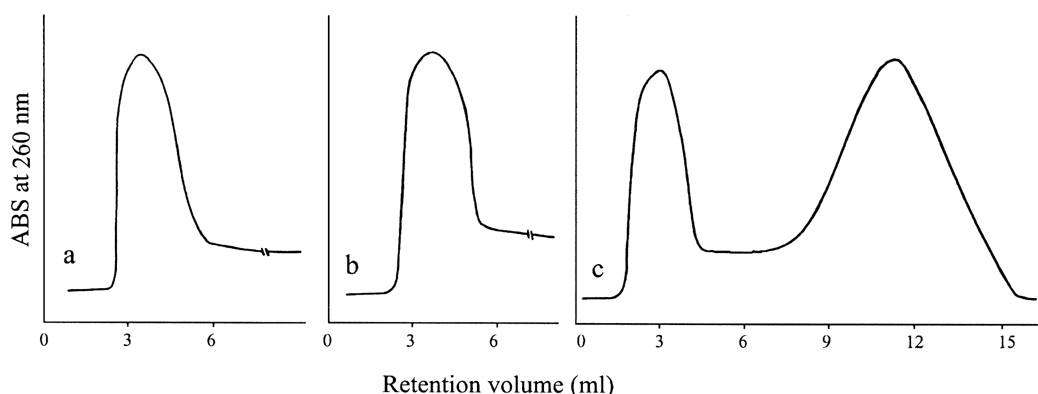

Fig. 7. Liquid chromatographic profiles of iodine/iodide solution on Pul–α-CyD (line a), Pul–β-CyD (line b) and Pul–γ-CyD (line c) stationary phase. Mobile phase, water; flow-rate, 0.3 ml/min; injected volume, 5 μl; solute concentration, 0.1 *M* I_2 in aqueous KI 10% w/v.

Table 3
Characterization of Pul–CyD microspheres loaded with different drugs

Ms type	Diclofenac (w/w, %)				Propranolol		Indol		3-ABA		Congo Red	
	after 1-st step	after 2-nd step	after 3-rd step	Eff[a] (%)	conc (%)	Eff (%)	conc (%)	Eff (%)	conc (%)	Eff (%)	conc (%)	Eff (%)
Pul–α-CyD	2.08	1.54	1.14	44.5	1.0	51.2	1.15	109.5	1.05	85.4	n.d.	–
Pul–β-CyD	2.65	2.02	1.52	47.9	1.2	37.9	n.d.	–	n.d.	–	n.d.	–
Pul–γ-CyD	2.23	1.76	1.16	38.0	1.0	52.1	n.d.	–	n.d.	–	16.9	93.0
Pul–MD	0.00	0.00	0.00	–	0.0	–	0.00	–	0.00	–	9.9	

Ms, microspheres; conc, concentration by weight; Eff, percentage of encapsulation efficiency.
Data are the mean of three independent experiments.

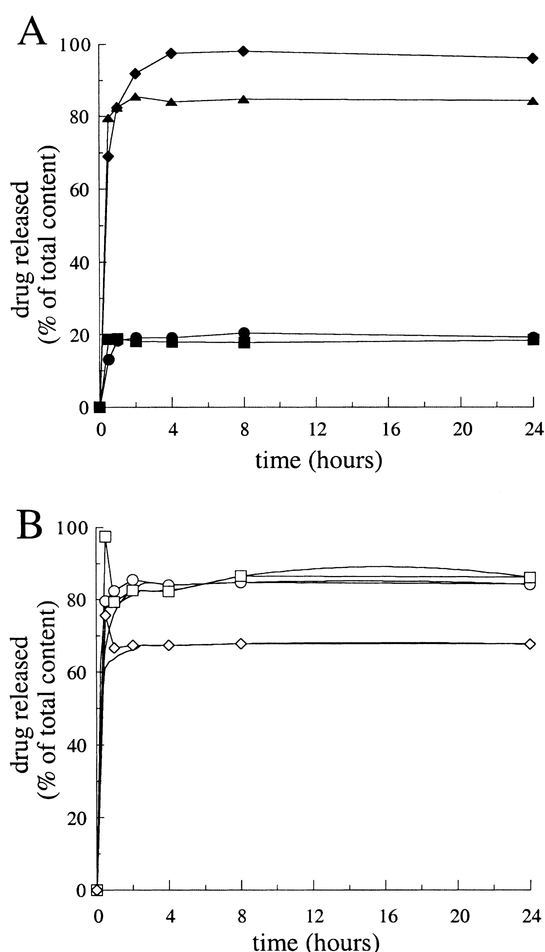

Fig. 8. Release profiles of different drugs from Pul–CyD microspheres. Panel A: indol (●), 3-aminobenzoic acid (■), propranolol (▲), and diclofenac (♦) released from Pul–α-CyD microspheres. Panel B: Release profile of propranolol from Pul–α-CyD (○), Pul–β-CyD (□), and Pul–γ-CyD (◊) microspheres. Experiments were performed and in phosphate buffer, pH = 7.4, at 37 °C.

3.6. In vitro drug release kinetics

In vitro dissociation kinetics gives important informations on the efficiency of a delivery system proposed for controlled drug release. The release kinetics was performed in phosphate buffer (pH 7.4) and 37 °C. Fig. 8A reports the release kinetics of drugs or TOCs characterized by different *K* values from Pul–CyD microparticles. In general, the most part of the drug is released within 60 min. However, after 1 h, the molecules characterized by the *K* values smaller than 3 (diclofenac, propranolol) showed an almost complete release of the included drug (82%) whereas the complexes of with *K* values higher than 4 (In, 3-ABA) showed a reduced (18%) release. Nevertheless, all the releases are characterized by a biphasic kinetics, a fast release is followed by a slower release and weak adsorption–desorption processes occurs, even if fresh medium was periodically added. Although release studies concerning complexes characterized by values of the interaction parameter *K* ranging between 3 and 4 were not performed, *K* values represent a measure of how drug is initially released, as confirmed by release studies of propranolol from the Pul–α-CyD, β-CyD, and γ-CyD stationary phase (see Fig. 8B).

4. Conclusions

CyD-containing pullulan microspheres were successfully obtained, using a single step procedure, by chemical cross-linking with epichlorohydrin of a 1:1 (w/w) mixture of the starch and α-, β-, or γ-CyD. The percentage of the ''active'' CyD in microspheres was determined by an original approach that consists

in the absorption of iodine or TOCs by the cavity of CyD. Based on the V_R of different drugs on the St/CyD stationary phase, accurate predictions can be done concerning drug–CyD interactions, drug loading and drug release profiles.

5. Nomenclature

3-ABA	3-aminobenzoic acid
4-HBA	4-hydroxybenzoic acid
BA	benzoic acid
CR	Congo Red
CyD	cyclodextrin
V_R	retention volume
ECH	epichlorohydrin
In	indol
ISEC	inverse size exclusion chromatography
MD	maltodextrin
Ms	microspheres
p-HB	*p*-hydroxybenzoate ethyl ester
Pul	pullulan
SD	standard deviation
TOC	typical organic compound

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