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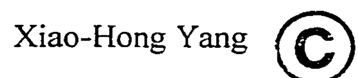
Characterization of a Novel β-Cyclodextrin-IUdR Prodrug Complex

by

Xiao-Hong Yang



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in

Pharmaceutical Sciences

Faculty of Pharmacy and Pharmaceutical Sciences

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Date: 21 Sept. 2000

Characterization of a Novel β-Cyclodextrin-IUdR Prodrug Complex

Abstract

The objective of this work was to prepare a suitable prodrug of 5-iodo-2'-deoxyuridine (IUdR) and to form the β-cyclodextrin-IUdR prodrug complex as a model for potential formulations. A novel adamantane-IUdR prodrug was prepared and the corresponding β-CyD complex was completely characterized. This project explores the possibility of utilizing a host-guest delivery strategy for delivery of radiopharmaceuticals for the treatment of hepatic cancers.

The solubility of the IUdR-prodrug was increased 317 times in the presence of one mole equivalent β-CyD. Detailed physicochemical analysis based on differential scanning calorimetry (DSC) and powder x-ray diffractometry was employed for detecting solid state interactions in the complex form. ^{1}H NMR spectroscopy was used for studying complexation in solution. The results described in this thesis clearly indicate IUdR-prodrug-β-cyclodextrin complex formation. The ^{1}H NMR spectra showed that a water soluble, 1:1 inclusion complex had indeed formed. The complex's formation constants were determined by HPLC, ^{1}H NMR spectroscopy, and capillary electrophoresis (CE). The binding constant was found to be 1.49×10^{5} M^{-1} by the HPLC method. An experiment similar to the determination of Log P for the prodrug was designed to estimate the rate of dissociation of the complex in aqueous solution into 1-octanol. The same experiment was repeated with a 1-octanol layer containing cholesterol at a 10 times higher concentration than the IUdR- prodrug complex. This study may be considered to be a greatly simplified model of the interaction at a cell surface. The stability of the IUdR-prodrug/β-CyD complex in plasma was also evaluated. The results obtained encourage further studies to establish the capability of drug delivery of therapeutic ^{131}I-radiolabelled IUdR-prodrug to liver tumors using inclusion complex formulation.

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Characterization of a Novel β-Cyclodextrin-IUdR Prodrug Complex** submitted by **Xiao-Hong Yang** in partial fulfillment of the requirements for the degree of Master of Science in Pharmaceutical Sciences.

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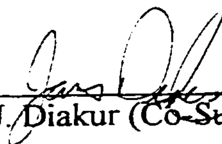

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Dr. Z. H. Xu (External Examiner)

Date: 20 Sept, 2000

DEDICATION

This thesis is dedicated with all my deeply love to my son, my brother, my mother, my father: Late Yongqing Yang

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Glossary of Abbreviations

μg	microgram
μL	microliter
μM	micro molar concentration
Å	Angstrom
α-CyD	alpha-cyclodextrin
β-CyD	beta-cyclodextrin
Alb	albumin
CE	capillary electrophoresis
Cl	chloride
CME-β-CD	carboxymethyl-ethyl-β-cyclodextrin
CM-β-CD	carboxymethyl-β-cylodextrin
DAD	direct absorbance detection
d	doublet
dd	doublet of doublets
ddd	doublet of doublet of doublets
DE-β-CD	diethyl-β-cyclodextrin
DM- β-CD	dimethyl- β-cyclodextrin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSC	differential scanning calorimetry
DTA	differential thermal analysis
dTMP	deoxythymidine-5'-monophosphate

dUDP	Deoxyuridine-5'-diphosphate
EOF	electroosmotic flow
γ-CyD	gamma-cyclodextrin
g	gram
g/L	gram(s) per liter
IAD	indirect absorbance detection
h	hour
HAI	hepatic artery infusion
HE-β-CD	hydroxyethyl-β-cyclodextrin
HPLC	high performance liquid chromatography
HP-β-CD	hydroxypropyl-β-cyclodextrin
IUdR	2'-deoxy-5-iodouridine
IdUMP	IUdR-monophosphate
i.v.	intravenous
kg	kilogram
L	liter
M	molar
m	multiplet
mg	milligram
min	minute
mL	milliliter
mM	millimolar concentration
mmol	millimole

mmol/L	millimole(s) per liter
mol	Mole(s)
μ	electrophoretic mobility
M-β-CD	methyl-β-cyclodextrin
n	number of observations
ng	nanogram
nm	nanometer
NMR	nuclear magnetic resonance
°C	degree celsius
pH	negative logarithm (base10) of the hydrogen ion
rpm	revolutions per minute
s	singlet
SD	standard deviation
SDE-β-CD	sulfobutylether-β-cyclodextrin
SE	standard error
TdR	thymidine
TLC	thin layer chromatography

1. Introduction

1.1. 5-Iodo-2'-deoxyuridine (IUdR)

1.1.1. Liver cancer therapy

Liver cancer encompasses a host of pathologies including hepatocellular carcinomas as well as metastases to this organ. From a global viewpoint, hepatocellular carcinoma (HCC) is the most common malignancy and within a demographic context, is linked to chronic liver cirrhosis and viral hepatitis. In particular, cirrhotic patients are at high risk, and one in five will develop HCC within five years (Bruix 1997). Although HCC is not a common form of cancer in North America, metastasis to the liver is the primary cause of mortality in patients with colorectal cancer. Portal vein drainage of the abdominal viscera is probably the route of dissemination of metastasis from distal sites including colon, stomach, pancreas as well as breast and lung.

In the event of HCC, surgical resection is contemplated for patients with solitary HCC who display no evidence of vascular invasion of extrahepatic metastasis. Even in situations where only a single mass is present, resection is restricted to tumors which are < 5 cm in diameter, since larger tumors may possess additional nodules making complete resection highly improbable. Thus, resection of liver tumors is possible in only a fraction (10 – 20 %) of the cases, however, at least half of these patients will experience recurrence. An even smaller number of patients are suitable candidates for liver transplantation, but again the high recurrence rate has led to the abandonment of this strategy (Johnson 1995). In the case of hepatic metastasis, multiple metastatic lesions are commonly observed, making it difficult to distinguish metastatic liver disease from multicentric hepatocellular carcinoma in patients whose primary site remains unidentified. Thus, resection can again only be suitable for treatment by resection or transplantation, while the recurrence rate for these attempts remains high.

For patients who are not candidates for surgical protocols, systemic chemotherapy has not offered significant improvement in the treatment of liver cancer. Presumably, this may in part be due to the fact that a vast number of anticancer drugs mediate cellular cytotoxicity by one of two mechanisms: (1) inhibition of DNA synthesis, or (2) disruption of the DNA template. Their action is therefore against cells that are in cycle, or in some instances, on a specific phase of the cell cycle, rather than selectively against tumor cells. There is good reason to believe, however, that significant reduction in tumor burden is possible at a dose level of two orders of magnitude higher than current clinical doses. Unfortunately, such a high dose level cannot be realized in practice due to the extreme toxicity towards normal rapidly dividing host tissues (i.e. gut mucosa and bone marrow). Solutions to this dilemma include the development of improved delivery agents for existing therapeutic agents.

The hypothesis of improving efficacy of drugs by increasing their local concentration has some validity, as evidenced by recent results obtained with surgical techniques for localization based on percutaneous and operative perfusion. Hepatic artery infusion (HAI), for example, is an approach that is based on the fact that liver neoplasms are primarily serviced by the hepatic artery. In theory, the administered agent can follow one of two pathways: (1) extraction by the liver, or (2) rapid clearance. This approach therefore, allows for the maintenance of a therapeutically meaningful regional concentration of the chemotherapeutic agent while reducing systemic exposure. Recent pharmacokinetic and biodistribution studies have supported this notion (Esminger 1989) and have further revealed that extensive metabolism of the agent on first pass through the liver has indeed occurred. Clinical trials with a limited number of patients (Remick 1989) have provided some promising results with respect to tumor response rate using HAI. Success in terms of survival remains to be assessed. Compilation of results from several randomized clinical trials which compared intravenous chemotherapy infusion of FUdR with HAI delivery of this agent revealed a 41% response rate (3% complete response, 38% partial response) (Meta analysis Group, 1996), thus implicating a significant benefit from localization

of the drug. However, the disadvantages of this technique relate to its invasiveness, the need for frequent replacement of the implanted infusion device and labor intensity. To address the lack of systemic regimens to complement surgery, we are currently exploring the possibility of utilizing a host-guest delivery strategy for selective hepatic delivery of radiopharmaceuticals for the treatment of hepatic cancers.

1.1.2. General properties of 5-iodo-2'-deoxyuridine (IUdR)

5-Iodo-2'-deoxyuridine (IUdR) is a halogenated nucleoside analog of thymidine (TdR) in which the 5-methyl group of TdR is replaced by iodine (Figure 1.1.2). The van der Waals radius of the 5-methyl group of thymidine is 2.0 Å, while that of the iodine atom is 2.15 Å. This similarity gives a compound that behaves remarkably like TdR (Eidinoff *et al.* 1959, Morris and Cramer, 1966, Commerford and Joel, 1979). Within the cell, both TdR and IUdR are phosphorylated by thymidine kinase to TdR-monophospate (dTMP) and IUdR-monophosphate (IdUMP), respectively. The former is then further phosphorylated in a stepwise reaction and incorporated into DNA. IdUMP, on the other hand, may either follow the fate of dTMP or be dehalogenated by thymidylate synthase (TS) to dUMP (Garrett *et al.* 1979) which is further converted to dTMP via the "de novo" TS-catalyzed reaction. This leads to the efficient incorporation of IUdR into the DNA of dividing cells with a thymidine replacement that can be as high as 50% (Szybalski, 1974). This substitution occurs only during the DNA synthetic (S) phase of the cell cycle (Eidinoff *et al.* 1959, Morris and Cramer, 1966) and during DNA repair of sublethal chromatin damage (G1 phase) (Iliakis and Nusse, 1983).

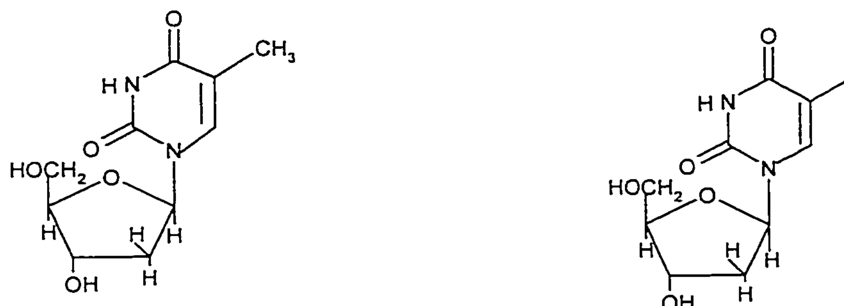


Figure 1.1.2. Thymidine (TdR) and 5-Iodo-2'deoxyuridine (IUdR).

Since at any given time, a certain fraction of cells within a tumor undergo DNA synthesis, administered IUdR can be incorporated. If radiolabelled, it is potentially suitable for diagnosis or therapy of certain types of cancer. It is important to note that most of the DNA-incorporated IUdR is retained for the life of the cell or its progeny (Commerford and Joel, 1979, Garrett *et al.* 1979). In contrast, unincorporated IUdR is rapidly catabolized to iodouracil or dehalogenated.

The serum half-life of IUdR in the human is < 5 minutes (Klecker *at al.* 1985) and in the mouse 7 min (Hampton and Eidinoff, 1961). This property compromises the delivery of IUdR radiotherapeutic doses to the tumors.

1.1.3. Human studies

Early studies with radioiodinated IUdR in humans have helped to determine that after intravenous administration, this agent is metabolized in a way similar to that of other radiolabeled nucleoside analogs in mammals (Eidinoff *et al.* 1959, Welch and

Prusoff, 1960, Kriss *et al.* 1963, Prusoff 1963). The very short biological half-life of IUdR in humans as well as in other mammals is due to its very rapid catabolism to 5-iodouracil and iodide, which accumulates in the thyroid and is excreted in the urine (Hampton and Eidinoff 1961, Kriss *et al.* 1962). As mentioned above, this high *in vivo* instability following systemic administration hampers efficient tumor targeting with radiolabeled IUdR, when employed with this route of administration. It is not surprising that attempts to use radiolabeled nucleoside analogs as diagnostic agents in patients suffering from cancer have largely failed (Philip *et al.* 1991). Kriss *et al.* (1963) have used the IUdR precursor, [^{131}I]-5-iodo-2-deoxycytidine for intravenous or locoregional administration (injection into the carotid artery homolateral to a brain tumor). No conclusion on the advantage of the locoregional versus the systemic route of administration could be made. This study provided important pharmacokinetic data.

1.1.4. Prodrugs of radiopharmaceuticals

A prodrug is a bioreversible modification of a structure of a therapeutic or diagnostic agent leading to the production of an inert precursor, which can be converted to an active drug by metabolic biotransformation. The prodrug concept was first introduced by Albert (1958) who suggested that this approach could be used to temporarily alter the pharmaceutical properties of a drug. The prodrug is used to solve pharmaceutical problems (such as stability, solubility), pharmacokinetic problems (such as retention time in plasma) or drug delivery problems (such as specific site targeting) (Roche 1987, Stella 1975, King 1994).

An ideal prodrug should fulfill certain requirements, which include ready conversion from the prodrug to active drug *in vivo*, adequate *in vitro* stability, low toxicity and other desired characteristics such as specific delivery and improved physicochemical properties. The prodrug-to-drug conversion can occur before, during, after absorption, or at a specific site in the body. For example, a prodrug designed to solve the solubility problem for making an intravenous injection solution should be readily converted to the parent drug once it is in the body. If a prodrug is

designed to produce a sustained drug action, the rate of conversion should match the desired rate of release from the formulation.

The interest in prodrugs as radiopharmaceuticals is for site-selectivity, which results from the metabolic trapping of the active agent. It involves the radiolabeled substrates (prodrug) that are enzymatically converted to radiolabeled products that are trapped and retained at the site of catalysis. Examples include:

1. Baranowska-Kortylewicz *et al.* (1997) designed several prodrugs for site-selective delivery of radiopharmaceuticals: Colon-directed ^{125}IUdR prodrugs, shown in Figure 1.1.4.A: include ^{125}IUdR-5'-β-D-glucopyranoside, ^{125}IUdR-5'-β-D-cellobioside, ^{125}IUdR-5'-β-D-glucuronide, and ^{125}IUdR-5'-β-D-galactopyranoside. These colon-targeting delivery systems are based on β-glycoside, that in this case release [^{125}I]-IUdR in the presence of bacterial glycosidases in the large intestine.
2. Lipophilic esters of [^{125}I]-IUdR for brain targeting. The structure is shown in Figure 1.1.4. B: [^{125}I]-IUdR-5'-N-methyldihydropyridyl ester.
3. Lipophilic esters of [^{125}I]-IUdR for ovarian cancer. The structure is shown in Figure 1.1.4. C: [^{125}I]-IUdR-3',5'-dioctanoyl ester.
4. Carrier-mediated delivery of [^{125}I]-IUdR prodrug. The structure is shown in Figure 1.1.4. D: N-hydroxysuccinimidyl ester (MMT = monomethoxytrityl).

Prodrugs of radiopharmaceuticals are an appealing option for a site-specific or local delivery of various radiotherapeutic agents. The main advantage of these approaches is accumulation of radioactivity in the target with reduced radiation burden to the whole body. Radiotherapeutic prodrugs activated selectively in the brain, colon, liver and cancer are possible by simply applying the basic concept of the metabolism as a controlling factor of prodrug targeting in conjunction with site-specific carriers or enzymes.

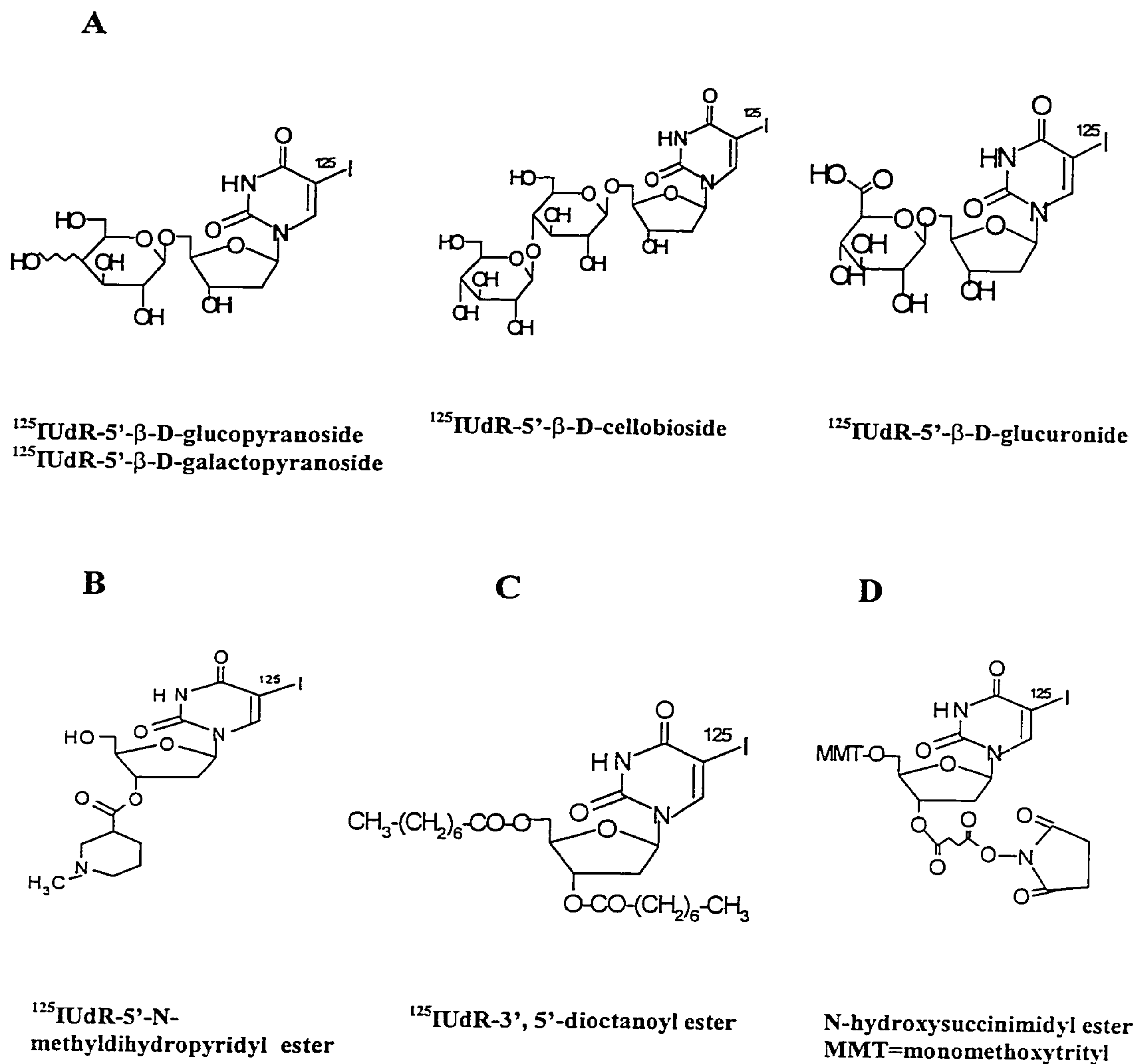
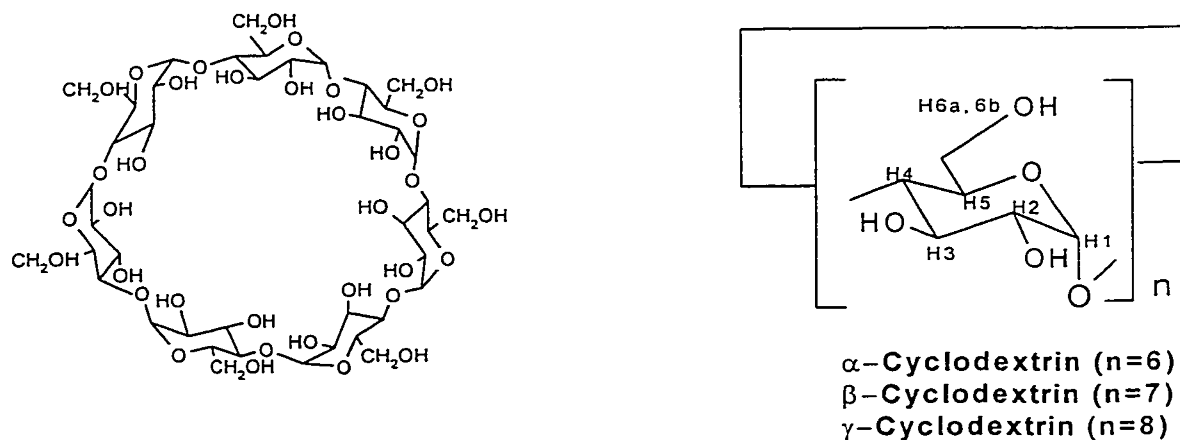


Figure 1.1.4. Chemical structures of prodrugs of 5-[^{125}I]iodo-2'deoxyuridine.

1.2. The cyclodextrins

1.2.1. General description of cyclodextrins (CyDs)

A cyclodextrin (CyD) is a cyclic oligomer of α-D-glucose composed of α(1-4)-linked glycopyranose units formed by the action of certain enzymes on starch. The three major cyclodextrins consist of six (α-CyD), seven (β-CyD), and eight (γ-CyD) glucopyranose units with cavity diameters of 4.9, 6.2 and 7.9 Å, respectively. This geometry gives the overall shape of a truncated cone with the wider side formed by secondary hydroxyl groups which are on the carbon 2 and carbon 3 atoms of glucose units and the narrow side formed by primary hydroxyl group which are on the carbon 6 atom of glucose units. Their interior structure consists of C-H bonds and ether-oxygen linkages and is distinctly hydrophobic. Conversely, the exterior surface of CyDs is populated with hydroxyl groups and is hydrophilic.



β-Cyclodextrin

Figure 1.2.1. Structure of the Cyclodextrins.

1.2.2. Review and applications of CyDs

Although cyclodextrins are considered to be a new group of pharmaceutical excipients, they have been known for 100 years (Loftsson and Brewster, 1996). Cyclodextrins were first isolated in 1891 by Villiers as degradation products of starch. In 1904, α-, β- and γ-CyD were characterized as cyclic oligosaccharides by Schardinger. In 1938, Freudenberg *et al.* reported that cyclodextrins are constructed from α(1→4)-linked glucose units; the molecular weights of the most common α-,β- and γ-CyD were determined by French and Freudenberg (1948). Freudenberg *et al.* (1948) were first to report that cyclodextrins can form inclusion complexes. In 1949, formation of the cyclodextrin guest complex was studied systematically by Cramer (1949). Pharmaceutical applications of cyclodextrin have been considered for over 30 years. There are over 2500 publications on the pharmaceutical aspects of the CyD, and 513 CyDs related patents have been filed in the U.S.A. alone. Cyclodextrins have now been widely used in the pharmaceuticals, foods, cosmetics, medicines and other industry (Szejtli, 1997).

1.2.3. Metabolism of CyDs

Cyclodextrins are slowly metabolized compared to starch. Linear dextrins are cleaved by α-1,4-glucanohydrase more easily than cyclodextrins and cyclodextrins are not hydrolyzed by enzymes that attack terminal groups. Andersen *et al.* (1963) fed ^{14}C-labeled-starch and cyclodextrins to rats and measured the radioactivity of $^{14}CO_2$ as a function of the time. On feeding ^{14}C-on starch the level of expired $^{14}CO_2$ reached a maximum after about 1h while ^{14}C-β-CyD reached the maximum after approximately 9h. The metabolism of degraded CyDs and starch is, however, comparable.

1.2.4. Toxicity of CyDs

Irie and Uekama (1997) have extensively reviewed the toxicity of the CyDs:

Oral safety: All toxicity tests have shown that orally administered cyclodextrin is harmless because of lack of absorption through the gastrointestinal tract. The acute toxicity of LD_{50} of β-CyD is 12500 mg/kg in mice, 12000 mg/kg in rats, 5000 mg/kg in dogs.

Parenteral safety: The parent CyDs in parenteral use have shown toxic effects on the kidneys. Parenteral administered of α- and β-CyDs causes necrosis of the proximal tubule of the kidney after glomerular filtration. The necrosis associated with administration of α- and β-CyDs is characterized by the presence of apical vacuoles and lysosomes in the epithelial cell of the proximal tubule. It was believed that the observed toxicity was related to the aqueous solubility of the respective cyclodextrin.

Cellular interactions: Irie *et al.* (1982) and Ohtani *et al.*(1989) found that CyDs can induce shape changes of membrane invaginations on human erythrocytes and, at higher concentrations, induce lysis. The hemolytic activity of the parent CyDs is reported to be in the order β-CyD > α-CyD > γ-CyD (Miyazawa *et al.* 1995).

1.3. Cyclodextrin inclusion complexes

1.3.1. Definition

Compounds that have interior cavities that are large enough to enclose organic 'guest' molecules can form *inclusion complexes*. Over the last decade there has been growing interest in the interaction of a variety of drugs with the α-, β- and γ-CyDs, which have cyclic structures with internal diameters of 0.6-1 nm. Uno *et al.* (1988) concluded on the basis of blue shifts in the spectra of amine N-oxides in the presence of CyDs, that the cavity environment is like methanol or ethanol, depending upon the probe. The cavity binds a hydrophobic portion of the 'guest' molecule, which is less hydrophilic than water, usually resulting in the formation of a 1:1 complex.

The minimum requirement for inclusion complex formation is size compatibility between host and guest molecules. The guest molecules must fit, entirely or at least partially, into the CyD cavity. In general, hydrophobic molecules or

residues, rather than hydrophilic ones, have a microheterogeneous matrix in such a polar solvent.

It has been shown that several forces for complex formation of CyDs act simultaneously. Various intermolecular forces are involved in complex formation, including van der Waals forces (Nishijo and Nagai 1991), hydrogen bonding (Tong *et al.* 1991, Jones *et al.* 1984), hydrophobic interactions (Niahijo and Nagai 1991; Tabushi *et al.* 1978), release of ring strain in the CyDs molecule (Jones *et al.* 1984), and change in solvent-surface tension (Orstan and Ross, 1987). These forces seem to work independently or in cooperation with each other.

1.3.2. Determination of inclusion complex formation

Various methods for the determination of inclusion complex formation have been described in the literature. These include optical spectroscopy: ultraviolet (UV), circular dichroism (CD), fluorescence, IR spectroscopies, nuclear magnetic resonance spectroscopy (NMR), single-crystal or powder X-ray diffraction combined with Differential Scanning Calorimetry (Saenger 1980), HPLC chromatography (Armstrong *et al.* 1986), potentiometry (Miyaji *et al.* 1976), thermal methods (Connors 1987) and capillary electrophoresis (Rundlett 1997).

1.3.2.1. Optical absorption spectroscopy

1.3.2.1.1. Ultraviolet absorption

UV spectroscopy is used to study the inclusion complex. For example, the close match of the UV spectra of 4-*tert*-butylphenol in dioxane and in an aqueous solution of α-CyD shows that the chromophore is included in the CyD cavity, which was considered to provide a molecular environment similar to dioxane (Etten *et al.* 1967). Toki *et al.* (1993) interpreted the disappearance of the charge-transfer band of the anthrancene-viologen as evidence of interruption of a donor-acceptor interaction between the two aromatic portions of the molecule, which in free solution takes place intramolecularly. Fujita *et al.* (1982) showed a modified β-CyD possessing a covalently attached chromophore as a "cap" exhibits blue shifts in the presence of

guest molecules, indicative of the chromophore's displacement upon guest binding to a less hydrophobic environment.

1.3.2.1.2. Circular dichroism

Although most guest molecules are achiral, in the chiral environment of a CyD a guest chromophore may exhibit induced circular dichroism (ICD), and this phenomenon has been widely applied as a means for deducing complex structure. The application to CyDs is based on the Kirkwood-Tinoco theory of polarizabilities, which when developed for the CyD case (Harata 1981) gives the following rule: if the transition dipole moment of the guest chromophore is aligned parallel to the axis of symmetry of the CyD (that is, the axis of the CyD cavity), then the sign of the ICD Cotton effect for that transition will be positive, whereas if the moment axis is aligned perpendicular to the cavity axis, the ICD sign will be negative. This rule applies to a chromophore that resides inside the cavity; if the chromophore is located outside the cavity the signs of the ICD are opposite to this. Examples of the use of ICD to deduce the guest orientation in CyD complexes include naphthalene derivatives (Harata *et al.* 1975), cyclohexenones (Bonora 1986)

1.3.2.1.3. Fluorescence

The effects of CyD complexation on emission spectra include information about the structure of the complex, but interpreting the information may not be straightforward because of disturbing effects. Fluorimetry can be used for the calculation of binding constant including changes in fluorescence polarization (Bright *et al.* 1985). Such changes reflect the relaxation time of molecules, thus providing direct evidence for complex formation and reduction (through quenching) in fluorescence intensity of the guest molecule on inclusion in the cyclodextrin cavity (Connors 1987). Inclusion reactions might be slow and it is essential that equilibrium (indicated by fluorescence reading that remains unchanged with time) be reached before the measurement of fluorescence intensity.

For example, Tung *et al* (1986) for a series of polymethylene-bis-β-naphthoates, $C_{10}H_7COO(CH_2)_nOCOC_{10}H_7$ (n=2, 3, 4, 5, 10), showed enhanced intramolecular eximer fluorescence in the presence of β-CyD or γ-CyD, indicating a favorable orientation for γ-CyD. This could occur by inclusion of both naphthyls, whereas in β-CyD it may take place through inclusion of the methylene chain and association of the naphthyls "exo" to the cavity. These results are consistent with the interpretation that γ-CyD protects against quenching of the fluorescence by sodium nitrite, whereas β-CyD does not (Tung *et al*, 1986).

1.3.2.2. Differential scanning calorimetry

In the DSC method, the sample and reference are maintained at the same temperature and the heat flow required to keep the temperatures equal is measured. Hence DSC plots are obtained as the differential rate of heating (in units of watts/second, calories/second, or Joules/second) against temperature. The area under a DSC peak is directly proportional to the heat absorbed or evolved by the thermal event, and integration of these peak areas yields the heat of reaction (in units of calories/second · gram or Joules/second · gram).

Sztatise *et al.* (1980) reported a complex thermal analysis of β-cyclodextrin using DSC. The DSC curve of β-cyclodextrin showed that most of the 14% water weight loss at about 100 ^{0}C and a minor endotherm at 220 ^{0}C corresponded to a reversible transformation within the molecule. Decomposition commenced at above 250 ^{0}C and was accompanied by melting at 300 ^{0}C prior to ignition of the sample. Uekama *et al.* (1983) examined diazepam-γ-cyclodextrin complex. Physical mixtures displayed an endotherm at 130 ^{0}C corresponding diazepam fusion and a broad endotherm at 290 ^{0}C due to cyclodextrin decomposition. The diazepam melting endotherm in the diazepam/γ-CyD complex was lost. Circular dichroism and X-ray diffraction confirmed the existence of the complex.

1.3.2.3. Single-crystal and powder X-ray analysis

X-rays are electromagnetic radiation lying between ultraviolet and gamma rays in the electromagnetic spectrum. The wavelength of X-rays is expressed in angstrom units (Å).

Diffraction is a scattering phenomenon. When X-rays are incident on crystalline solids, they are scattered in all directions. In some of these directions, the scattered beams are completely in phase and reinforce one another to form the diffracted beam (Klug and Alexander, 1974)

Single-crystal X-ray structure analysis provides much valuable information about cyclodextrin complexation. The detailed inclusion structure and mode of interaction have been elucidated by single-crystal X-ray analysis. Comparison of the structures, α-, β- and γ-cyclodextrin complexes reveals that the cyclodextrins always have a "round", slightly conical form whose narrower opening contains the O(6) H groups whereas the wider opening is occupied by the O(2) H and O(3) H groups. The glucose units always have a C1-chair conformation, the C(6) – O(6) bonds are preferentially directed away from the center of the ring (torsion angle O(5) – C(5) – O(6) is (-)-gauche) these bonds can, however, turn "inward" (torsion angle O(5) – C(5) – C(6)- O(6) (+)-gauche) after formation of hydrogen bonds between the O(6) H group and the guest molecule. Intramolecular hydrogen bonds O(3) – H······O(2) and O(3)······H – O(2) exist between the secondary hydroxyl groups of adjacent glucose units. These interactions stabilize the cyclodextrin ring (Saenger 1980).

Although single-crystal X-ray analysis provides much valuable information about cyclodextrin complexation, this technique is somewhat complicated for routine use. Difficulties are often encountered in preparing crystals with a suitable size for analysis, and crystals of many inclusion complexes are difficult to obtain.

Powder X-ray diffractometry has widespread use in the analysis of pharmaceutical solids. Powder X-ray diffractometry is a useful method for the characterization of cyclodextrin in powder or microcrystalline states (Saenger 1980). The X-ray power pattern of every crystalline form of a compound is unique, making

this technique particularly suited for the identification of different phases. The diffraction pattern of the complex is clearly distinct from that of the superposition of each component if a true complex exists. However this technique has limited utility in the identification of noncrytalline (amorphous) materials since the powder patterns consist of one or more broad diffuse peaks.

1.3.2.4. Potentiometry

Electrodes whose potential varies in response to changes in the concentration of a specific analyte in solution or in the gas phase can be used to measure cell voltage to extract chemical information is called potentiometry. Potentiometry is applicable to the systems involved in the acid-base equilibrium in the complexation. Potentiometric studies of binding can be carried out in the usual manner in which separate solution are prepared each to contain the same total substrate concentration and varying total ligand concentrations to conduced the experiment as a titration. Temperature control is particularly important in the potentiometric method.

Uekama and Hirayama (1978) have used the potentiometric method to studied inclusion complexation of prostaglandin $F_{2\alpha}$ with α- and β-cyclodextrins. Miyaji *et al.* (1976) has studied barbiture-β-cyclodextrin system.

1.3.2.5. Capillary electrophoresis

Capillary electrophoresis (CE) is a powerful separation technique and a probe for molecular interaction technique for small molecules, especially pharmaceutical compounds. Pharmaceutical compounds usually have been used routine separation technique – HPLC. However, CE has several advantages over traditional HPLC for some analysis. CE separations have four advantages: they are generally more efficient, they can be performed on a faster time scale, they require only nanoliter injection volumes, and they usually happen under aqueous conditions. CE has been a useful tool for studying molecular associations. CE binding studies involve measuring the change

in solute electrophoretic mobility causees the addition of another entity to the system. The mobility change must be due only to the association of the solute and substrate.

In the capillary electrophoretic separation, CyDs are carried towards the cathode and the neutral CyDs are carried towards the cathode and the detector by the electroosmotic flow (EOF) with the mobility. The EOF is caused by cations attracted to the negatively charged silanol groups on the inside of the fused silica capillary. These cations move towards the cathode, dragging the solute in the capillary in the same direction. The aromatic actions in the background electrolyte (BGE) move towards the anode against the EOF and away from the detector with the mobility. Equilibrium is formed between free CyDs being dragged towards the cathode by the EOF and CyD-aromatic anion complexes migrating towards the anode with the mobility. These results in the CyDs being separated from other neutral species not able to form complexes with the aromatic cations as BGE, the CyD-aromatic cation complexes will cause the CDs to move towards the detector ahead of the EOF with the mobility.

CE has been used for the estimation of the binding constants between CyD (host) and drug molecules (guest). The change in mobility of the CyD at a given concentration of the drug compared to that without the drug in the electrophoresis buffer, the change in the mobility at a concentration of a drug that will saturate the active site of the CyD. Measuring changes in migration time of the CyD can obtain binding constant of inclusion complex.

Lee and Lin (1996) have developed two methods for determining binding constant, the direct absorbance detection method (DAD) and the indirect absorbance detection (IAD). DAD estimates the inclusion complex binding constant between CyD and a charged guest molecule by measuring the change of mobility of the guest molecule in buffers containing various concentrations of CyD while IAD the CyD is run as an analyte and the mobility of the CyD is measured as a function of the concentration of the charged guest molecule.

Lemesle-Lamache *et al* (1996) determined the binding constant of salbutamol to unmodified and ethylated cyclodextrins by affinity capillary electrophoresis. The results show that several parameters influence calculation of binding constants: temperature, pH of the running buffer and voltage. Wren and Rowe (1992) explained theoretical aspects of chiral separation in capillary electrophoresis.

1.3.2.6. ^{1}H NMR spectroscopy

^{1}H NMR and ^{13}C NMR are already known as powerful tools for the characterization of inclusion complexes. Information provided by these methods, both in solution and in the solid state, includes: confirmation of complex formation, calculation of the stoichiometry of the complex and binding constant, and establishment of the geometrical structure of the inclusion complex (Loukas 1997, Djedaini 1990, Hirayama 1992, Csabai 1993).

Thakkar and Demarco (1971) were first to use NMR to study the CyD inclusion complexes. They noticed ^{1}H chemical shift changes of CyD H (3) and H (5) in the presence of many guest molecules, and implied that inclusion in the CyD cavity had taken place. The values of $\Delta\delta$ are critical functions of the position of the guest molecule in the host, the cavity size of the CyD and the [G]/[H] ratio. In general, the inclusion of an aromatic ligand into the β-CyD cavity causes significant chemical shift changes for the H3 and H5 protons located inside the β-CyD cavity. These shift changes are due to anisotropic shielding by the aromatic molecules, and their extent reflects the location of the corresponding protons with respect to the aromatic ring of the guest molecule. The changes in chemical shifts of the H3 and H5 resonances of CyDs induced by an aromatic guest can be interpreted qualitatively in terms of the spatial relationship between these protons and the centre of the benzene ring of the guest, based on the Johnson-Bovey theory of the ring-current effect (Johnson 1958). Nakajima *et al.* (1984) studied bencyclane/β-CyD inclusion complex. They found that the aromatic ring entered from the small-diameter side, since the relative magnitude of $\Delta\delta$ for H3 and H5 is contrary to the case of α-CyD, $\Delta\delta$ (H5) > $\Delta\delta$ (H3). This behavior

of H3 and H5 is the same as the reported by Thakkar *et al.* (1971). These authors assumed that association took place by approach of the guest molecule from the primary hydroxyl side of CyD, based on comparison of the relative magnitude of the Δδ for H5 and H3 in the barbiturate/β-CyD inclusion complex system. It strongly indicates inclusion of the aromatic moiety into in the cavity. (Rekharsky *et al.* 1995). For non-aromatic compounds, penetration of the guest into the β-CyD cavity also induces chemical shift changes for the β-CyD H3 and H5 protons, but to a lesser extent than aromatic compounds (Takahashi *et al.* 1971). The other β-CyD protons are located outside the cavity (H1, H2, H4, H6), thus are not usually affected by complexation.

Many other inclusion complexes have been used ^{1}H NMR as investigational tools: barbiturates (Pecca *et al*, 1970), adamantanes (Jaime 1990, Jaime, 1991), alicyclic and aromatic substrates (Gadre *et al*, 1997), gliclazide (Moyano *et al*, 1997), propofol (Trapani *et al*, 1998), mebendazole (Diaz *et al*, 1999), phenethylamine and ephedrins (Rekharsky *et al*, 1995), fenoprofen calcium (Diaz *et al*, 1999), rennin inhibitor (Kitamura *et al*, 1999), cyclosporin A (Miyake *et al*, 1999), cyclohexane derivatives (Rekharsky *et al*, 1994).

During complexation the chemical environment of some proton changes and this results in the chemical shifts. The shift changes are caused by shielding or deshielding effects. Usually the β-CyD H3 and H5 protons, which are located inside the cavity, and the protons of the guest that are included in the β-CyD cavity are affected the most due to the hydrophobic interactions. Therefore the observed difference Δδ between the chemical shifts of free and fully complexed states is used to comparison and calculated as functions of the depth of insertion of the ring of the guest molecule into the CyD cavity. Djedaini *et al.* (1990) and Loukas (1997) developed linear and non-linear methods, respectively, to use ^{1}H NMR spectroscopy to determine the binding constant of the CyD inclusion complex.

^{1}H NMR can provide information on the structure and geometry of cyclodextrin inclusion complexes as well as their dynamic properties (Inoue 1993).

Furthermore, through the NMR information, the chemical and physical nature of cyclodextrins, especially their cavities, is possible to investigation of the formation mechanisms of inclusion complexes. NMR has indeed played an important role in the study of cyclodextrin inclusion complexes.

1.4. Applications of inclusion complexes

There are two important areas of application of CyDs in pharmaceuticals:

- Improvement of drug properties.
- Drug delivery.

1.4.1. Improvement of drug properties

Cyclodextrin inclusion complexation can enhance the bioavailability of certain drugs and can also stabilize substances against oxidation, decomposition, and hydrolysis, and reduce side effects. Complexation can change physical and chemical properties of the guest molecule. The pharmaceutical effects and pharmaceutical potential are shown in Table 1.4.1.1

Some inclusion complex dosage forms have been introduced to the market in the Europe and Japan. Table 1.4.1.2 given a list of approved pharmaceutical products containing CyDs (Stella and Rajewski, 1997).

There are now several excellent books on cyclodextrins and their physicochemical properties. Fromming's *Cyclodextrin in Pharmacy* (1994), Duchene's *New Trends in Cyclodextrins and Derivatives* (1991), Szejtli's *Cyclodextrin Technology* (1988), and Duchene's *Cyclodextrin and their Industrial Uses*.

Table 1.4.1.1. Improvements of drug properties by cyclodextrin complexation (Uekama and Otagiri 1987)

Pharmaceutical Application	Drug
Improved dissolution rate and solubility	Nonsteroidal anti-inflammatory drug, steroid hormones, cardiac glycosides, oral antidiabetics, fat-soluble vitamins, barbiturates, sulfonamides, disulfiram, benzodiazepine, cinnarizine.
Suppression of volatility	Iodine, isosorbide dinitrate, menadione, salicylic acid, chlorobutanol, menthol, vanillin.
Powdering of liquid drugs	Nitroglycerin, benzaldehyde, fat-soluble vitamins,ONO-802
Suppression of unpleasant tastes	chloral hydrate, clofibrate, phenbufen, flurbiprofen, pirprofen.
Reduction of local hemolysis	Chlorpromazine, flurbiprofen, imipramine, benzyl alcohol, fulfenamic acid, antibiotic.
Deceleration of chemical reactions:	
Hydrolysis	Prostacyclin, carmoful, indomethacin, cardiac glycosides, procaine, benzocain, hydrocortisone-17-varelate.
Oxidation and thermal decomposition	Benzaldehyde, nitroglycerin, vitamins, Isosorbide dinitrate.
Photodecomposition	Phenothiazines, fat-soluble vitamins, Benzaldehyde, metronidazole
Dehydration	Prostaglandin E_1 and E_2
Enhancement of drug absorption	
Oral route	Phenytoin, digoxin. diazepam, carmoful, salicylic acid, prednisolone, flurbiprofen, spironolactone, indomethacin, ketoprofen.
Rectal route	Acetohexamide, carmoful, flurbiprofen, phenobarbital., prednisolone.
Transdermal	Predonisolone, betamethasone beclomethazone dipropionate.
Reduction of local irritancy:	
GI muscular	Indomethacin, flurbiprofen, biphenyl acetic acid.
Muscular tissue damage:	Chlorpromazine, tiamulin, predonisolone

Table 1.4.1.2. A listing of approved pharmaceutical products containing cyclodextrins (Stella and Rajewski 1997)

Drug	Trade name	Dosage form	Company	Country
PGE1/α-CD 20μg/ampoule	prostavasin	i.a.	Ono Schwarz	Japan Germany
PGE1/α-CyD 500μg/ampoule	Prostandin 500	Infusion	Ono	Japan
PGE/β-CyD	prostarmon	Sublingual tablet	Ono	Japan
OP-1206/α-CyD	Opalmon	Sublingual table	Ono	Japan
Piroxicam/β-CyD	Brexin	Tablet	Chiesi Robapharm Promedica	Italy France France
	Brexidal		Nycomed Lauder	Scandanavia German
	Cycladol	Suppository	Masterpharm	Italy Belgium Netherlands Switzerland
Benexate/β-CyD	Ulgut Lonmiel	Capsule capsule	Shionogi teiloku	Japan Japan
Iodine	Mena-Garge	gargline solution	kyushin	Japan
DexamethasoneGlyteer/β-CyD	Glymesason ointment	Ointment	Fujinage	Japan
Nitroglycerin/β-CyD	Nitropen	Sublingual	Nippon Kayaku	Japan
Cefotian hexetil HCL/α-CyD	Pansporin T	Tablet	Takeda	Japan
ME 1207/β-CD new cephalosporin	Meiact	Tablet	Meiji Seila	Japan
Thyaprofeinic acid/β-CyD	Suramyl	Tablet	Roussel-maestrelli	Italy
Chlordiazepoxide /β-CyD	Thansillium	Tablet	Gador	Argentina
Hydrocortisone/HP-β-CyD	Dexocot	mouth wash	Icelandic pharm	Iceland
Itraconazole/HP/β-CyD	Sporanox	Liquid	Janssen	Belgium
Carlic oil/β-CyD	Xund,tegra Allidex Garlessence	Dragees Tablet	Bipharm, Hermes Phaarma-fontana CTD	Germany U.S.A.

1.4.2. Cyclodextrins in drug delivery systems

Cyclodextrins (CyDs) are capable of improving the undesirable properties of drug molecules for various routes of administration through the formation of inclusion complexes, for example, oral formulation, parenteral formulation, transdermal, nasal, rectal, pulmonary formation. In an oral drug delivery system, hydrophilic and ionizable CyDs can serve as potent drug carriers for immediate release and delayed release formations while the release rate of water-soluble drugs can be retarded by hydrophobic CyDs. Because CyDs are able to extend the function of pharmaceutical additives, the combination of molecular encapsulation with other carrier materials will become an effective and valuable tool in the improvement of drug formulation. Also, the most desirable characteristic for the cyclodextrin-based site-specific drug delivery includes colon targeting and brain targeting.

1.4.2.1. Controlled release in oral drug delivery systems

1.4.2.1.1. Immediate release

The hydrophilic and hydrophobic CyDs are used for the immediate release and the prolonged release type formations, respectively.

Immediate release formulations of analgesics, antipyretics, coronary vasodilators are particularly useful in emergency situations. Since the dissolution rate of the poorly water-soluble drugs is mainly responsible for both the rate and extent of oral bioavailability of a drug, various hydrophilic materials are used to gain an immediate release formulation. The hydrophilic CyDs have been extensively used to enhance the oral bioavailability of steroids, cardiac glycosides, nonsteroidal anti-inflammatory drugs, barbiturates, antiepileptics, benzodiazepines, anti-diabetics (Szejtli 1988, Uekama and Otagiri 1987, Brewster 1991).

The rate and extent of oral bioavailability of a poorly water-soluble drug from its CyD complex can be optimized by adjusting various factors that affect the dissociation equilibrium of the complex both in the formulation and in the biophase

in which the complex is administered (Stella and Rajewski, 1997, Uekama, 1987). Only a free form of the drug, which is in equilibrium with the complex form of the drug in solution, is capable of penetrating lipophilic barriers consisting of either mucosal epithelia or stratified cell layers and eventually entering the systemic circulation.

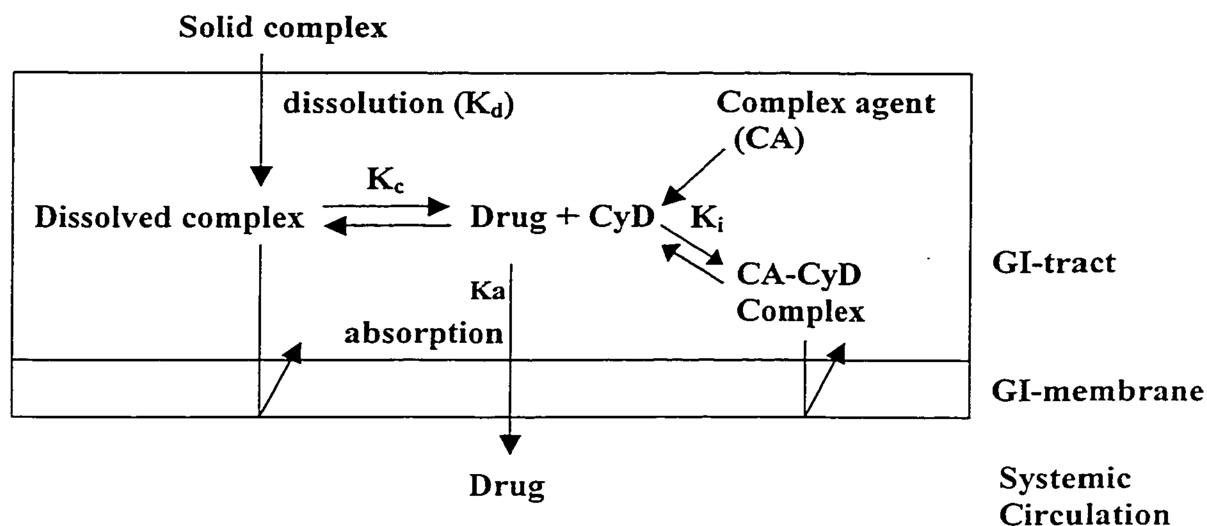


Figure 1.4.2.1. The process of drug absorption from an inclusion complex following dissolution and dissociation in the gastrointestinal tract.

Modified from Uekama and Otagiri (1987). K_d, dissolution rate constant of drug-CyD complex; K_c, stability constant of drug-CyD complex; K_i, stability constant of competing agent-CyD complex; K_a, absorption rate constant of drug.

As shown in Figure 1.4.2.1, CyDs are supposed to act only as carrier materials and help to transport the drug through an aqueous medium to the lipophilic absorption surface in the gastrointestinal tracts. Therefore, such applications have been successful when the rate-limiting step in drug absorption is dissolution of the drug itself and not absorption across the gastrointestinal tract. Recent studies have shown that complexation of β-CyD with imidazole anti-fungal agent, such as ketoconazole and econazole, provided an enhancement of oral bioavailability (Esclusa-Diaz *et al*, 1996, Pedersen *et al*, 1998).

1.4.2.1.2. Delayed release

An enteric preparation can be classified as time-controlled release, since the drug is preferentially released in the intestinal tract. Hydrophobic excipients having a weak acidic group are preferable because they are less soluble in water at low pH, but are soluble in neutral and alkaline regions due to the ionization of the acidic group. Under the control of this pH dependence, the delayed release dosage form, which passes from the stomach into the higher pH environment of the upper small intestine, would experience increased drug release. For this purpose, CME-β-CyD was developed which exhibits pH-dependent solubility for use in selective dissolution of the drug-CyD complex (Uekama 1989). CME-β-CyD displays limited solubility under acidic conditions, such as those found in the stomach, with the complex solubility increasing as the pH passes through the pKa (3.7) of the CME-β-CyD. CME-β-CyD complexes have been used *in vitro* and *in vivo* studies with diltiazem, a calcium-channel antagonist, and molsidomine, a peripheral vasodilator. The diltiazem studies were carried out in gastric acidity-controlled fasting dogs with the gastric pH being controlled to less than two and greater than six. Diltiazem absorption was slower at high gastric acidity (t_{max}, 4.0 ± 0.5 h) than at low gastric acidity (t_{max}, 2.3 ± 0.2 h). The *in vitro* release data measured using a pH changeable dissolution apparatus were in good agreement with this *in vivo* data (Uekama 1993). Molsidomine absorption from tablets containing CME-β-CyD was also studied in both gastric acidity-controlled dogs in the fasted and fed states, Under high gastric acidity, molsidomine absorption was significantly retarded relative to that found under low gastric acidity conditions. In high gastric acidity, the delayed absorption effect was more pronounced under fasted conditions. As in the diltiazem studies, a high degree of correlation was noted between the *in vivo* studies and the *in vivo* release measured with the pH-changeable dissolution apparatus.

1.4.2.2. Cyclodextrins in parenteral drug delivery systems

The purpose of cyclodextrins in parenteral drug delivery is to solubilized the drug, enabling rapid and quantitative delivery of sparingly water soluble drugs for intravenous dosing, reducing irritation at the site of administration of paternally administered drugs, and stabilization of drugs.

The earlier attempts to use CyDs in parenteral formulations were not successful because β-CyD is parental nephrotoxicity. Recently scientists tried to use SBE4-β-CyD or HP-β-CyD for parental drug delivery. Frijlink *et al.* (1991) studied the effects of cyclodextrins on the disposition of intravenously injected into the rat. Naproxen and flurbiprofen HP-CyD complexes (binding constants are 2207 and 12515 M^{-1}, respectively), were administered intravenously to rats. This study evaluated the tissue distribution in rats of naproxen and flurbiprofen form HP-β-CyD containing solution as compared to solutions of the drugs dissolved in the rat plasma. The disposition in the body of naproxen was not significantly altered by the complexation. This indicates that immediately after administration the cyclodextrin complexes dissociated completely. However, the initial distribution of flurbiprofen was changed upon complexation. Drug concentrations in the liver, brain, kidney, and spleen were increased, indicating that HP-β-CyD may improve the presentation of flurbiprofen to biomembranes, as compared to plasma protein. Frijlink *et al.* (1997) hypothesized that the high levels in some tissues reflected a transitory change in protein binding when HP-β-CyD was used as the deliver vehicle. In an *in vitro* study, HP-β-CyD was able to compete to protein binding for both naproxen and flurbiprofen, but the effect was more noticeable with the flurbiprofen. This result can be explained by the simultaneous displacement of the drug from the cyclodextrin carrier by plasma cholesterol (the binding constant of cholesterol/HP-β-CyD inclusion complex is 19,000 M^{-1}).

1.4.2.3. Cyclodextrin-based site-specific drug delivery

Recently, pharmaceutical researchers have tried to design a system that is able to deliver drugs more efficiently to specific organs, tissues and cells. CyD complexes are in equilibrium with guest and host molecules in aqueous solution, with the degree of the dissociation being dependent on the magnitude of the stability constant of the complex. This property is a desirable quality because the complex dissociates to give free CyD and drug at the absorption site, and only the drug in free form enters into the systemic circulation. However, in some cases, the complex can dissociate before it reaches target organs or tissues.

1.4.2.4. Cyclodextrin-drug conjugates as colon targeting carriers

Colon targeting involve a delayed release with a fairly long lag-time because the time required to reach the colon after oral administration is expected to be about 8 h (Friend 1992) in man. The CyDs are not suitable for colon-specific delivery because of release of the drug before it reaches the colon. In order to solve this problem, the drug is covalently bound to CyD. The advantage of CyD-drug conjugates is that they can survive passage through the stomach and small intestine. Under this condition, the drug release will be triggered by enzymatic degradation of CyDs in the colon. Uekama *et al.* (1997) and Hirayama *et al.* (1996) have designed amide- and ester-type conjugates of the anti-inflammatory drug biphenylacetic acid (BPAA) with three natural CyDs, anticipating a new candidate for a colon-targeting product. Figure 1.4.4.1 shown the release mechanism of BPAA from CyD prodrugs in rat cecum and colon. In the case of ester-type conjugates, drug release is triggered by the ring opening of CyDs, which consequently provides site-specific drug delivery in the colon while the amide conjugates do not release the drug even in the cecum and colon, despite the ring opening of CyDs. The amide linkage of the small saccharide drug conjugates may be resistant to the bacterial enzymes and poorly absorbable from the intestinal tract due to high hydrophilicity. Therefore, the esters type conjugate is

preferable as a delayed release-type prodrug that can release a parent drug selectively in the cecum and colon.

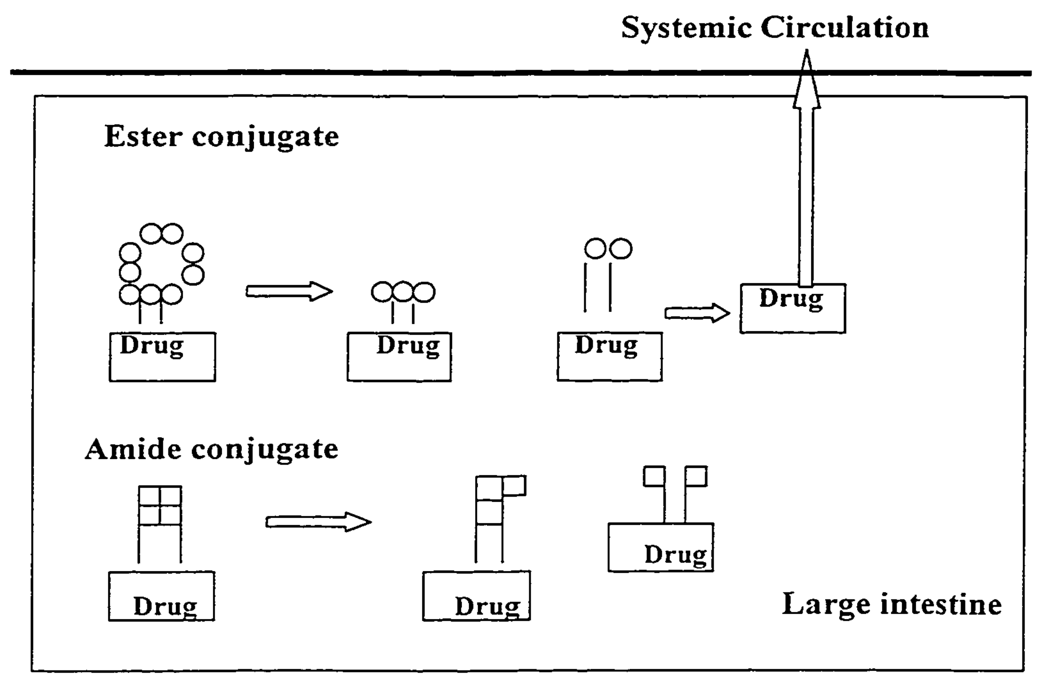


Figure 1.4.2.4.1. Release mechanism of drug from γ-CYD-drug conjugates in rat cecum and colon (Minami 1998).

1.4.2.5. Cyclodextrin-drug conjugates as brain targeting carriers

The specific delivery of drugs to the brain is obstructed by the presence of the blood-brain barrier. One of the strategies to overcome this transport problem is to prepare prodrugs having high lipophilicity that pass through the blood-brain barrier. Begley (1996) has found when a drug is covalently coupled to 1-methyl-1, 4-dihydronicotinic acid through an enzymatically labile linkage, its lipophilicity increases and allows for selective delivery of drug molecules into the brain across the blood–brain barrier. This barrier is characterized by endothelial cells of cerebral

capillaries that have tight continuous circumferential junctions, thus restricting the passage of polar drugs to the brain. After entry into the brain, the dihydropyridine moiety is oxidized by oxidoreductase to 1-methyl-pyridinium cation. Thus, the polar drug 1-methylpyridinium derivative is trapped in the brain since it cannot pass through the blood-brain barrier. Subsequently, the parent drug is released from the prodrug by the action of enzymes. This is an essential concept of Bodor's chemical delivery system and is applied to brain targeting of drugs such as steroids, anti-tumor agents (Bodor 1985). Brewster (1991) used modified β-CyD to form the soluble inclusion complexes to solve the prodrugs of the chemical delivery system that are poorly water-soluble due to the presence of the lipophilic moiety. The improvement in chemical delivery systems using HB-β-CyD with testosterone was reported by Anderson *et al.* (1988) dexamethasone (Pop *et al.* 1991) and benzyl penicillin (Siegal *et al.* 1997).

2. Experimental hypothesis and objectives

The overall effectiveness of certain anticancer radiopharmaceuticals, such as rediolabelled IUdR, can be improved by improving *in vivo* stability, which may be optimized by complexation with cyclodextrins. Such a non-invasive approach may prove beneficial for cases of hepatic cancer where surgery is not an option. We have chosen to couple IUdR with adamantane prior to complexation with CyDs, in order to test whether a non-covalent complex is suitable for *in vivo* clinical application. The advantage of such a system is that characterization of the chemical and physical properties of the complex can be accomplished with the non-radioactive form.

The rationale for employing molecular encapsulation as a delivery strategy is the expectation of improvements in the physiochemical properties of encapsulated therapeutic agents including (i) increased stability of variable drugs, (ii) enhancement of aqueous solubility and (iii) improvement in bioavailability through slow release action. A fundamental prerequisite to the evaluation of such therapeutic complexes for *in vivo* application is a thorough understanding of the structure and stability of such potential therapeutic complexes.

The first phase of the project is therefore predicated on the following goals.

- Preparation and characterization of the cold IUdR-adamantane prodrug;
- Assessment of the binding constant of the prodrug-β-CyD complex by suitable physical means (i.e. HPLC, Capillary Electrophoresis, ^{1}H NMR Spectroscopy).
- Preliminary assessment of the stability of the complex in aqueous solution;
- Preliminary assessment of the stability of the complex to cholesterol challenge (as β-CyD is known to possess affinity for cholesterol);
- Preliminary assessment of the stability of the complex to serum challenge was also carried out.

3. Experiments

3.1. Materials

3.1.1. Chemicals, solvents and equipments

β-Cyclodextrin (β-CyD), 1-adamantanamine hydrochloride, IUdR and 1-octanol (99.99% spectroscopic grade) were purchased from Sigma Aldrich Canada Ltd. β-CyD was dried over P_2O_5 at 60 ^{0}C prior to use. Chloroform was distilled from P_2O_5 and pyridine was distilled from CaH_2 and stored over 3 Å molecular sieves. All solvents used for synthesis were reagent grade unless otherwise specified. All other chemicals and solvents were of analytical reagent grade.

A Dubnoff Metabolic Shaking Incubator was used to establish equilibrium in the partition coefficient determinations, the phase solubility tests, and the stability tests of the inclusion complexes. A Vortex Genie ™ (Scientific Industries, Inc. Bohemia, N.Y. U.S.A) was used for mixing samples. A Branson 2200 sonicator was used for sonication. Eppendorf micropipettes were used for measurement of micro quantities of liquids. All centrifugation was performed using an Eppendorf 5412 microcentrifuge and Eppendorf microcentrifuge tubes. Samples were passed through a 0.45 μm membrane (Millipore) filter fixed in a Nucleopore holder for phase solubility tests and sample preparation for capillary electrophoresis. A sterile, low protein binding 0.22 μm Filter Unit (MILLEX ®-GV, MILLIPORE) was used for filtering plasma samples. Thin-Layer Chromatography (TLC) was performed on Kieselgel 60 F_{254} (Merck) and visualization was accomplished by charring with 5% methanolic sulfuric acid. Column chromatography was performed using Merck 9385 silica gel (40-63 μ).

3.1.2. Instrumentation

^{1}H NMR spectra were record on a Bruker AM 300 spectrometer. Chemical shifts in $CDCl_3$ solution are reported in parts per million downfield from TMS, or in the case of D_2O solution, HOD was set at δ 4.82 (25 ^{0}C) unless otherwise specified. A PU 8740 UV/VIS Scanning Spectrophotometer (PHILIPS) was used to determine partition coefficients. A Labconco Freeze Dryer 3 was used to lyophilize the inclusion complexes. A differential scanning calorimeter (DSC 120, SEIKO SII, model

SSC/5200) was used to study the formation of the inclusion complex formed between prodrug 7 and β-CyD. An automated X-ray diffractometer (Rigaku Geigaflex Vertical Coriometer X-ray Diffraction Unit, Toronto, Canada) was to use to obtain the solid state diffraction patterns of the inclusion complex. A Beckman P/ACE 5500 capillary electrophoresis system equipped with a Diode Array Detector (Beckman Instrument Inc., Fullerton, CA) was used for determination of the binding constant for the inclusion complex formed between compound **5** and β-CyD. Electrophoresis and electropherogram analysis was performed using the System Gold version 8.10 software (Beckman Instrument Inc., Fullerton, CA). HPLC analytical systems comprised of a Waters system equipped with two Waters 501 solvent delivery pumps, a Waters model U6K injector, and a Waters 486 tunable absorbance detector. Analysis was carried out at a HP 79994A workstation using Waters 810 software for data analysis. Chromatographic separations were performed using a reverse-phase column (Phenyl radial-pak cartridge; 10 μm, particle size, 8 mm id x 100 mm length) equipped with a connected guard column (μBondapak™ phenyl Guard-pak™).

3.1.3. Animals

Sprague Dawley male rats (350-400 g) were purchased from the Health Sciences Animal Service, University of Alberta, and their plasma was used for determination of the stability of the inclusion complex. Animals were handled in accordance with guidelines of the Canadian Council on Animal Care, and experimental protocols were approved by the University of Alberta Health Science Animal Welfare Committee.

3.2. Methods

3.2.1. Chemical syntheses

3.2.1.1. Mono-6-deoxy-6-*N*-benzoyl-β-cyclodextrin 5

Mono-6-deoxy-6-(*p*-toluenesulfonyl)-β-cyclodextrin (1). Compound **1** was synthesized according to the procedure published by Petter and Salek (1990). β-CyD (6000 mg, 5.29 mmol.) was suspended in 50 mL of water, and NaOH (657 mg, 16.4

mmol) in 2 mL of water was added dropwise to the stirred CyD solution. The suspension became homogeneous during the addition of the base. A solution of *p*-toluenesulfonyl chloride (1200 mg, 6.35 mmol) in acetonitrile (3 mL) was then added dropwise to the reaction mixture. After stirring at room temperature for 3 h, the precipitate was removed by filtration, and the filtrate kept at 4 ^{0}C overnight. The resulting white precipitate was collected by filtration to give 604 mg of white solid after drying *in vacuo*. A second crop was obtain after an additional 12 h at 4^{0} C for a combined yield of 11 %: ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.74 (d, J = 8.07 Hz, 2H aromatic), 7.42 (d, J = 8.02 Hz, 2H, aromatic), 5.87-5.58 (m, 14H), 4.82 (br s, 4H), 4.76 (br m, 3H) 4.55-4.13 (m, 6H), 3.74-3.43 (m, 28H), 3.42-3.18 (m, overlaps with HOD), 2.42 (s, 3H).

Per-*O*-acetyl-mono-6-deoxy-6-azido-β-cyclodextrin (2). Compound 1 (515 mg, 0.4 mmol) in dry *N,N*-dimethylformamide (10 mL) was treated with sodium azide (260 mg, 4.0 mmol) at 120 ^{0}C for 2 h. After cooling, the reaction mixture was filtered and evaporated to give crude mono-6-deoxy-6-azido-β-cyclodextrin. Pyridine (10 mL) and acetic anhydride (8 mL) were added, and the mixture was stirred for 48 h at room temperature, then evaporated. Column chromatography of the residue over silica gel, using 3:2 hexane: acetone v/v as the eluant, gave 400 mg (46%) of 2 as an amorphous white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.30-5.08 (m, 7H), 5.07-4.90 (m, 7H), 4.78-4.65 (m, 7H), 4.55-4.40 (m, 7H), 4.25-3.94 (m, 14H), 3.74-3.54 (m, 7H), 2.10-1.90 (m, 60 H, $COCH_3$).

Mono-6-deoxy-6-azido-β-cyclodextrin (3). Per-*O*-acetyl-β-cyclodextrin derivative **2** (400 mg, 0.2 mmol) was treated with sodium methoxide (11 mg, 0.2 mmol) in methanol (20 mL) at room temperature for 4 h. The reaction mixture was neutralized with acetic acid (5 mL) and the solvents were removed. The resulting crude solid was purified by column chromatography over silica gel using 2:1:1 v/v/v ethyl acetate-methanol- water as eluant to give 162 mg (75 %) of **3** as a white solid.

Mono-6-deoxy-6-amino-β-cyclodextrin (4). A mixture of the compound **3** (162 mg, 0.14 mmol) and triphenylphosphine (140 mg, 0.56 mmol) was dissolved in *N,N*-dimethylformamide (3 mL) and the reaction was stirred at room temperature for 45 min. Ammonium hydroxide (3 mL) was then added dropwise over 5 min. Precipitation occurred, therefore methanol (2 mL) was added to dissolve the precipitate and the reaction was stirred at room temperature for 72 h. Analysis by the TLC: 7:2:2 v/v/v 2-propanol-water-ammonium hydroxide revealed that the reaction was complete. The solvent was removed and the residue was diluted with water (20 mL) and the aqueous layer was extracted several times with chloroform until the organic layer did not display UV activity. The aqueous layer was concentrated, and dissolved in water (3 mL) and lyophilized to give 34 mg (21 %) of **4**.

Mono-6-deoxy-6-*N*-benzoyl-β-cyclodextrin (5). Compound **4** (34 mg, 0.03 mmol) was dissolved in dry N, N-dimethylformamide (1 mL) and K_2CO_3 (16.5 mg, 0.12 mmol) and benzoic anhydride (8.5 mg, 0.038 mmol) were added. The reaction was stirred at room temperature for 48 h. The reaction was then concentrated and purified by column chromatography over silica gel using 6:2:2 v/v/v 2-propanol-water-ammonium hydroxide to give 19 mg (51 %) of **5** as a white solid. ^{1}H NMR (500 MHz, D_2O), δ 7.88 (d, 2H, J_d = 7.3 Hz), 7.77 (t, 1H, J_t = 7.3 Hz), 7.63 (t, 2H,Jt = 7.3 Hz), 5.21(d, 1H, J_d = 7.30 Hz, H-1), 5.20-5.15 (m, 5H, H-1), 5.03 (d, 1H, J_d = 3.0 Hz), 4.25 (d, 1H, J_d = 13.0 Hz, H-6), 4.12 (m, 1H), 4.10-3.99 (m, 3H), 3.22 (bd, 1H, J_d =13.0 Hz, H-6).

3.2.1.2. *N*-(1-Adamantyl) benzamide (6)

A mixture of 1-adamantanamine hydrochloride (187 mg, 1 mmol) and benzoic anhydride (565 mg, 2.5 mmol) in 1:1v/v chloroform-pyridine (10 mL) was stirred for 48 h at room temperature and then evaporated. The residue was dissolved in chloroform (50 mL) and washed with 2 % aqueous sulfuric acid, water, saturated aqueous sodium hydrogen carbonate and saturated aqueous sodium chloride,

respectively. The solvent was removed, and the resulting material was purified by column chromatography over silica gel using 12:1 v/v hexane-ethyl acetate to give 90 mg (35 %) of 6. UV: λmax 224 nm; ^{1}H NMR (300 MHz, $CDCl_3$), δ 7.55 (m, 2H, aromatic), 7.50-7.38 (m, 3H, aromatic), 5.78 (s, NH), 2.13 (s, 9H, adamantane protons), 1.7 (s, 6H, adamantane protons)

3.2.1.3. 5-Iodo-5'-adamantoyl-2'-deoxyuridine (7)

To a solution of 1-adamantanecarboxylate acid (540 mg, 3 mmol) in toluene (15 mL) was added oxalyl chloride (525 μL, 6 mmol), and the reaction was stirred for 12 h at room temperature. The reaction mixture was concentrated and the resultant oil was dissolved in chloroform (6 mL) and then added dropwise to solution of 5-iodo-2'-deoxyuridine (500 mg, 1.5 mmol) in pyridine (10 mL) at 0 ^{0}C. The mixture was then allowed to warm to room temperature and the reaction was monitored by TLC (4:1 v/v chloroform-methanol). Upon completion of the reaction, pyridine was removed under reduced pressure. The residue was dissolved in chloroform (75 mL) and successively washed with saturated aqueous sodium hydrogen carbonate, water saturated sodium chloride, and then dried (Na_2SO_4), filtered and concentrated. Column chromatography over silica gel using 12:12:1v/v/v hexane-ethyl acetate-ethanol gave first the diadamantanyl derivative **8** 61 mg (8 %) followed by 498 mg (68 %) of the desired prodrug 7. Data for the compound 7: Rf = 0.18 (solvent: 12:12:1 v/v/v hexane-ethyl acetate-ethanol); UV: λmax 207, 285 nm; ^{1}H NMR ($CDCl_3$) δ 9.05 (s, 1H, NH), 7.91 (s, 1H, H-6), 6.25 (dd, 1H, J_d = 7.32 Hz, J_d = 5.8 Hz, H-1'), 4.4 (dd, 1h, J_d = 12.7 Hz, J_d = 12.7 Hz, J_d = 1.9 Hz, H-5$_b$'), 4.38 (m, 1H, H-3'), 4.25 (dd, 1H, J_d = 12.7 Hz, J_d = 1.9 Hz, H-5a'), 4.23 (s, 1H, OH), 2.53 (ddd, 1H, J_d = 13.4 Hz, J_d = 8.5 Hz, J_d = 2.8Hz, H-2b'), 2.06 (dd, 1H, J_d = 13.7 Hz, J_d = 7.02, H-2a'), 2.06 (m, 3H adamantane γ-protons), 1.93 (m, 6H, adamantane α-protons), 1.74 (m, 6H, adamantane β-protons).

Data for compound (8): R_f = 0.36 (solvent same as the compound 7); ^{1}H NMR ($CDCl_3$) δ 8.39 (s, 1H, NH), 7.89 (s, 1H, H-6), 6.22 (dd, 1H, J_d = 8.9 Hz, J_d = 5.0 Hz, H-1'), 5.18 (d, 1H, J_d = 6.1 Hz, H-3'), 4.45 (dd, 1H, J_d = 12.5 Hz, J_d = 3.4 Hz, H-5b'), 4.29 (dd, 1H, J_d = 12.5 Hz, J_d =2.4, H-5a'), 4.23 (m, 1H, H-4'), 2.54 (dd, 1H, J_d = 14.0

Hz, J_d = 5.2 Hz, H-2'), 2.1 (m, 1H, H-2', obscured by adamantane γ-protons), 2.04 (m, 6H, adamantane γ-protons), 1.92 (m, 6H, adamantane α-protons), 1.87 (m,6H, adamantane α-protons), 1.72 (m, 12H, adamantane β-protons).

3.2.2. Physicochemical properties of the prodrug 5-Iodo-5'-adamantoyl-2'deoxyuridine (7)

3.2.2.1. Determination of partition coefficient (LogP) of the prodrug (7)

The partition coefficient of prodrug **7** was determined using standard 1-octanol/ water and 1-octanol/phosphate-buffer solution (0.02 M, pH = 7.4) systems. Mechanical shaking was used to achieve equilibration, and UV spectroscopy was used for the quantitation of the analyte in the 1-octanol phase. Equal volumes of 1-octanol and buffer were stirred for 24 h at room temperature to prepare water-saturated 1-octanol and 1-octanol-saturated-buffer. The two phases were then separated using a separatory funnel and each fraction was stored in a dark bottle at room temperature for subsequent use. A standard solution of the prodrug was prepared in water-saturated 1-octanol. The standard sample was analyzed by UV spectrophotometry at 282 nm and a standard curve was prepared by plotting UV absorbance *vs* concentration of the standard solution.

The compound **7** (0.5 mg/mL) was partitioned between equal volumes (2 mL) of presaturated 1-octanol-water and 1-octanol:phosphate buffer, respectively.

The solutions were gently shaken for 24 h at room temperature. The two phases were then separated and the concentration of compound **7** in the 1-octanol phase was determined by UV spectrophotometry, before and after partitioning. The partition coefficient (P) was calculated as the molar ratio of the concentration in the 1-octanol to the concentration in the water or phosphate buffer phase, respectively, ($P = C_{n\text{-octanol}} / C_{\text{water or buffer}}$).

3.2.2.2. Computer calculation of the Log P of prodrug (7)

A theoretical Log P was calculated by energy optimization of the chemical structure of prodrug 7 by Alchemy 32; Version 2 (1997), Tripos. Inc. Louis, MD

(U.S.A), then using subsequent analysis with SciQSAR, version 3.0 (1995-1998 Scivision, Inc.) to provide the theoretical Log P values.

3.2.2.3. Solubility of prodrug 7 in aqueous solution and in aqueous β-CyD solution

Excess prodrug 7 was added to water and an aqueous solution of β-CyD (1.5 mL) in Eppendorf centrifuge tubes, respectively. After 10 min of water bath sonication, the samples were placed in a shaking water bath at 25 ^{0}C and mixing was continued overnight. The samples were analyzed by HPLC at a fixed wavelength of 282 nm. HPLC analyses were performed using a Waters reverse phase Phenyl radial-pak cartridge (10 μL, particle size, 8 mm id x 10 cm length) using 4:1 v/v methanol-water as solvent with a flow rate of 1 mL/min. The retention time for prodrug 7 was 4.4 min.

3.2.3. Characterization of the prodrug 7/β-CyD inclusion complex

3.2.3.1. Preparation of a solid dosage form of the prodrug inclusion complex with β -CyD

A suspension of prodrug 7 and β-CyD (molar ratio 1:1) in water (2 mL) was stirred at 60 ^{0}C for 5 h. The solution was then filtered and lyophilized to give a white solid.

3.2.3.2 Confirmation of the inclusion complex formation by differential scanning calorimetery

The samples of free prodrug 7, β-CyD, the physical mixture of prodrug 7 and β-CyD and the prodrug 7/β-CyD inclusion complex (2 mg) were loaded into aluminum pans and measured using a sealed aluminum pan as the reference. The instrument was calibrated with Indium (156 ^{0}C). Thermograms were obtained at a heating rate of 10 ^{0}C/min from 40 ^{0}C to 180 ^{0}C and 5 ^{0}C/min from180 ^{0}C to 260 ^{0}C. A nitrogen purge was maintained throughout each run.

3.2.3.3 Confirmation of the prodrug 7/β-CyD inclusion complex by x-ray diffractometry

The powder samples of prodrug 7, β-CyD, the physical mixture of the prodrug a 7 and β-CyD, and the prodrug 7/β-CyD inclusion complex were pressed onto a sample slide, and then covered with double-sided cellophane tape. An automated X-ray diffractometer equipped with a 2θ compensating slit, a nickel filter and Cobalt tube (40 kV, 30 mA). Co (λ_{α} = 1.7962A^{0}) was to use to obtain the diffraction patterns of the samples. The patterns were recorded from 2 ^{0}C - 90 ^{0}C (2θ) / min.

3.2.3.4. ^{1}H NMR spectroscopy of inclusion complexes

3.2.3.4.1. β-CyD Inclusion complexes

^{1}H NMR spectroscopy was used for studying complexation in solution. Three model compounds: 1-adamantandine hydrochloride, sodium 1-adamantanecarboxylate and 1-adamantanecarboxylic acid were prepared as 1:1 molar ratio complexes with β-CyD. The samples were dissolved in 1 mL D_2O and were analyzed by ^{1}H NMR. The ^{1}H NMR analysis of the prodrug 7 and β-CyD inclusion complexes were carried out under the same conditions as used for the model compounds.

3.2.4. Binding constant determination of inclusion complexes

3.2.4.1. Prodrug 7/ β-CyD inclusion complex binding constant determining by HPLC method

For the HPLC determination of the host-guest binding constant, a β-CyD stock solution of 1.59 x 10^{-2} M was prepared. β-CyD solutions (8.8 x 10^{-3} to 1.59 x 10 $^{-2}$ M) were put into 1.5 mL Eppendorf centrifuge tubes and excess prodrug 7 was added. After 30 min of water bath sonication, the samples were placed in a shaking water bath and kept at 25 ^{0}C for 24 h. The samples were then filtered through a 0.45 μm membrane filter and were then analyzed by HPLC. The retention time for prodrug 7 under these conditions was 7.4 min (solvent: 80:20 v/v methanol-water).

3.2.4.2. Determination of the 1-adamantanamine hydrochloride / compound (5) inclusion complex binding constant determining by the CE method

Capillary electrophoresis was performed using a Beckman P/ACE 5510. The capillary tubing was of uncoated fused silica with an internal diameter of 75 μm, 47 cm in length (40 cm to the detector). The following conditions were applied: voltage 10 kV; capillary thermostat set at 25 ^{0}C; UV detection at 230 nm; 5 s pressure injection (5 nL) of 1 mM of compound **5** in 25 mM phosphate buffer, pH = 7.4. 1-Adamantanamine hydrochloride concentrations were varied from 3 mM to 15 mM. The capillary was rinsed for 5 min with 1 mM NaOH followed by a 5 min rinse with the running buffer between injections. All samples were filtered through a 0.45 μm filter prior to injection.

3.2.4.3. β-CyD inclusion complex binding constant determination by ^{1}H NMR

3.2.4.3.1. Inclusion complex binding constants for adamantane/β-CyD complex

The binding constants for three adamantane compounds, namely 1-adamantanamine hydrochloride, sodium 1-adamantancarboxylate and 1-adamantanecarboxylic acid were determined under the following conditions: 1-adamantanamine concentration was kept at $5x10^{-3}$ M while the β-CyD concentration was varied between $3x10^{-3}$ to $1.2x10^{-2}$ M. ^{1}H NMR spectra were recorded at 300 MHz in D_2O and were recorded both in the absence and in the presence of increasing concentrations of β-CyD. The binding constants were determined by plotting $\Delta\delta_{obs}$ *vs* concentration of β-CyD [M], and data analysis were performed using S-PLUS.

In a similar way, spectra were also recorded for solutions containing sodium 1-adamantanecarboxylate $8.2x10^{-3}$ M with various β-CyD concentrations between 3 $x10^3$ to $1.0x10^{-2}$ M, and for 1-adamantanecarboxylic acid solutions ($5x10^{-3}$ M) with varied β-CyD concentrations between $5x10^{-3}$ to $1.5x10^{-2}$ M.

3.2.4.3.2. Prodrug 7/β-CyD inclusion complex binding constant determining by ^{1}H NMR

The previously described procedure was repeated with Prodrug 7 over a concentration range of $1.0x10^{-3}$ to $1.5x10^{-2}$ M while the β-CyD concentration was kept at $1.5x10^{-2}$ M.

3.2.5. The stability of the inclusion compound of prodrug 7/β-CyD in aqueous solution

The 1:1 molar ratio of prodrug 7 /β-CyD inclusion complex was prepared as described in section 3.2.3.1.

A solution of the inclusion complex (2 mg/mL, 1.2 mM, 0.5 mL) and 1-octanol (0.5 mL) was placed into a shaking water bath and kept at 37 ^{0}C. Aliquots (20 μL) from the 1-octanol layer were taken at 15, 45, 75, 120, 180 min after mild shaking and analyzed for prodrug 7 content in the 1-octanol phase by HPLC. The retention time for prodrug 7 was 7.4 min (solvent: 80:20 v/v methanol-water).

3.2.6. Stability of the prodrug 7/β-CyD inclusion complex to cholesterol challenge

The 1:1 molar ratio of inclusion complex (10.2 mg/mL, 6.2 mM) in water (1 mL) was added into to 1-octanol (1 mL); 1-octanol (1 mL) containing 2.4 mg (6.2 mM) cholesterol) and 1-octanol (1mL) containing 24 mg (62 mM) cholesterol, respectively. The mixture was placed into shaking water bath and kept at 37 ^{0}C. Aliquots were taken at 0, 20, 60, 90, 120, 150, 180, 210, 240, 270 min after mild shaking, and the aqueous layer was analyzed by HPLC under the same conditions as above.

3.2.7. Diffusion of prodrug 7 (in complex form) from plasma into 1-octanol

A solution of a 1:1 molar ratio prodrug 7 complex (10.2 mg/mL, 6.2 mM) in plasma was prepared, and 1.5 mL of this mixture was then added to 1-octanol (1.5 mL). The solution was placed into shaking water bath and kept at 37 ^{0}C. The samples were taken at 0, 20, 60, 90, 120, 150, 180, 210, 240, 270 min after mild shaking and the 1-octanol layer was analyzed by HPLC under the same conditions as above.

3.2.8. The stability of the prodrug (7)/β-CyD inclusion complex in plasma

Two concentrations of the 1:1 molar ratio inclusion complex were investigated. The inclusion complex 17 mg/mL (1.0 mM) and 10.2 mg/ml (6.2 mM) in plasma was transferred to a 1.5 mL vial and was then placed into shaking water bath and kept at 37 ^{0}C, respectively. The samples were taken at 0, 15, 45, 75, 120, 180, 240 min after shaking. The plasma samples (50 μL) were treated with methanol (400 uL) then centrifuged at 2500 rpm for 25 min. The supernatant was filtered through 0.22 um MILLEX. GV filter. An 80 μL aliquot of the filtrate was analyzed by HPLC. The mobile phase consisted of 80 % methanol (A) and 20 % water (B) using the gradient elution profile shown in table 3.3.7.4. Using a flow rate of 1 mL/min. The effluent was monitored at 288 nm with a detector sensitivity of 0.05 a.u.f.s.

Table 3.3.7.4. Gradient composition of solvent systems A and B for HPLC separation of prodrug 7 and IUdR in the plasma samples

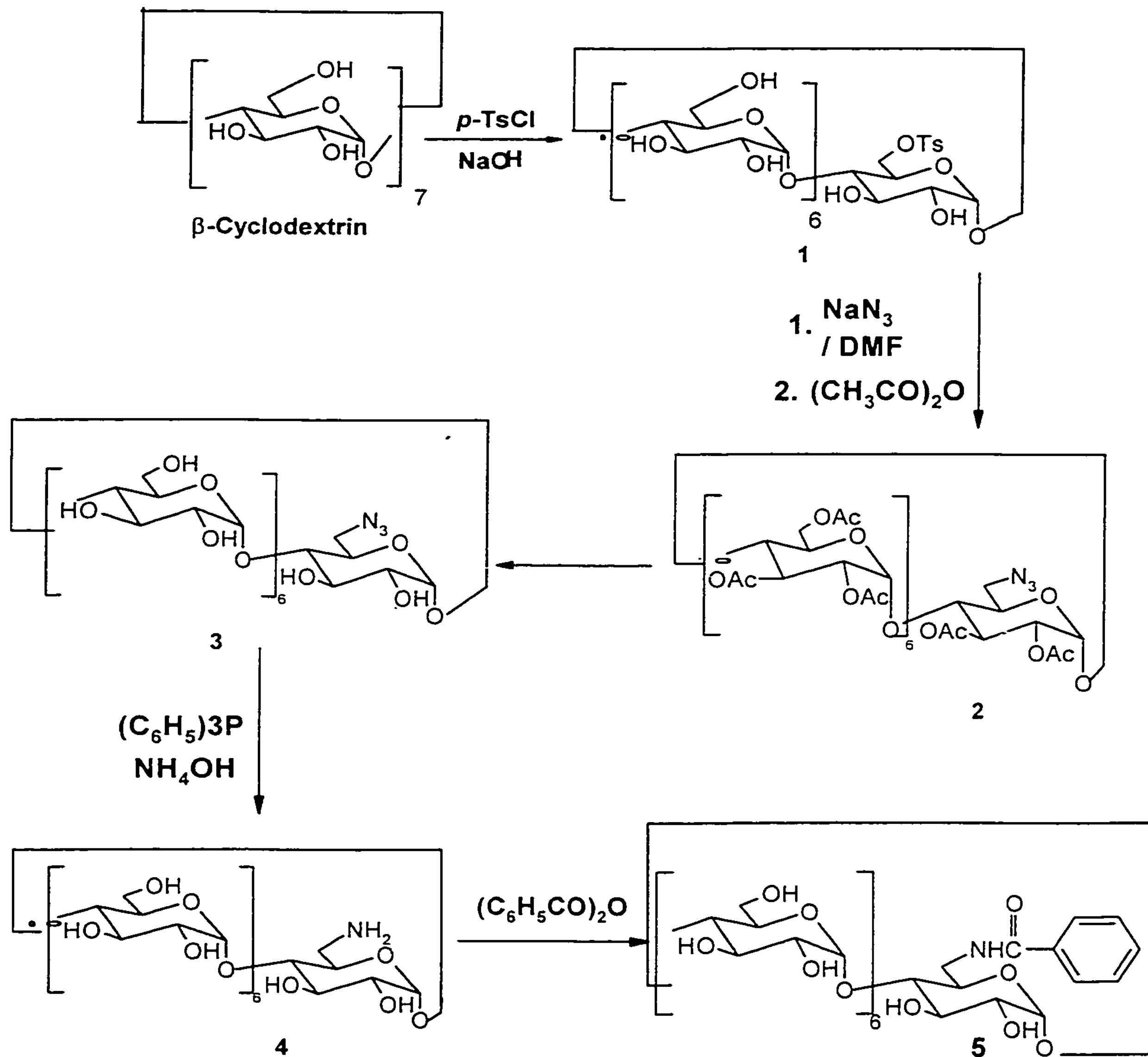
Time (min)	Flow (ml/min)	% A	% B	Crv*
0	1.0	20	80	*
9	1.0	20	80	6
10	1.0	90	10	6
20	1.0	90	10	6
22	1.0	20	80	6

* In the baseline 810 chromatography processing system, the Crv "6" program performs the linear change of the elution solvent gradient.

4. Results and Discussion

4.1. Chemical synthesis

4.1.1. Synthesis of mono-6-deoxy-6-*N*-benzoyl-β-cyclodextrin 5

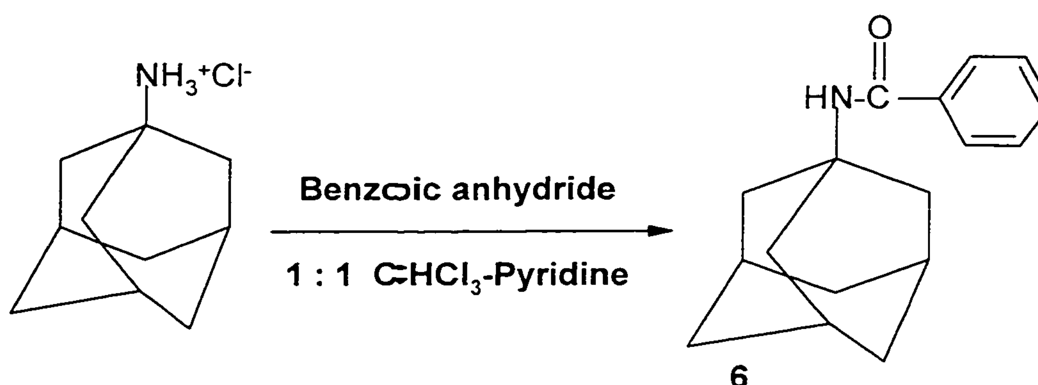


Scheme 1. Synthesis of mono-6-deoxy-6-N-benzoyl-β-cyclodextrin 5

The synthesis of mono-6-deoxy-6-*N*-benzoyl-β-cyclodextrin **5** was carried out in order to provide an UV active cyclodextrin derivative for the determination of host-guest binding constants by capillary electrophoresis. The synthesis proceeded as shown in Scheme 1. Thus, β-cyclodextrin was first treated with sodium hydroxide (3 equivalent) and then reacted with *p*-toluenesulfonyl chloride (1 equivalent) in aqueous solution. The monotosylate (1) was precipitated from the aqueous solution by cooling at 4 ^{0}C overnight. The precipitate was collected by filtration, and a second crop could be obtained after cooling to 4 ^{0}C for an additional 24 h. The combined yield of **1** was 11% and the ^{1}H NMR spectral data for this compound were in good agreement with previously published data (Petter and Salek 1990). Cyclodextrin derivative **1** was next treated with sodium azide (10 equivalent) in *N,N*-dimethylformamide at 120 ^{0}C for 2 h. Evaporation of solvents gave the crude monoazide derivative, which was directly treated with 2:1 v/v pyridine-acetic anhydride at room temperature for 48 h. Evaporation of solvent followed by chromatography over silica gel using 3: 2 v/v hexane-acetone provided pure **2** as an amorphous solid in 46% yield. The proton signals for the β-cyclodextrin ring protons were difficult to assign due the unsymmetrical nature of monosubstitution. Twenty acetate methyls were found to be present, indicating monosubstitution had occurred. De-O-acetylation of **2** was accomplished by treatment with methanolic sodium methoxide. Neutralization, followed by chromatography over silica gel using 2:1:1 v/v/v ethyl acetate-methanol-water as eluant, gave pure mono-6-deoxy-6-azido-β-cyclodextrin **3** (75%) as a white solid. Reduction of the azide moiety was accomplished by first treating a solution of **3** in *N, N*-dimethylformamide with excess triphenylphosphine (4 equiv.) for 45 min at room temperature followed by addition of ammonium hydroxide to liberate the free primary amine. The reaction mixture was diluted with water and the aqueous layer was extracted with chloroform until the organic layer did not display UV activity. The aqueous layer was then concentrated and the residue was lyophilized from water to give crude **4**. This material was of sufficient purity and was therefore used directly in

the next step without further purification. Thus, a solution of 4 in dry *N, N*-dimethylformamide was treated with potassium carbonate and excess benzoic anhydride (1.26 equiv.) for 48 h at room temperature. Removal of solvents followed by column chromatography over silica gel using 6:2:2 v/v/v 2-propanol-water-ammonium hydroxide gave **5** as a white solid in 51 % yield. The ^{1}H NMR spectrum clearly showed the presence of a single aromatic moiety with three - resonance signals at 7.89 (2H), 7.75 (1H) and 7.62 (2H) ppm respectively. The UV activity of **5** (λ = 230 nm) was sufficient for subsequent capillary electrophoresis work.

4.1.2. Synthesis of N- (1-adamantyl) benzamide 6

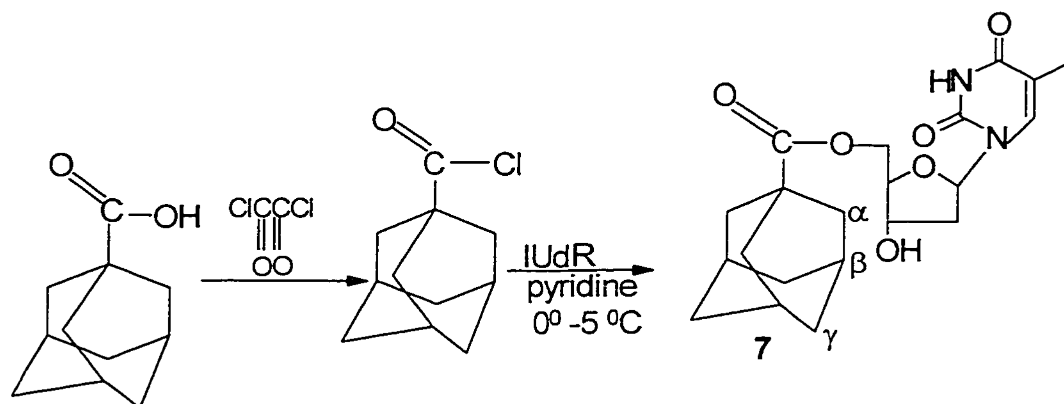


Scheme 2. Synthesis of N- (1-adamantyl) benzamide 6

The synthesis of an UV active adamantane derivative for capillary electrophoresis studies was carried out as shown in Scheme 2. Thus, a solution of 1-adamantanamine hydrochloride and benzoic anhydride (2.5 equivalent) in 1:1 chloroform: pyridine was stirred at room temperature for 48 h. The solvent was removed and the residue was taken up in chloroform and washed with dilute acid, saturated aqueous sodium hydrogen carbonate, saturated sodium chloride, dried (Na_2SO_4), filtered and concentrated. Column chromatography of the residue over silica gel using 12:1 hexane - ethyl acetate gave (6) in 35 % yield. The ^{1}H NMR

spectrum of this adamantane derivative was consistent with the proposed structure and its UV activity (λ=224 nm) was suitable for subsequent capillary electrophoresis work.

4.1.3. Synthesis of 5-Iodo-5'-adamantoyl-2'-deoxyuridine 7



Scheme 3. Synthesis of 5-Iodo-5'-adamantoyl-2'-deoxyuridine (7)

The synthesis of IUdR-adamantane prodrug (7) for complexation with β-cyclodextrin was carried out as shown in Scheme 3. Thus, a solution of freshly prepared 1-adamantanecarbonyl chloride (2 equiv.) in chloroform was added dropwise to a solution of IUdR in pyridine at 0 °C. The reaction was monitored by TLC (4:1 v/v chloroform-methanol) for consumption of IUdR. Upon completion of the reaction, the solvents were evaporated and washed with saturated aqueous sodium chloride, dried (Na_2SO_4), filtered and evaporated. The residue was purified by column

chromatography over silica gel using 12:12:1 v/v/v hexane-ethyl acetate-ethanol as eluant. Di-adamantoyl IUdR **8** (71 mg, 10 %) eluted first followed by the desired product (498 mg, 68 %). The ^1H NMR spectrum of **7** was in excellent agreement with the proposed structure and only a single set of adamantane proton was observed along with a corresponding downfield shift for the H-5'protons of IUdR to 4.4 and 4.25 ppm. The ^1H NMR data for **8** revealed two sets of adamantane signals, and in addition to the downfield shift for the H-5'protons, the H-3'proton signal was shifted downfield to 5.18 ppm.

4.2. Partition coefficients

The partition coefficient P is a known parameter for expressing the lipophilicity of a drug and is routinely used for evaluating aqueous solubility, bioaccumulation, bioconcentration, and pharmacokinetics of drugs (Jackson 1993). The P value is established from the equilibrated concentration ratio for a given compound between an aqueous and a nonaqueous phase (Biagi 1994). By definition, $P = C_{octanol} / C_{water}$, where the concentration $C_{octanol} / C_{water}$ ratio is expressed in Log units.

De Bruijn and Hermens (1991) reported that octanol /water is a useful reference solvent system as the P correlates well with biological activity. Octanol is considered a surrogate for biotic lipid, as these substances have similar polarities.
The prodrug **7** partition coefficient results are shown in Table 4.2.

Table 4.2. Partition coefficient (Log P) of prodrug (7) measured in different aqueous solutions and as determined by computer calculation

Condition	Log P value
octanol/ water	1.91
octanol/20 mM phosphate buffer (pH = 7.4)	1.97
computer calculated	1.85

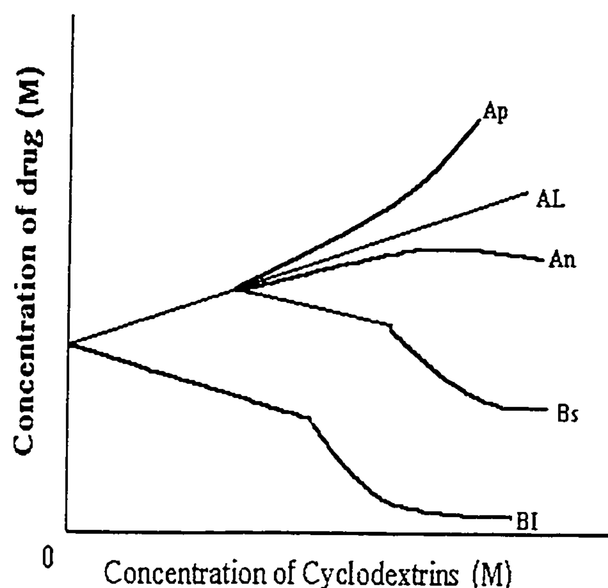
4.3. Characterization of the prodrug 7/β-CyD inclusion complex

4.3.1. Solubility of prodrug (7) at various concentrations of β-CyD

The most common pharmaceutical application of cyclodextrins is to enhance drug solubility in aqueous solutions. The aqueous solubility of prodrug 7 was found to be 26 μg/mL while in the aqueous β-CyD solution its solubility is dramatically increased to 8.26 mg/mL thus, the enhancement factor is 317 (enhancement factor is equal to the solubility in the aqueous cyclodextrin solution divided by the solubility in pure water) (Loftsson *et al.* 1996).

For the study of guest solubility in the presence of a host, changes in solubility of the guest are plotted as a function of the cyclodextrin concentration. The phase solubility diagrams thus obtained are generally classified into the type A (soluble complex) and B (complex with a definite solubility), as established by Higuchi and Connors (1965). From Figure 4.3.1.1., type A was divided into A_L, A_p and A_N while type B was divided into Bs and B_I. The A_L system implies a linear increase with unchanged stoichiometry. A_N means the system is even more complicated, because it can point either to an increase in the stoichiometry of the complex or a change in the solute-solvent interaction (hydration, ionization of the guest) or both. Ap systems

generally reflect high order complexation, meaning that more than one cyclodextrin molecule is involved in the complexation of the guest. If a complex formed between a drug and cyclodextrin is not soluble, a B_I type curve is generated, and a complex of limited solubility gives Bs type relationships. For A and B type systems, the initial



linear portion of the curve can be useful in examining the efficiency of complexation, and binding constant. These parameters can often be obtained from the phase diagram.

Figure 4.3.1.1. Phase solubility diagrams for complexation with β-CyD
(Higuchi, T. and Connors, K. 1965)

The phase solubility diagram obtained for prodrug 7 with β-CyD at 25 ^{0} C is shown in Fig. 4.3.1.1. This diagram shows that a linear relationship exists between the amount of drug solubilized and the concentration of β-CyD in solution. The apparent stability constant (K) was estimated from the slope of the straight line of the phase-solubility diagrams according to the equation of Higuchi and Connors (1965). The

binding constant of the prodrug 7/β-CyD complex at 298 K was found to be 1.49×10^5 M^{-1}.

A binding constant of prodrug (7) with β-CyD inclusion complex achieves 10^5 M^{-1} indicating strong binding. If a binding constant were to reach a value of 1×10^5 M^{-1}, then a 1: 4,200 dilution results in dissociation of only 32.7 % of the complex due to dilution effects alone (Stella *et al*, 1997)

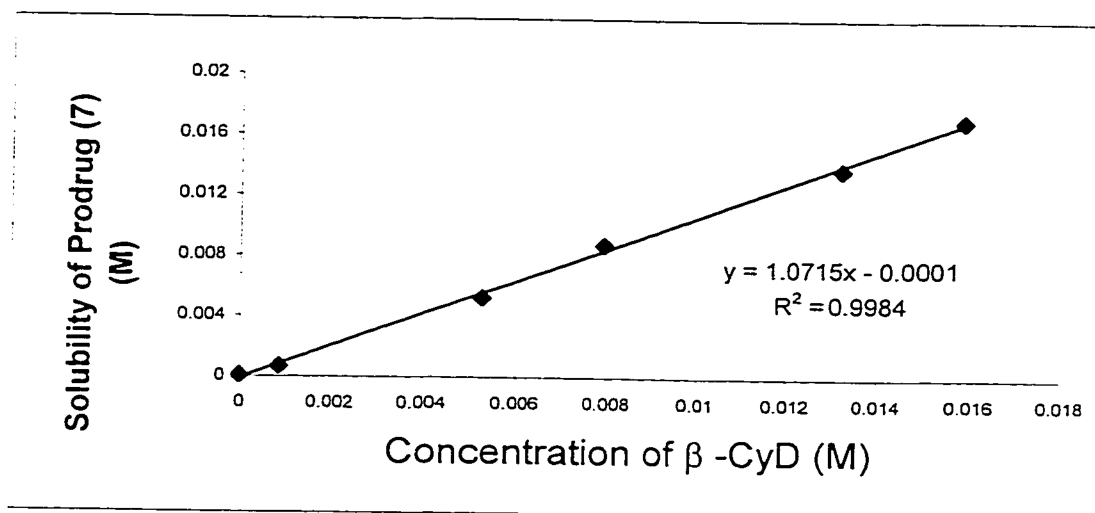


Figure 4.3.1.2. The phase solubility study of prodrug (7) in presense of β-CyD

4.3.2. Differential scanning calorimetry

Characterization of the β-CyD complex with prodrug **7** in the solid state was carried out using DSC. DSC scans were obtained in the temperature range from 40 – 250 ^{0}C for prodrug 7, β-CyD, their mixture and prodrug 7/β-CyD complex. The thermogram of pure prodrug **7** shows one exothermic peak at 208 ^{0}C. β-CyD has a very broad transition range between 70 – 180 ^{0}C and a small endothermic peak at 220 ^{0}C is also observed. The mixture of prodrug **7** and β-CyD consists of two endothermic peaks at 220 ^{0}C and 227 ^{0}C and an exothermic peak at 240 ^{0}C. The prodrug 7/β-CyD complex has an endothermic peak at 235 ^{0}C (Figure 4.3.2).

The thermogram of pure prodrug (7) showed one exothermic peak at 208 ^{0}C. Decomposition has occurred of this temperature because TLC showed a few peaks. Prodrug 7 was heated to 208 ^{0}C which is the same temperature as the exothermic peak, was analyzed by TLC.

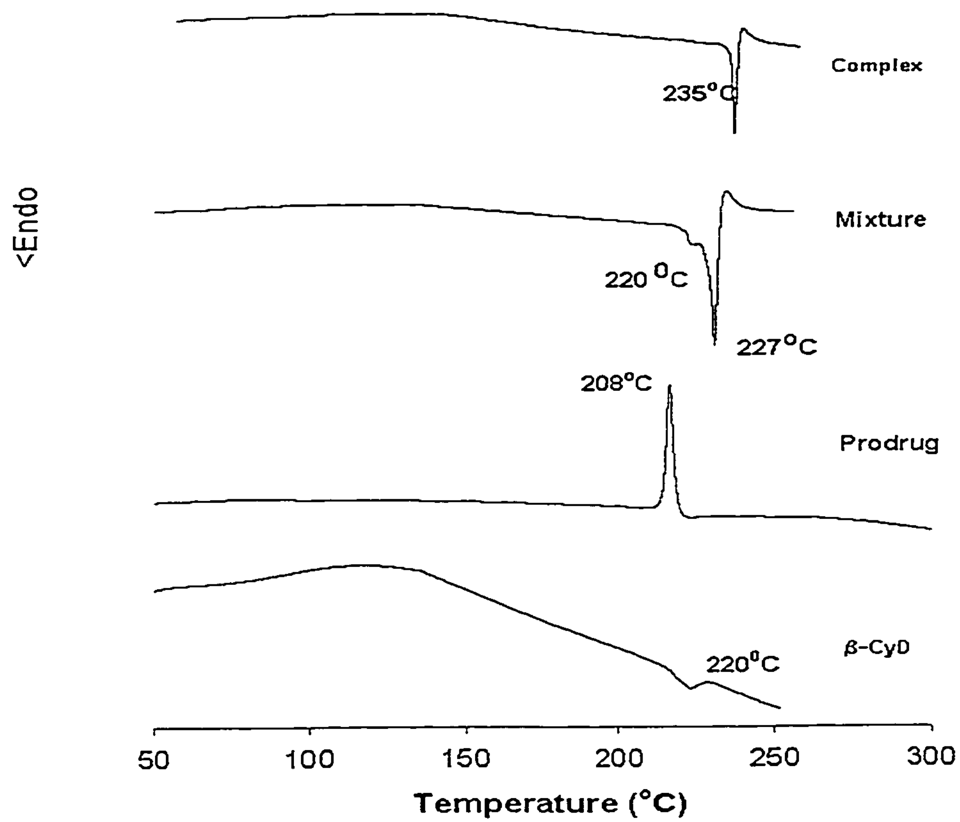


Figure 4.3.2. DSC traces of β-CyD, prodrug 7, physical mixture of prodrug 7 and β-CyD, and prodrug7/β-CyD inclusion complex.

4.3.3. X-ray Diffractometry

X-ray powder diffractometry is widely used for the identification of solid phase. The x-ray powder pattern of every crystalline form of a compound is unique, making this technique particularly suited for the identification of different phases. However this technique has limited utility in the identification of noncrytalline (amorphous) materials since the powder patterns consist of one or more broad diffuse peaks.

Further evidence of complex formation was obtained from X-ray power diffraction studies. The X-ray powder diffractograms of β-CyD, pure compound **7**, the physical mixture and inclusion complex are shown in Fig. 4.3. The diffraction pattern of the physical mixture is simply the superposition of each component with the peaks having lower intensity. This may be attributed to the physical mixture. On the other hand, the diffraction pattern of the complex only consists of one broad diffuse peak which indicates that the inclusion complex is markedly noncrystalline (amorphous). This shows that the mode of interaction of β-CyD with prodrug **7** was different from that of natural β-CyD in the physical mixture, and this agrees with DSC results. Therefore it is may indicate that a new phase has been produced.

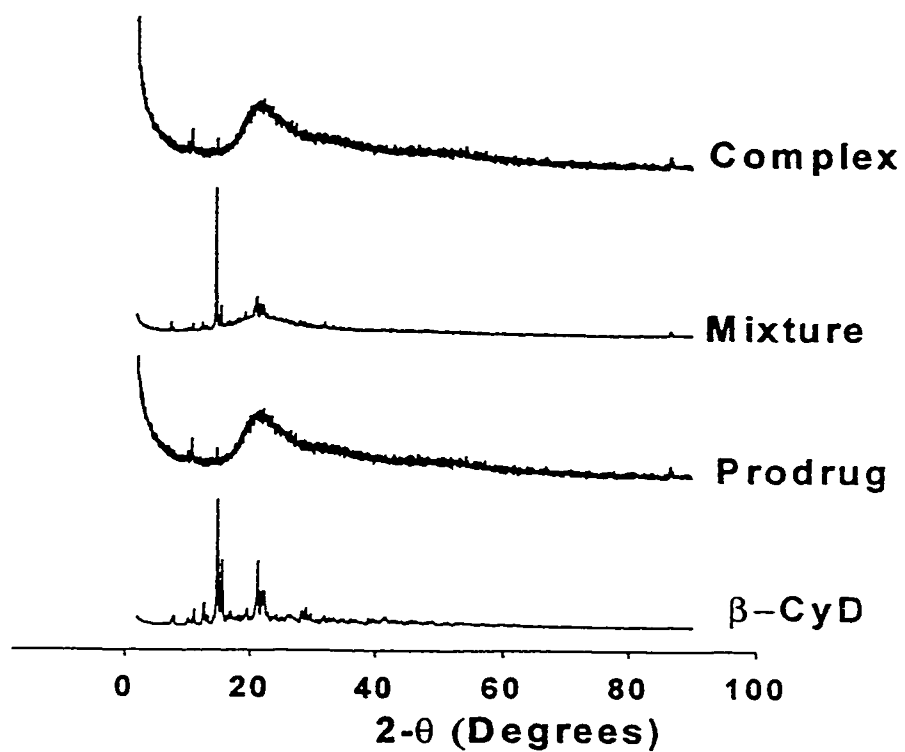
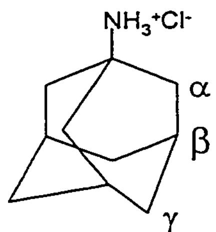


Figure 4.3.3. The X-ray powder diffractograms of the β-CyD, prodrug 7, the physical mixture and the prodrug 7/β-CyD inclusion complex.

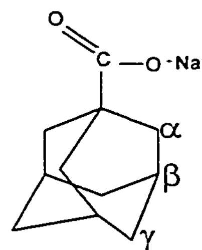
4.4. ^{1}H NMR spectroscopy

4.4.1. ^{1}H NMR spectroscopy of inclusion complex

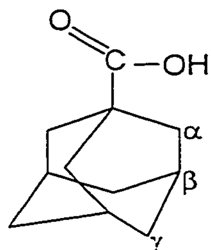
Proton NMR has been used to investigate the nature of cyclodextrin complexes in solution (Thakkar and Demarco 1971). For the aqueous soluble complexes described in this thesis, the observed resonances were the time-averaged peaks of free and complexed compounds (fast exchange on the NMR time scale at 303 °K except for prodrug 7, which was studied at 310 °K). The complexation of the following adamantine model compounds by β-CyD was examined by NMR spectroscopy: 1-adamantanamine hydrochloride, sodium 1-adamantanecarboxylate and 1-adamantanecarboxylic acid. Their structures are shown in Figure 4.4.1.1. A sequence of typical NMR spectra for the complexation of adamantine model compounds with β-CyD at varying concentration are shown in Tables 4.4.1.1 – Table 4.4.1.8. The spectral data obtained clearly show that changes in chemical shifts ($\Delta\delta$) occur for both the guest (drug) and the host (β-CyD) protons. The values of $\Delta\delta$ are critical functions of the position of the guest molecule in the host, the cavity size of the CyD and the [G]/[H] ratio. In general, the inclusion of an aromatic ligand into the β-CyD cavity causes detectable shifts of the H3 and H5 protons located inside the β-CyD cavity. In the case of an aromatic guest, this is caused by the anisotropic effect of an aromatic ring current, strongly indicating the inclusion of aromatic moiety into in the cavity. (Rekharsky *et al.* 1995). For non-aromatic compounds, penetration of the guest into the β-CyD cavity also induces chemical shift changes for the β-CyD H3 and H5 protons, but to a lesser extent than aromatic compounds (Takahashi *et al.* 1971). The other β-CyD protons are located outside the cavity (H1, H2, H4, H6), thus are not usually affected by complexation.



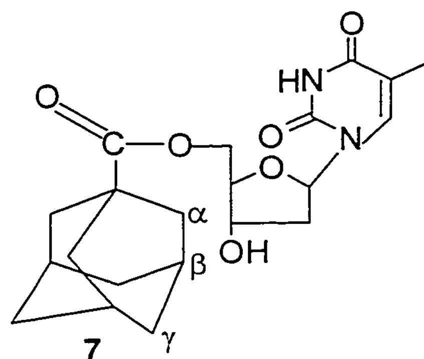
1-adamantanamine hydrochloride



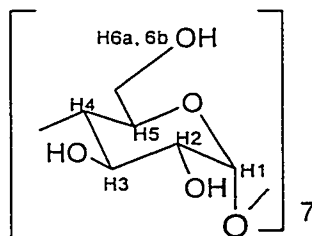
sodium 1-adamantanecarboxylate



1-adamantanecaboxylic acid



prodrug 7



β-cyclodextrin

Figure 4.4.1.1. β-CyD host and adamantine guests

β-**Cyclodextrin complexes with:**

1-Adamantanamine hydrochloride: The values for the chemical shift changes, $\Delta\delta$, of the β-CyD protons (H3, H5) at various 1-adamanatanamine hydrochloride/β-CyD ratios and temperature at 293 K are shown in Table 4.4.1.1. The H3 and H5 protons show a significant upfield shift upon complexation from 4.02 and 3.87 to 3.89 and 3.73 ppm, respectively. The notable H5 shielding implies reasonable penetration of the guest into the cavity (Diakur *at al.* 1999) and this observation is consistent with inclusion of the adamantine moiety into the β-CyD cavity. Complexation is further demonstrated by the notable values for chemical shift changes of the damantine protons as shown in Table 4.4.1.2. The maximum observed chemical shift change for the α-proton was found to be $|\Delta\delta| = 0.13$ ppm, for the β-proton $|\Delta\delta| = 0.2$ ppm and for the γ-proton $|\Delta\delta| = 0.14$ ppm. Figure 4.4.1.2 and 4.4.1.3 show the spectral region of the guest protons of a 1:1 adamantanamine hydrochloride/β-CyD inclusion complex. The observed shifts are consistent with those previously reported (Jaime and Redondo 1990) for the β-CyD/1-bromoadamantane host-guest complex. These authors found that irradiation of the guest's α- and γ-protons resulted in NOE enhancement (1.1% and < 0.3% for the H3 and H5 protons, respectively) which strongly indicates inclusion into the cavity.

Sodium 1-adamantanecarboxylate: The values for the chemical shift changes for hosts H3 and H5 protons at various sodium 1-adamantanecarboxylate/β-CyD ratios and temperature at 293 K are shown in Table 4.4.1.3. The H3 protons display a slight upfield shift upon complexation, from 4.08 to 4.03 ppm. The H5 protons show a greater upfield shift upon complexation, from 3.95 to 3.86 ppm, and the induced chemical shift change is concentration dependent. The notable H5 shielding implies reasonable penetration of the guest into the cavity and this hypothesis is further supported by the observed chemical shift changes of the sodium 1-adamantancarboxylate protons as shown in Table 4.4.1.4. The maximum observed downfield chemical shift change for the α-proton was found to be $\Delta\delta = -0.12$ ppm, for

the β-protons Δδ = -0.17 ppm and for γ-protons Δδ = -0.10 ppm. Figure 4.4.1.4 and 4.4.1.5 shows the ^{1}H NMR spectrum of a 1:1 sodium 1-adamantanecarboxylate/β-CyD inclusion complex.

1-Adamantanecarboxlic acid: The values of the chemical shift changes for host H3 and H5 protons at various 1-adamantanecarboxlic acid/β-CyD ratios and the temperature at 293 K are shown in Table 4.4.1.5. Once again, the H3 and H5 protons show a significant upfield shift upon complexation, from 4.09 and 3.95 to 3.98 and 3.91 ppm, respectively. This notable H3 shielding implies reasonable penetration of the guest into the cavity and is consistent with the well-recognized upfield shift due to hydrophobic interactions (Pecca *et al.* 1971). This is further supported by the observed chemical changes of the 1-adamantanecaboxylic acid protons as shown in Table 4.4.1.6. It is interesting to note that upon complexation, the α-proton and γ-proton are downfield shifted to a greater extent than for ether 1-adamantanamine hydrochloride or sodium 1-adamantanecarboxylate guests. The maximum observed chemical shift change for the α-proton was found to be Δδ = -0.11 ppm, for β-proton Δδ = -0.22 ppm and for γ-proton Δδ= -0.09 ppm, and again indicates complex formation. Figure 4.4.1.6 and 4.4.1.7 shows the ^{1}H NMR spectrum of a 1:1 1-adamantanecarboxlic acid/β-CyD inclusion complex.

Prodrug 7: The values for the chemical shift changes of the β-CyD H3 and H5 protons and various prodrug **7**/β-CyD ratios and temperature at 310 K are shown in Table 4.4.1.7. The H3 and H5 protons show a significant upfield shift upon complexation from 4.18 and 4.06 to 4.10 and 3.97 ppm, respectively. The notable H3 shielding implies reasonable penetration of the guest into the cavity as seen with the model damantine compounds. This observation is consistent with inclusion of the damantine moiety into the β-CyD cavity. The chemical changes of the damantine protons are shown in Table 4.4.1.8. The maximum observed chemical shift change for the α-proton was found to be Δδ = -0.12 ppm, for β-proton, Δδ = -0.21 ppm and for γ-proton, Δδ= -0.1 ppm. The results are similar to 1-adamantanamine hydrochloride and

1-adamantanecarboxylic acid chemical shift changes. The chemical shifts for the IudR portions of the prodrug remain unchanged at all host/guest ratios. The Figure 4.4.1.8 and 4.4.1.9 shows the ^{1}H NMR spectra of the 1:1 prodrug **7**/β-CyD complex.

Table 4.4.1.1. Complexation-induced chemical shift changes of the β-CyD protons at various 1-adamantanamine hydrochloride/β-CyD ratios

[β-CyD] (M)	Compound 1 * (M)	β-CyD H3 δ (ppm)	β-CyD ΔδH3 (ppm)	β-CyD H5 δ (ppm)	β-CyD Δδ H5 (ppm)
5.0E-3	0.000	4.020	0.000	3.873	0.000
3.0E-3	5.0E-3	3.888	0.132	3.728	0.145
4.0E-3	5.0E-3	3.899	0.121	3.749	0.124
5.0E-3	5.0E-3	3.900	0.120	3.758	0.115
6.0E-3	5.0E-3	3.906	0.114	3.773	0.100
8.0E-3	5.0E-3	3.936	0.084	3.796	0.077
1.0E-2	5.0E-3	3.948	0.072	3.809	0.064
1.2E-2	5.0E-3	3.957	0.063	3.818	0.055

Table 4.4.1.2. Complexation-induced chemical shift changes of the guest protons at various 1-adamantanamine hydrochloride/β-CyD ratios

[β-CyD] (M)	[Compound 1*] (M)	α-H δ (ppm)	α-H Δδ (ppm)	β-H δ (ppm)	β-H Δδ (ppm)	γ-H δ (ppm)	γ-H Δδ (ppm)
0.000	5.0E-3	1.882	0.000	2.274	0.000	1.682	0.000
3.0E-3	5.0E-3	1.959	-0.077	2.286	-0.112	1.765	-0.083
4.0E-3	5.0E-3	1.982	-0.100	2.325	-0.151	1.794	-0.112
5.0E-3	5.0E-3	1.995	-0.113	2.339	-0.165	1.809	-0.127
6.0E-3	5.0E-3	2.004	-0.122	2.353	-0.179	1.817	-0.135
8.0E-3	5.0E-3	2.009	-0.127	2.361	-0.187	1.822	-0.140
1.0E-2	5.0E-3	2.008	-0.126	2.365	-0.191	1.822	-0.140
1.2E-2	5.0E-3	2.009	-0.127	2.371	-0.197	1.823	-0.141

Compound 1 = 1-adamantanamine hydrochloride

Δδ: "+" means upfield shift, "-" means downfield shift

Table 4.4.1.3. Complexation-induced chemical shift changes of the β-CyD protons at various sodium 1-adamantanecarboxylate/ β-CyD ratios

[β-CyD] (M)	[Compound 2*] (M)	β-CyD H3 δ (ppm)	β-CyD ΔδH3 δ (ppm)	β-CyD δ H5 (ppm)	β-CyD Δδ H5 (ppm)
8.0E-3	0.000	4.079	0.000	3.952	0.00
3.0E-3	8.2E-3	4.029	0.050	3.859	0.093
4.0E-3	8.2E-3	4.031	0.048	3.860	0.092
5.0E-3	8.2E-3	4.031	0.048	3.865	0.087
6.0E-3	8.2E-3	4.030	0.049	3.871	0.081
8.0E-3	8.2E-3	4.034	0.045	3.883	0.069
1.0E-2	8.2E-3	4.035	0.044	3.893	0.059

Table 4.4.1.4. Complexation-induced chemical shift changes of the guest protons at various sodium 1-adamantanecarboxylate/ β-CyD ratios

[β-CyD] (M)	[compound 2] (M)	α-H δ (ppm)	α-H Δδ (ppm)	β-H δ (ppm)	β-H Δδ (ppm)	γ-H δ (ppm)	γ-H Δδ (ppm)
0.000	8.2E-3	1.888	0.000	2.055	0.000	1.777	0.000
3.0E-3	8.2E-3	1.931	-0.043	2.106	-0.051	1.806	-0.029
4.0E-3	8.2E-3	1.941	-0.053	2.119	-0.064	1.810	-0.033
5.0E-3	8.2E-3	1.953	-0.065	2.134	-0.079	1.819	-0.042
6.0E-3	8.2E-3	1.965	-0.077	2.135	-0.080	1.856	-0.079
8.0E-3	8.2E-3	1.989	-0.101	2.190	-0.135	1.834	-0.057
1.0E-2	8.2E-3	2.008	-0.120	2.225	-0.170	1.873	-0.096

Compound 2 = sodium 1-adamantanecarboxylate

Table 4.4.1.5. Complexation-induced chemical shift changes of the β-CyD protons at various 1-adamantanecarboxylic acid/ β-CyD ratios

[β-CyD] (M)	[Compound 3*] (M)	β-CyD H3 δ (ppm)	β-CyD Δδ H3 (ppm)	β-CyD H5 (ppm)	β-CyD Δδ H5 (ppm)
1.5E-2	0.000	4.078	0.000	3.950	0.000
5.0E-3	5.0E-3	3.981	0.097	3.912	0.038
6.0E-3	5.0E-3	3.994	0.084	3.916	0.034
8.0E-3	5.0E-3	4.015	0.063	3.925	0.025
1.0E-2	5.0E-3	4.025	0.053	3.936	0.014
1.2E-2	5.0E-3	4.034	0.044	3.937	0.013
1.5E-2	5.0E-3	4.044	0.034	3.940	0.010

Table 4.4.1.6. Complexation-induced chemical shift changes of the guest protons at various 1-adamantanecarboxylic acid/ β-CyD ratios

[β-CyD] (M)	[Compound 3*] (M)	α-H δ (ppm)	α-H Δδ (ppm)	β-H δ (ppm)	β-H Δδ (ppm)	γ-H δ (ppm)	γ-H δ (ppm)
0.000	need	1.914	0.000	2.067	0.000	1.805	0.000
5.0E-3	5.0E-3	2.014	0.100	2.269	-0.202	1.882	-0.077
6.0E-3	5.0E-3	2.020	0.106	2.279	-0.212	1.888	-0.083
8.0E-3	5.0E-3	2.020	0.106	2.279	-0.212	1.890	-0.085
1.0E-2	5.0E-3	2.021	0.107	2.282	-0.215	1.889	-0.084
1.2E-2	5.0E-3	2.020	0.106	2.280	-0.213	1.888	-0.085
1.5E-2	5.0E-3	2.021	0.107	2.282	-0.215	1.889	-0.084

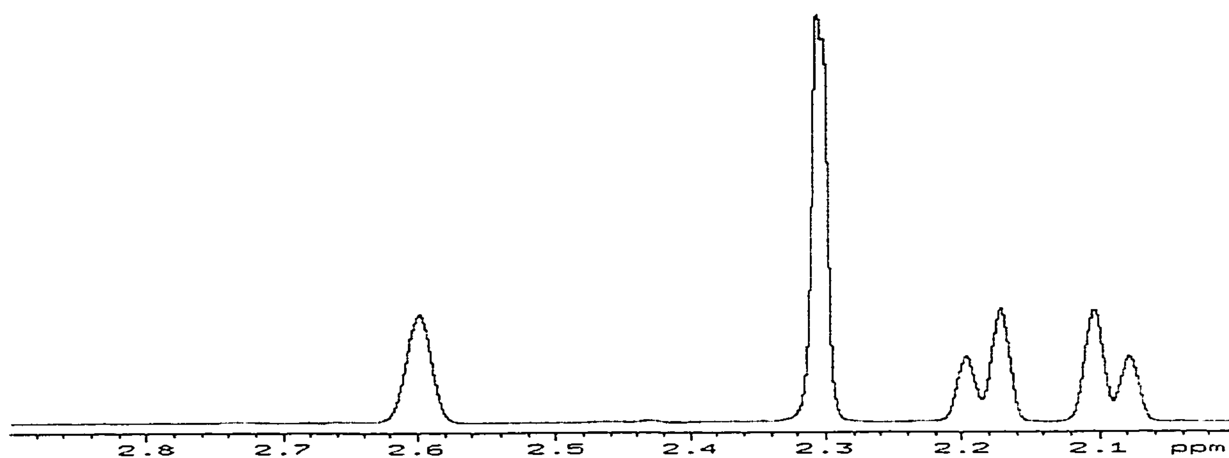
Compound 3 = 1-adamantanecarboxylic acid

Table 4.4.1.7. Complexation-induced chemical shift changes of the β CyD protons at various prodrug 7/β-CyD ratios

[β-CyD] (M)	[Prodrug 7] (M)	β-CyD H3 δ (ppm)	β-CyD ΔδH3 (ppm)	β-CyD H5 δ (ppm)	β-CyD Δδ H3 (ppm)
1.5E-2	0.000	4.183	0.000	4.055	0.000
1.5E-2	7.0E-3	4.142	0.041	4.020	0.035
1.5E-2	8.0E-3	4.120	0.063	4.002	0.053
1.5E-2	1.0E-2	4.114	0.069	3.987	0.068
1.5E-2	1.2E-2	4.103	0.080	3.977	0.078
1.5E-2	1.4E-2	4.098	0.085	3.971	0.084
1.5E-2	1.5E-2	4.095	0.088	3.968	0.087

Table 4.4.1.8. Complexation-induced chemical shift changes of the guest protons at various prodrug 7/β-CyD ratios

[β-CyD] (M)	Prodrug 7* (M)	Prodrug 7 α-H δ (ppm)	Prodrug 7 α-H Δδ (ppm)	Prodrug 7 β-H δ (ppm)	Prodrug 7 β-H Δδ (ppm)	Prodrug 7 γ-H δ (ppm)	Prodrug 7 γ-H Δδ (ppm)
0.000	5.0E-5	2.022	0.000	2.201	0.000	1.890	0.000
1.5E-2	7.0E-3	2.139	-0.117	2.407	-0.206	1.988	-0.098
1.5E-2	8.0E-3	2.138	-0.116	2.406	-0.205	1.988	-0.098
1.5E-2	1.0E-2	2.138	-0.116	2.407	-0.206	1.989	-0.099
1.5E-2	1.2E-2	2.138	-0.116	2.408	-0.207	1.991	-0.101
1.5E-2	1.4E-2	2.137	-0.115	2.406	-0.205	1.989	-0.099
1.5E-2	1.5E-2	2.138	-0.116	2.408	-0.207	1.992	-0.102



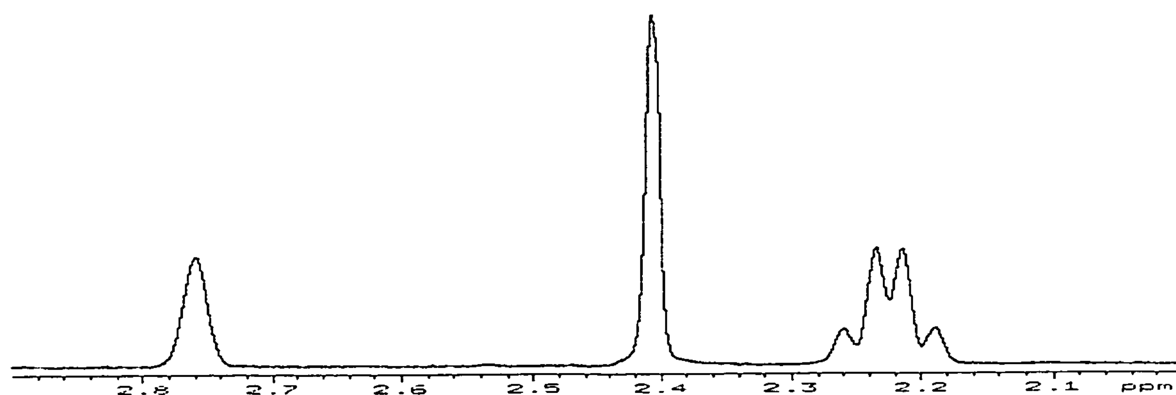


Figure 4.4.1.2. [1]**H NMR spectrum (D_2O) of 1-adamantanamine hydrochloride (top) and (1:1) 1-adamantanamine hydrochloride/β-CyD complex (bottom)**

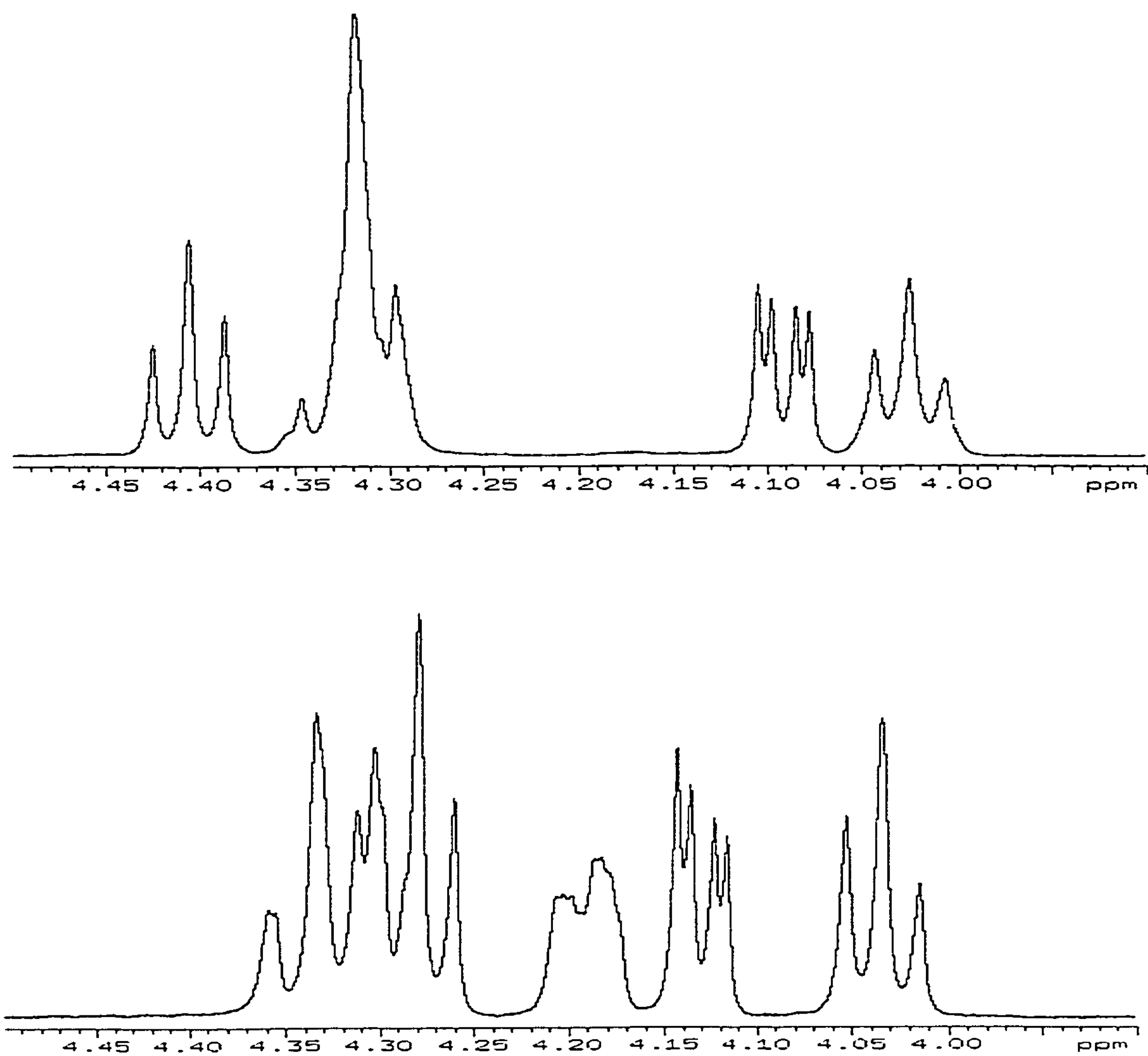
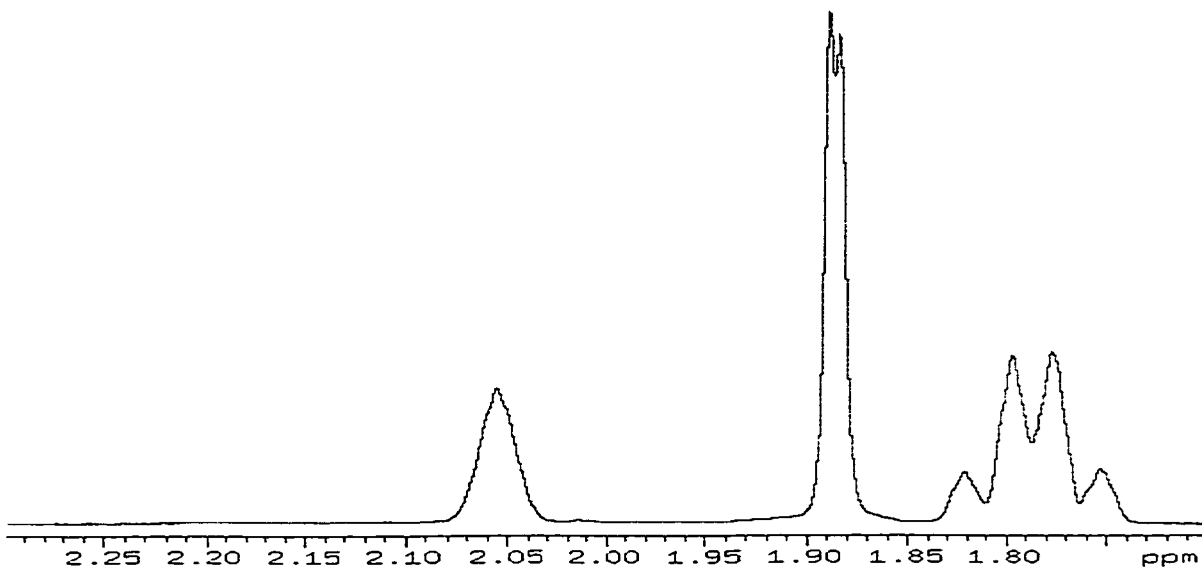


Figure 4.4.1.3. ^{1}H NMR spectrum (D_2O) of β-CyD (top) and (1:1)1-adamantanamine hydrochloride/ β-CyD complex (bottom)



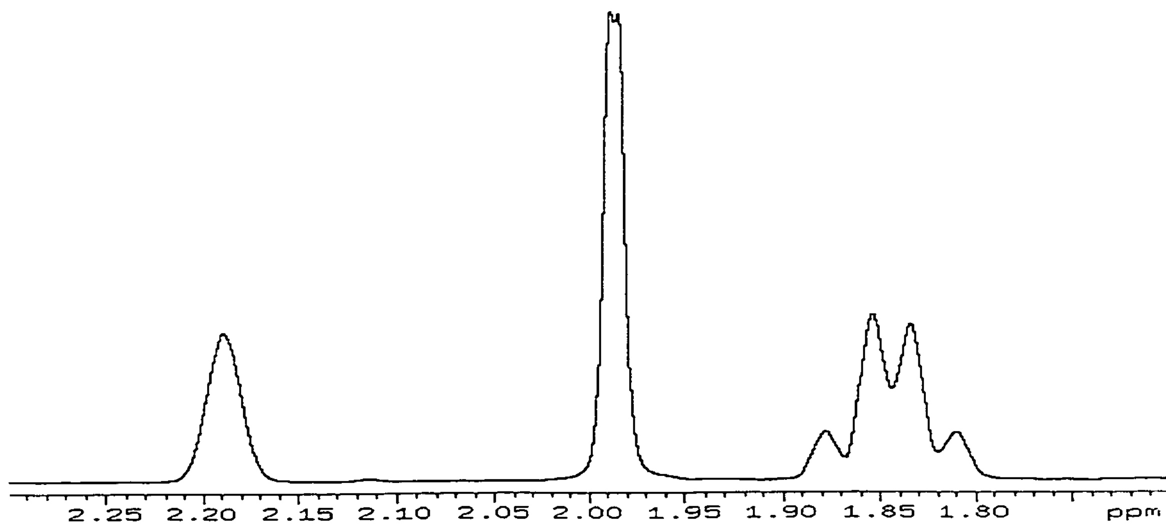
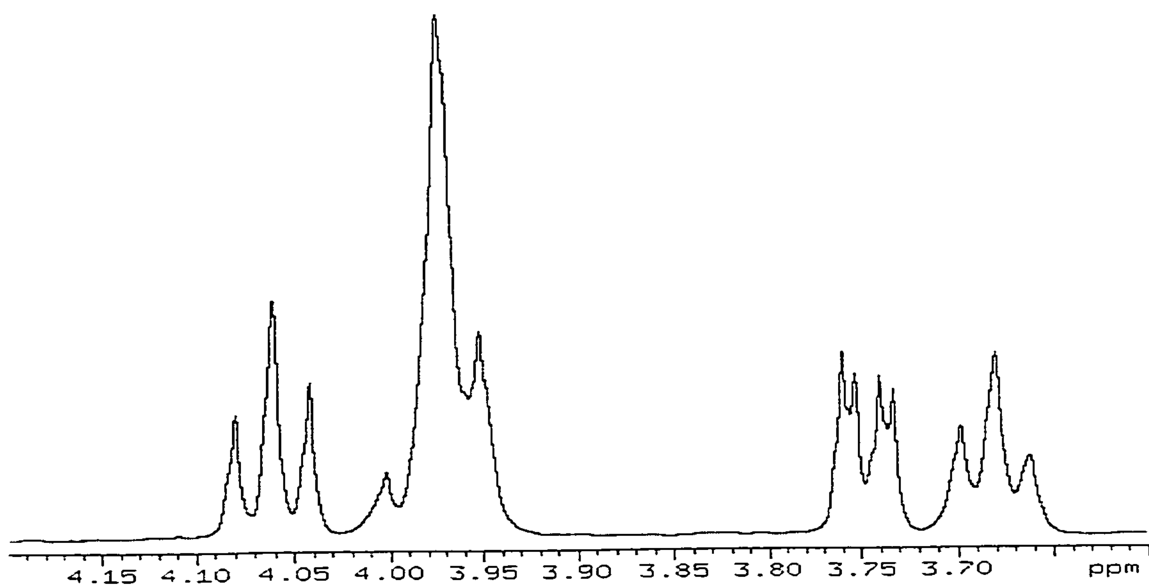


Figure 4.4.1.4. ^{1}H NMR spectrum (D_2O) of sodium 1-adamantanecarboxylate (top) and (1:1) sodium 1-adamantanecarboxylate/β-CyD complex (bottom)



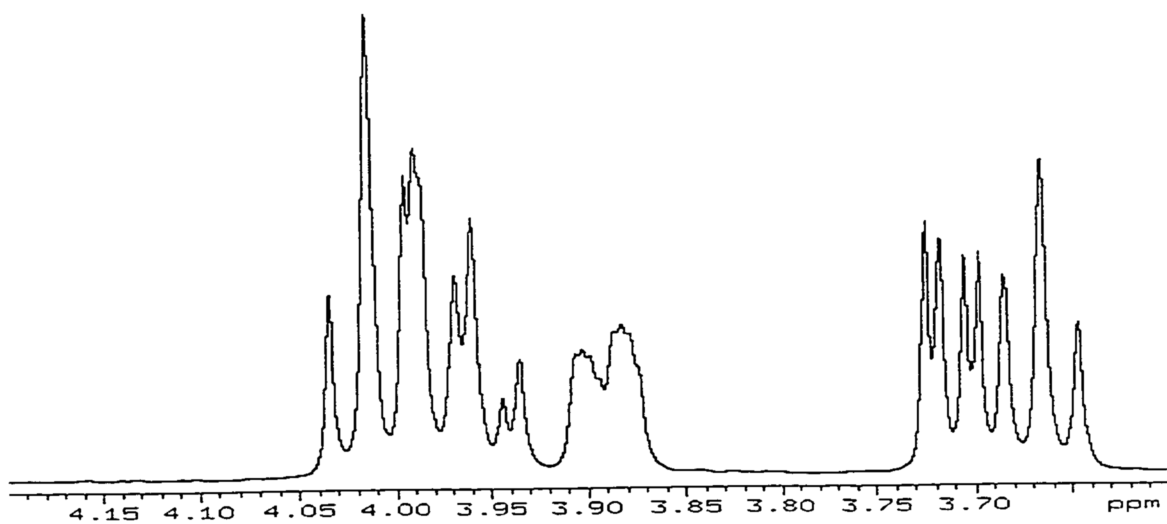


Figure 4.4.1.5. ^{1}H **NMR spectrum (D_2O) of β-CyD (top) and (1:1) sodium 1-adamantanecarboxylate/β-CyD complex (bottom)**

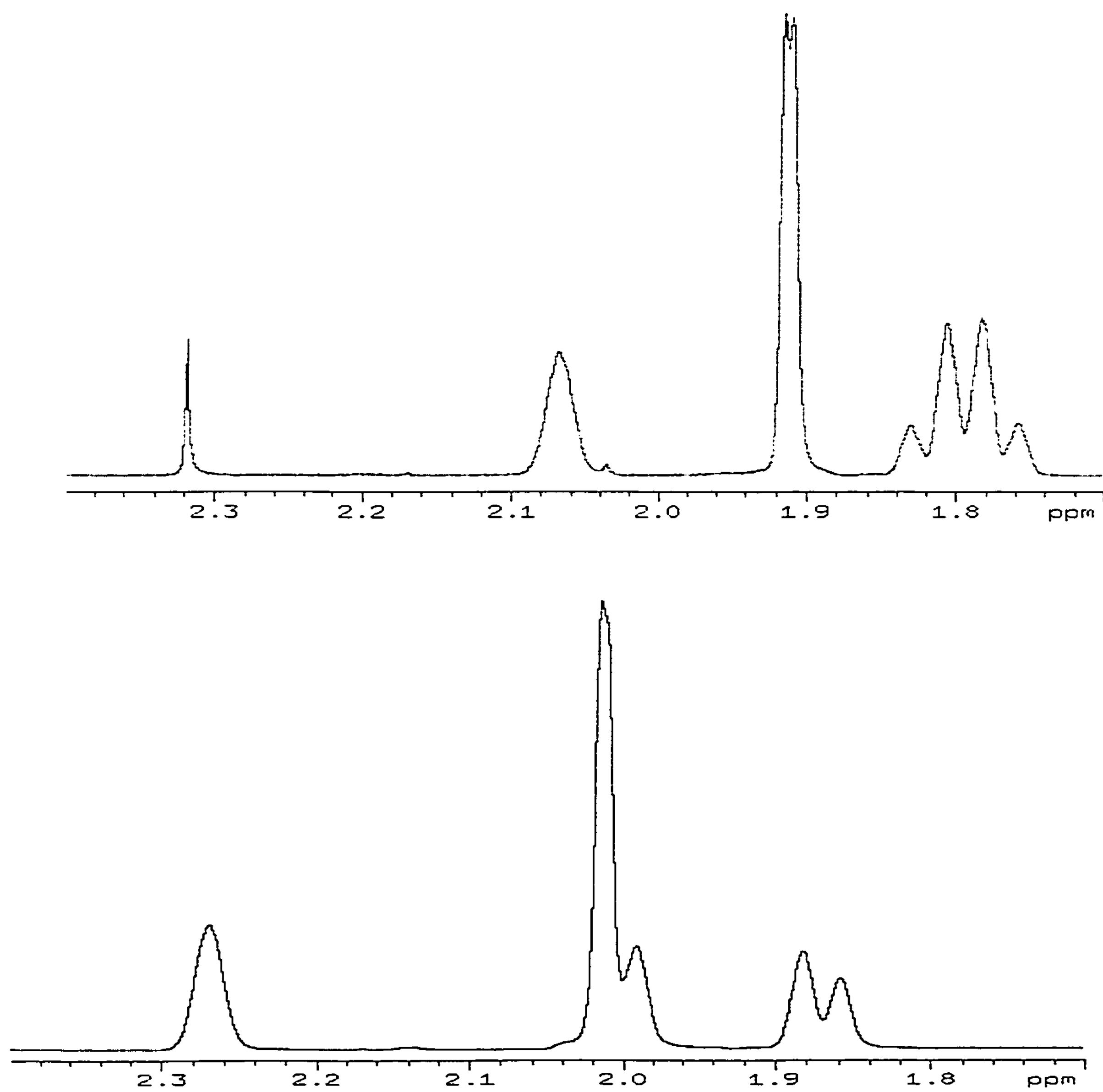


Figure 4.4.1.6. ^{1}H **NMR spectrum (D_2O) of 1-adamantanecarboxylic acid (top) and 1-adamantanecarboxylic acid/β-CyD complex (bottom).**

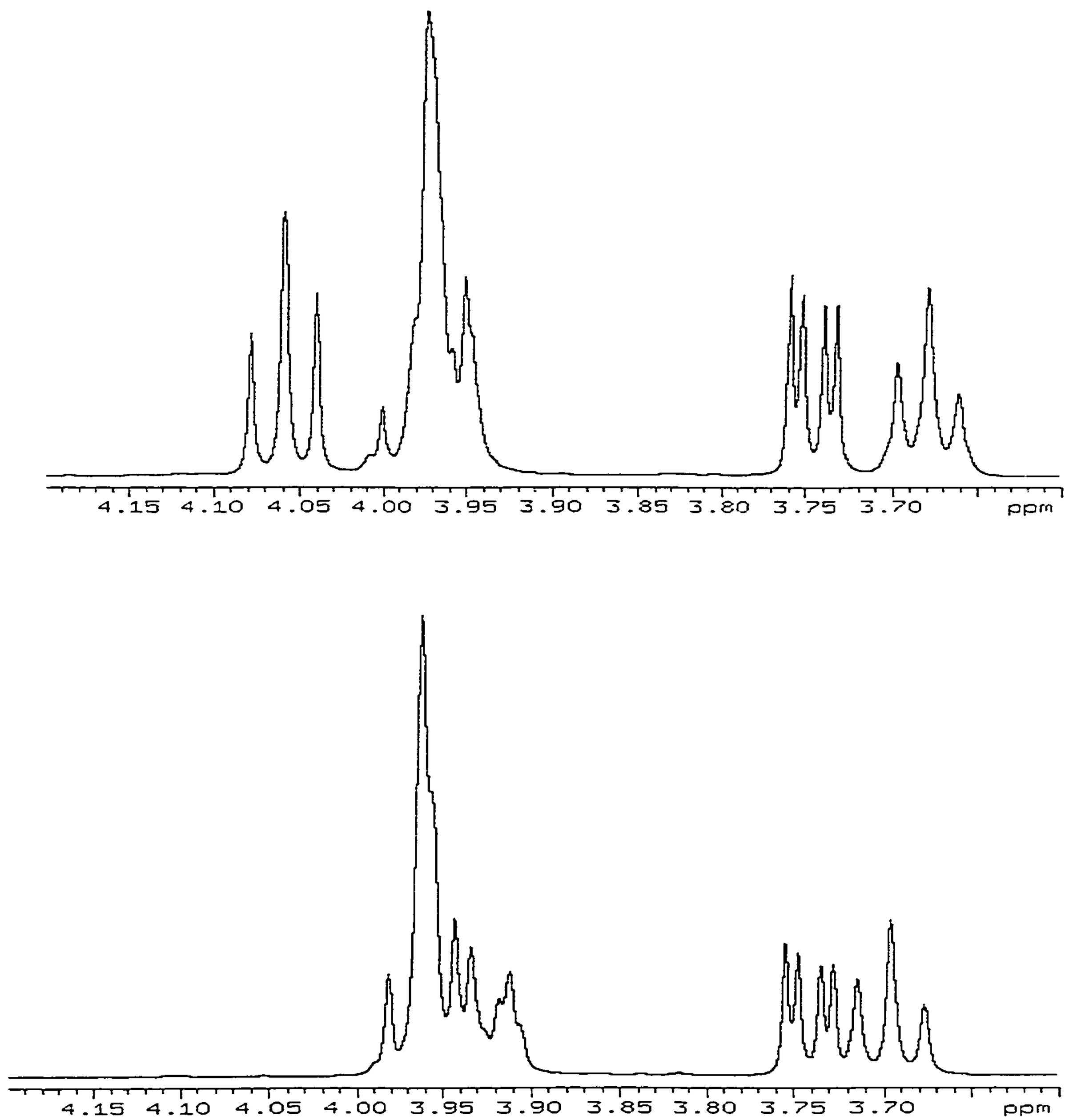
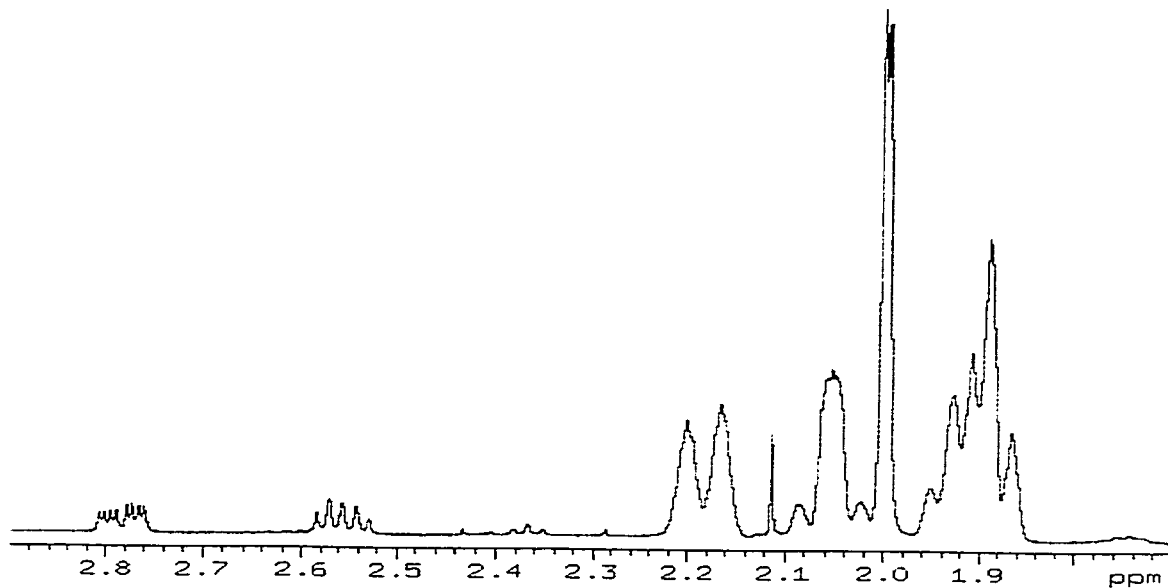


Figure 4.4.1.7. ^{1}H NMR spectrum (D_2O) of β- CyD (top) and (1:1) 1-adamantanecarboxylic acid/β-CyD complex (bottom)



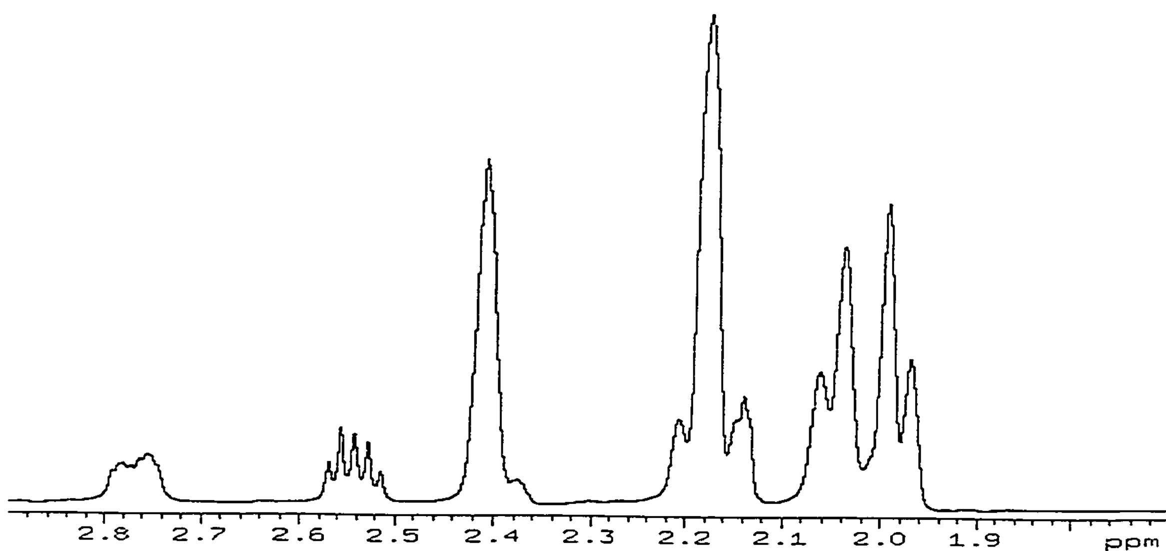
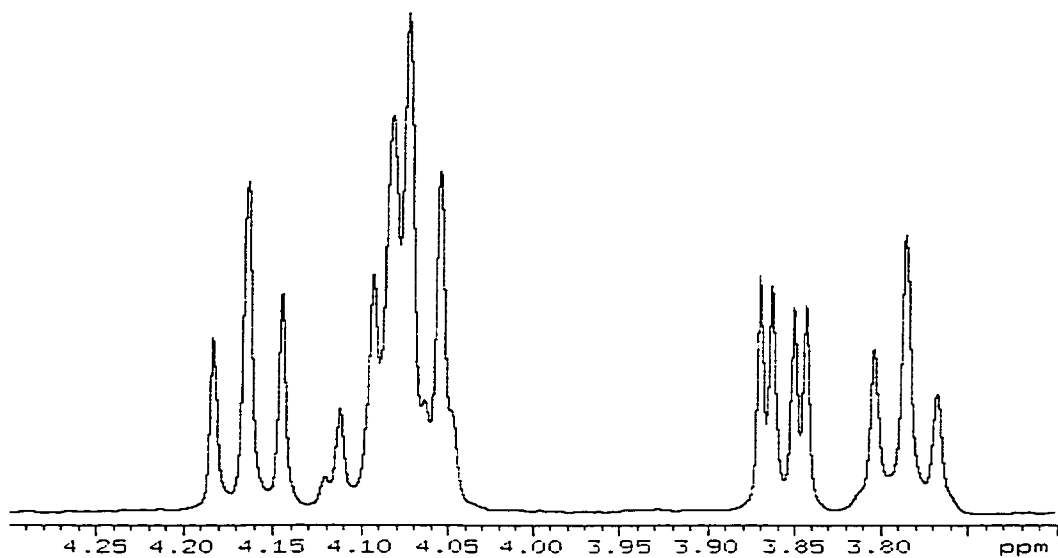


Figure 4.4.1.8. **^{1}H NMR spectrum (D_2O) of the prodrug 7 (top) and (1:1) prodrug 7/β-CyD complex (bottom)**



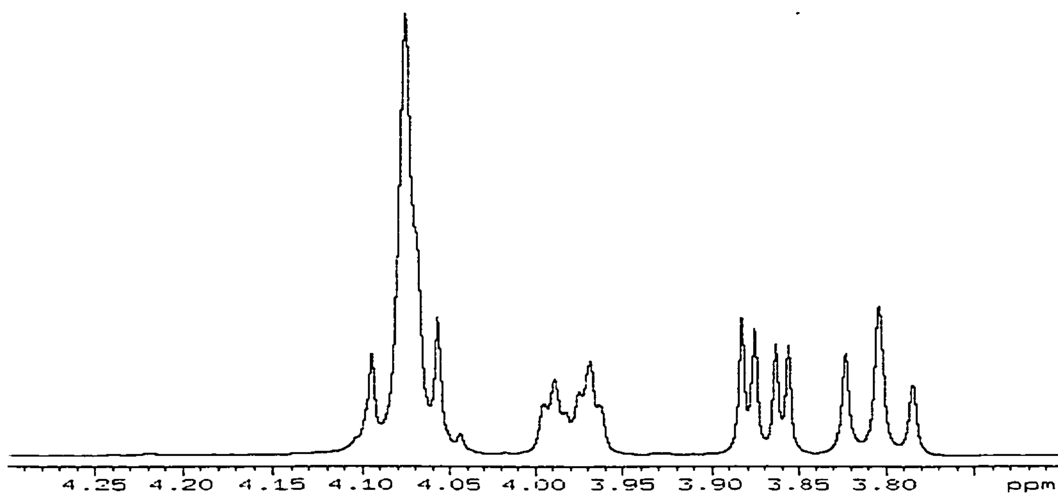


Figure 4.4.1.9. ^{1}**H NMR spectrum (D_2O) of β-CyD (top) and (1:1) prodrug 7/β-CyD complex (bottom)**

4.4.2. ^{1}H NMR estimation of inclusion complex binding constants

During complexation, the chemical environment of some protons changes and this results in changes in the chemical shifts. The shift changes are caused by shielding or deshielding effects. Usually the β-CyD H3 and H5 protons, which are located inside the cavity, and the protons of the guest that are included in the β-CyD cavity are affected the most due to the hydrophobic interactions.

For a 1:1 stoichiometry, the binding (equilibrium) constant equals:

$$K = [C] \times ([A]\times[B])^{-1} \quad (1)$$

where [A] and [B] are the concentrations of free drug and β-CyD, respectively. [C] is the concentration of the complex.

There are two mathematical models to use to determine the binding constant: linear least-squares regression analysis and non-linear least–squares regression analysis. Linear least-squares regression analysis has been applied to known mathematical models (Benesi-Hildebrand 1949 and Jedaini 1990). Most models employ assumptions. For example, they assume one of the species, A or B, is observed in the presence of a large excess of the other component. Under these conditions, from the Benesi-Hildebrand model, equation **1** can be derived as follows:

$$(\Delta\delta_{obs})^{-1} = (k.\bullet \Delta\delta_c)^{-1} \bullet([B]_t)^{-1} + (\Delta\delta_c)^{-1} \quad (2)$$

Plots of $(\Delta\delta_{obs})^{-1}$ against $([B_t])^{-1}$ are linear. The slope, abscissa, and ordinate intercepts are $(k.\bullet \Delta\delta_c)^{-1}$, -k, and $(\Delta\delta_c)^{-1}$, respectively.

In this study, the adamantine derivatives and prodrug **7** do not meet the condition that one of the species, A or B, is observed in the presence of a large excess

of the other component, because the ratio of prodrug to β-CyD if over 1:1 the chemical shift doesn't change.

Schneider et al. (1984), Ferguson and Diederich (1986), and Diedrich(1988) have all applied the non-linear least–squares regression analysis procedure to 1H NMR data. This approach is free from the above mentioned assumptions. The non-linear least–squares regression analysis has found widespread application and is likely to replace evaluations by use of linear models.

Loukas (1997) has applied non-linear least-squares regression analysis to ^1H NMR in order to measure the binding constant of a host/guest complex. This author derives the following equation:

$$\Delta\delta_{obs} = \{K_{1:1}\,(CyD_t - \tilde{n}\,G_t)\,/1 + K_{1:1}\,(CyD_t - \tilde{n}\,G_t)\}\ \Delta\delta_c \quad (3)$$

$\Delta\delta_{obs}$: the chemical shift different between free A (obtained in the absence of B) and the observed value for a given ratio.

$K_{1:1}$: the binding constant.

CyD_t: the total concentration of cyclodextrin.

G_t : the total concentration of drug.

$\Delta\delta_c$: the chemical shift difference between the free molecule and the pure complex

ñ : percentage host and guest form the complex

The author emphasizes that these models are free from any assumption and from practical or theoretical shortcomings.

1-Adamantanamine hydrochloride/β-CyD complex binding constant: The ^1H NMR chemical shifts of the β-protons of 1-adamantanamine hydrochloride, as a function of the concentration of β-CyD, are shown in Table 4.4.1.1 and Table 4.4.1.2. The binding constant was calculated by using the mathematical models described above, by plotting $\Delta\delta_{obs}$ *vs* concentration of β-CyD [M]. Data analysis was performed using mathematical program S-PLUS. The binding constant for the 1-adamantanamine

hydrochloride/β-CyD complex was found to be $K_{1:1} = 1.4 \times 10^3 M^{-1}$. For comparison the spectrophotometric method gave a binding constant of $8.4 \times 10^3 M^{-1}$ (Robert, 1983) for the same complex.

From the Table 4.4.1.1 and 4.4.1.2, it can be seen that upon increasing the host/guest ratio above 1:1 the chemical shift changes become very small, which indicates the formation of a 1:1 inclusion complex.

The non-linear estimation of the parameters $K_{1:1}$ and ñ based on an iteration procedure following specific algorithms (Loukas, 1997). The proposed mathematical models were examined with different algorithms to observe any possible deviation of the calculated value. Figure 4.4.2.1 shows the curve fitting of the experimental data using equation (3).

Sodium 1-adamantanecarboxylate/β-CyD, 1-adamantanecarboxylic acid/β-CyD and prodrug 7/β-CyD complex binding constants: The ^1H NMR chemical shifts of sodium 1-adamantanecarboxylate and 1-adamantanecarboxylic acid, as a function of the concentration of β-CyD, are shown in Table 4.4.1.3 to Table 4.4.1.8. The binding constants were calculated using the same equations described above, however, reasonable values for binding constants could not be obtained because of a large standard error. Application of other curve fitting programs, including S-Plus, SigmaPlot, and Excel, all yielded large standard errors. This observation may indicate that the equation has some limitations.

$$\Delta\delta_{obs} = \{K_{1:1} (CyD_t - \tilde{n}\, G_t) / 1 + K_{1:1} (CyD_t - \tilde{n}\, G_t)\}\, \Delta\delta_c \quad (3)$$

Failure to obtain the binding constants for the above mentioned complexes can be attributed to the error propagation (Liu, 1999) inherent in eq (1). In cases where the $K_{1:1}$ is very large and $K_{1:1} (CyD_t\text{-}\tilde{n}G_t) >> 1$, $K_{1:1} (CyD_t\text{-}\tilde{n}G_t)+1 \approx (CyD_t\text{-}\tilde{n}G_t)$ and eq (3) reduces to $\Delta\delta_{obs} \approx \Delta\delta_c$. Thus the K value obtained from this equation becomes nearly arbitrary. The error propagation can be expressed as follows:

Rearranging eq (1), we have:

$$K=[1/(CyD_t-\tilde{n}G_t)]\{(\Delta\delta_{obs}/\Delta\delta_c)/[1-(\Delta\delta_{obs}/\Delta\delta_c)]\} \quad (4)$$

For clarity, we define:

$$A=(CyD_t-\tilde{n}G_t) \quad (5)$$

And

$$B=(\Delta\delta_{obs}/\Delta\delta_c) \quad (6)$$

Equation (2) can now be rewritten as:

$$K=(1/A)[B/(1-B)] \quad (7)$$

Applying the logarithm to both sides gives:

$$\ln K=-\ln A+\ln B-\ln(1-B) \quad (8)$$

Differentiating (6), we have:

$$(\Delta K/K)=-(\Delta A/A)+(\Delta B/B)+[\Delta B/(1-B)] \quad (9)$$

Rearranging (7), we have:

$$(\Delta K/K)=-(\Delta A/A)+[1/(1-B)]\bullet(\Delta B/B) \quad (10)$$

Substituting (4) into (8), (8) can be rewritten as:

$$(\Delta K/K)=-(\Delta A/A)+[1/(1-B)]\bullet[\Delta(\Delta\delta_{obs}/\Delta\delta_c)/(\Delta\delta_{obs}/\Delta\delta_c)] \quad (11)$$

or

$$(\Delta K/K) = -(\Delta A/A) + [1/(1-B)] \bullet [\Delta(\Delta\delta_{obs})/\Delta\delta_{obs}] \qquad (12)$$

where $(\Delta K/K)$ is the relative deviation of K, $(\Delta A/A)$ is the relative deviation of $(CyD_t\text{-}\tilde{n}G_t)$, and $[\Delta(\Delta\delta_{obs})/\Delta\delta_{obs}]$ is the relative deviation of $\Delta\delta_{obs}$. As a rule in error propagation from equation (9), the relative deviation of K must be larger than $[1/(1-B)]$ times the relative deviation of $\Delta\delta_{obs}$.

In cases where the K is very large and $K(CyD_t\text{-}\tilde{n}G_t) >> 1$, $K(CyD_t\text{-}\tilde{n}G_t)+1 \approx (CyD_t\text{-}\tilde{n}G_t)$ and $\Delta\delta_{obs} \approx \Delta\delta_c$, thus $B \approx 1$ and $[1/(1-B)] >> 1$. Therefore the error in measurement of $\Delta\delta_{obs}$ translates to a greatly magnified error in calculated K. In our experiments, $(CyD_t\text{-}\tilde{n}G_t)$ is $10^{-3} \sim 10^{-2}$.

When $(CyD_t\text{-}\tilde{n}G_t) = 10^{-3}$,

$B = (\Delta\delta_{obs}/\Delta\delta_c) = K(CyD_t\text{-}\tilde{n}G_t)/K(CyD_t\text{-}\tilde{n}G_t)+1] = (10^6)(10^{-3})/[1+(10^6)(10^{-3})] = 0.999$

Or

$1/(1-B) = 1001.$

Therefore, the relative error in K must be more than 1000 times the relative error in $\Delta\delta_{obs}$. The error on the measurement of $\Delta\delta_{obs}$ was about 0.005 ppm, while the highest $\Delta\delta_{obs}$ value measured was 0.088 ppm. Thus, in the best case, the relative deviation in $\Delta\delta_{obs}$ was 6% and the relative deviation in K is 1001 times 6%, or 6006%. In other words, the error in K could be over 60 times its true value. Therfore, the Loukas model can not be used to determine K if K is very large.

For the 1-adamantanamine hydrochloride, the experimental results fit this model very well, and the calculated equilibrium constant K is 1.4×10^3, which is in the effective range of the equation. In the other three compounds however, the experimental results did not fit the equation at all. This was because the equilibrium constant was very large, and the basis of this equation became invalid. The equation

suggests that in such cases, $\Delta\delta_{obs}$ is independent of CyD_t, ñ or G_t. Nevertheless, the experimental data clearly showed a trend not indicated by the equation. Gelb and Schwartz (1983) used spectrophotometric and pH potentiometric methods to study binding constants of the sodium 1-adamantanecarboxylate and 1-adamantanecarboxylic acid, and found that both were $> 10^6$. For prodrug 7 the binding constant was found to be 1.49×10^5 as measured by HPLC. This strongly indicated that there might be some other factors, whose effects were negligible when K was small, that played a very strong role in the process when K was large.

All of the approaches tried were based on the assumption that there is just one mechanism for the interaction between host and guest molecules. Therefore, the K value is independent of other factors including concentration or ratio of the concentration. However, if certain concentrations, ratios, or the mechanism of the interaction changes, the value of K also changes. In this situation, none of the previous models can explain the relationship of K and change chemical shifts. Other factors may be involved, including guest molecule orientation in the cavity (which side of the guest), and formation of hydrogen bonds between the host/guest (increases binding) leading to a high binding constant. Therefore, the chemical shift change is not the only factor, which determines the binding constant.

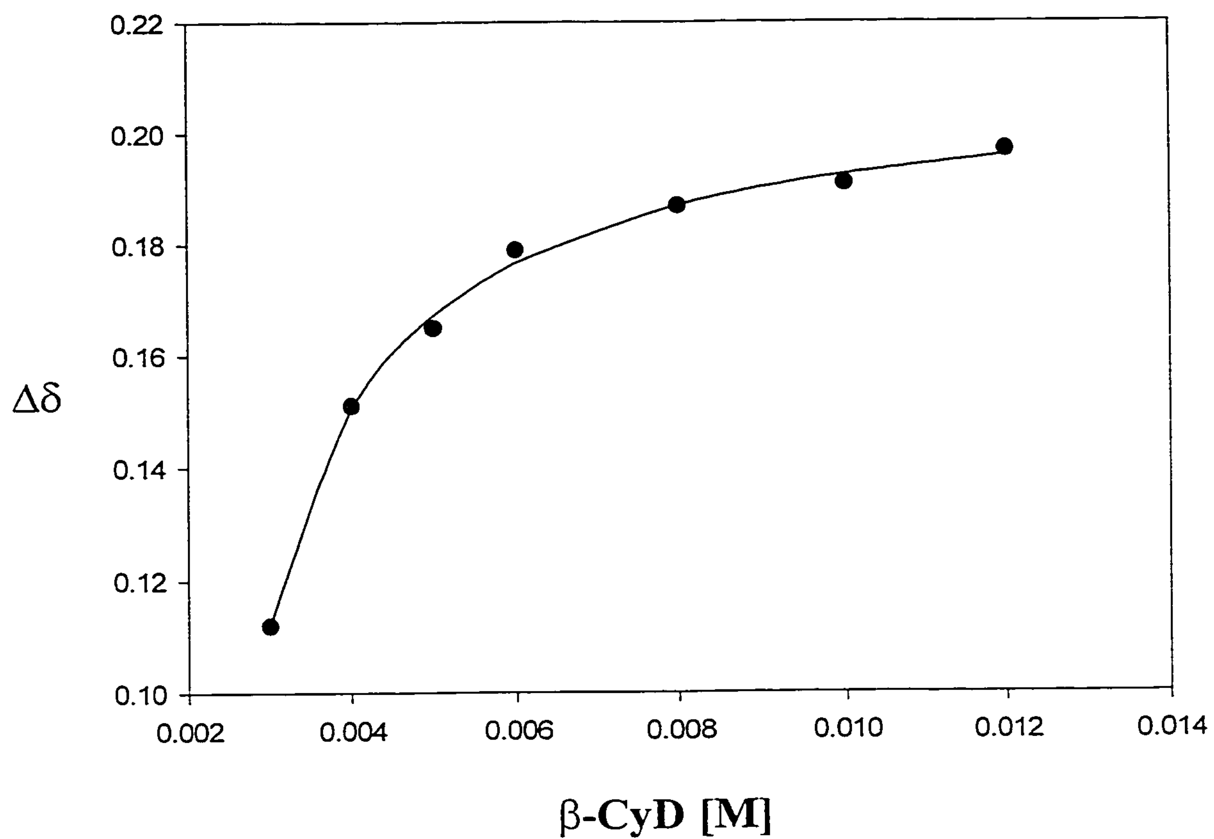


Figure 4.4.2.1. Curve fitting of 1-adamantanamine hydrochloride/β-CyD binding constant experimental data

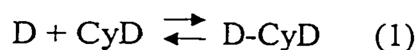
4.5. CE determination of inclusion complex binding constants

Electrophoresis has long been recognized as a useful tool for examining the nature and strength of biological interactions (Cann, 1996 and Takeo, 1995). Capillary electrophoresis can be described as a method for the high efficiency separation of molecules in narrow bore (I.D. 10-100 μm) capillary tubes filled with an electrolyte solution under the influence of an electric field. The separations are based on the differences in the mobility of ionic species or differences in the affinity of charged or neutral molecules to charged electrolytes. Highly specific separations, analysis of molecules and intermolecular interactions are based on affinity effects (for example, hydrogen bonding, electrostatic interactions, hydrophobic interactions and Van der Waals forces). This technique is termed affinity capillary electrophoresis (ACE). ACE has been widely used for the study and determination of physico-chemical parameters, such as binding constants in the study of drug–protein interactions, protein-protein and protein-carbohydrate interactions, and cyclodextrin-drug interactions. The application of CyDs as chiral and achiral discriminators in CE has been reviewed (Li and Purdy, 1992, Luong, and Nguyen, 1997, and Rundlett, 1997).

In the capillary electrophoretic separation of CyDs, the neutral CyDs are carried towards the cathode by the electroosmotic flow (EOF) with the mobility μ_{EOF}. The EOF is caused by cations attracted to the negatively charged silanol groups on the inside of the fused silica capillary. These cations move towards the cathode, dragging the solute in the capillary in the same direction. Anions in the background electrolyte (BEG) move towards the anode against the EOF and away from the detector with the mobility μ_{A^-}. An equilibrium is formed between free CyDs migrating towards the cathode by the EOF and CyD-anion complexes migrating towards the anode with the mobility μ_{CyDA^-}. This results in the CyD carrier being separated from other neutral species which are not able to form complexes with the anions. When applying cations as BEG the CyD moved towards the detector ahead of the EOF with the mobility μ_{CyD^+}.

The separation of CyD by CE relies on the formation of inclusion complexes between the CyD and a charged BEG. Differences in the formation constants between various CyDs and the BEGs leads to their separation. In general, two methods have been applied (Lee and Lin 1996). The direct absorbance detection method (DAD) estimates the inclusion complex binding constant between CyD and a charged guest molecule, by measuring the change of mobility of the guest molecule in buffers containing various concentrations of CyD. In the indirect absorbance detection method (IAD), the CyD is run as an analyte and the mobility of the CyD is measured as a function of the concentration of the charged guest molecule. In this study, we have measured the binding constant between 1-adamantanamine hydrochloride and compound **5** (an UV active derivative β-CyD) using the IAD method.

For a typical 1:1 equilibrium between drug [D] and β-CyD [CyD], if the total concentrations are denoted as $[D]_t$ and $[CyD]_t$ respectively, the fractions of free (f_0) and complexed CyD (f_1) in such equilibrium are expressed as follows (Loukas, 1997 and Wan, 1999):



$$D + CyD \rightleftarrows D\text{-}CyD \quad (1)$$

$$K = [D\text{-}CyD]/[D]\bullet[CyD] \quad (2)$$

$$\begin{aligned} f_0 &= [CyD]/[CyD]_t \\ &= [CyD]/\{[CyD]+[D\text{-}CyD] \\ &= [CyD]/\{[CyD]+K\,[D]\,[CyD] \\ &= 1/\{1+K\,[D]\} \qquad (3) \end{aligned}$$

$$\begin{aligned} f_1 &= [D\text{-}CyD]/[CyD]_t \\ &=[D\text{-}CyD]/\{[CyD]+[D\text{-}CyD] \\ &= K\,[D][CyD]/\{[CyD]+K\,[D][CyD]\} \\ &= K\,[D]/\,1+K\,[D] \qquad (4) \end{aligned}$$

and

$$f = f_0 + f_1 = 1 \qquad (5)$$

where $K_{1:1}$ is the binding (equilibrium) constant and [D] and [CyD] are the concentrations of the uncomplexed (free) drug and β-CyD, respectively.

In this ideal system, a change in solute mobility, is observed when the ligand is introduced into the system, is caused solely by complexation of the solute with the ligand. The experimentally measured electrophoretic mobility of the solute in a solution containing the ligand is the weighted average of the mobilities of the solute in the free and complexed states (Rundlett and Armstrong, 1997 and Wang 1999):

$$\mu_{ob} = \mu_{eo} + f_0\,\mu_{CyD} + f_1\,\mu_C \quad (6)$$

where μ_{ob} is the experimentally measured electrophoretic mobility, μ_{CyD} is the mobility of the free (uncomplexed) β-CyD, μ_C is the mobility of the complexes and μ_{eo} is the mobility of the electroosmotic flow. Equation (6) can be expressed as the migration time formula and gives the following relationships:

$$1/T_{ob} = 1/T_{eo} + K\,[D]/\{1+[D]\} \bullet 1/T_c \quad (7)$$

Equation (7) can be rearranged to equation (8) as follows:

$$1/T_{ob} - 1/T_{eo} = K\,[D]/\{1+[D]\} \bullet 1/T_c \qquad (8)$$

The experimental parameters T_{ob}, T_{eo}, T_c are the measured migration times of β-CyD and neutral marker (EO) peaks in the presence and the absence of the drug in the electrophoresis buffer, respectively. Binding constants based on corrected electrophoretic mobility for β-CyD can then be determined using equation (8), which can be solved by non-linear least-squares regression analysis.

Determination of the binding constant for the 1-adamantanamine hydrochloride/ CyD 5 inclusion complex: CyD **5** was run as an analyte, and the mobility of the compound was measured as a function of the concentration of the charged guest molecule. Migration times of compound **5** and neutral marker (EO) peaks in the presence and the absence of the drug in the electrophoresis buffer (pH = 7.4) are shown in Table 4.5.1. The binding constant was calculated using the mathematical model described above. By plotting $1/T_{ob} - 1/T_{eo}$ *vs.* concentration of 1-adamantanamine hydrochloride [D], the binding constant was found to be $K_{1:1}$ 1.7 x 10^3 M^{-1}.

Table 4.5.1. Migration times of β-CyD 5 and neutral marker (EO) Peaks in the presence and absence of the drug in the electrophoresis buffer (Phosphate buffer pH = 7.4).

Drug (mM)	T_{ob} (min)	T_{eo} (min)	$T_{ob} - T_{eo}$ (min)
0.00	7.32	7.32	0.00
3.00	7.13	7.65	0.52
5.00	7.33	7.91	0.58
10.00	7.56	8.18	0.62
12.00	7.61	8.23	0.62
15.00	7.83	8.45	0.62

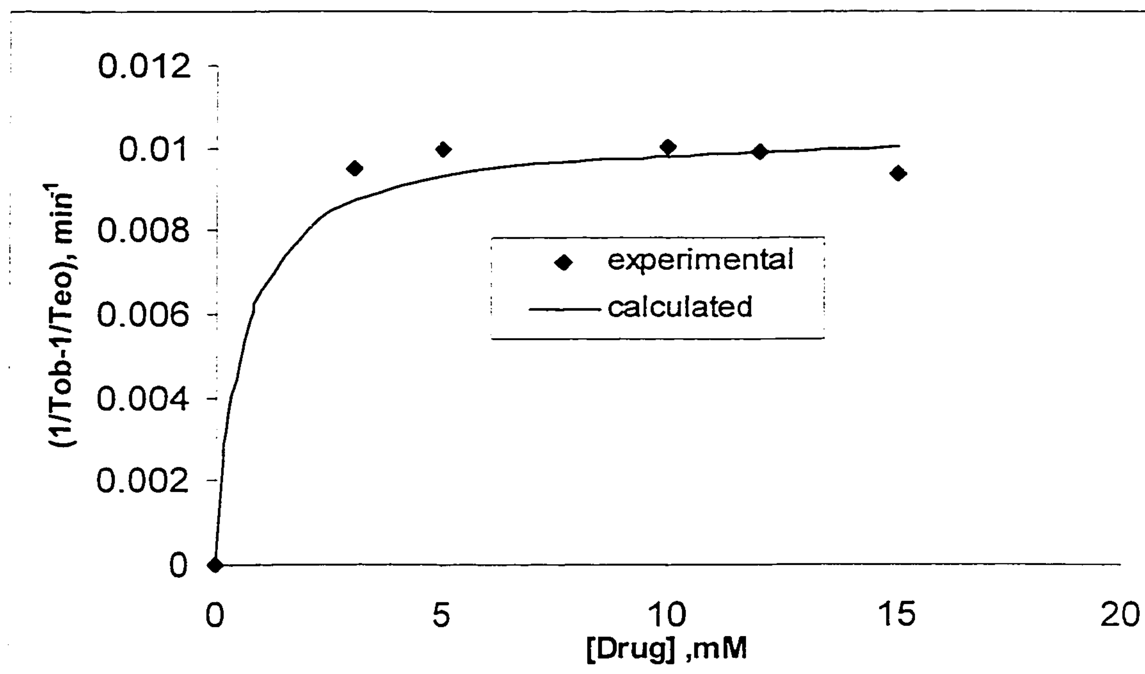


Figure 4.5.1. Curve fitting of 1-adamantanamine hydrochloride/β-CyD 5 binding constant using equation 8 to the experiment data.

4.6. Stability of the prodrug 7/β-CyD inclusion compound in aqueous solution

Inclusion complexes formed between a host molecule and a guest molecule are non-covalent. The complex is held together by physical forces, such as Van der Waals forces, hydrophobic interactions, and possibly the formation of intermolecular hydrogen bonds. Therefore, during β-CyD/drug complex formation, no covalent

bonds are formed or broken (Connors, 1997). It should be noted that the β-CyD complex in solution is rapidly exchanging with its free components. The association of β-CyD and drug molecules and the dissociation of the formed β-CyD/drug complex are controlled by kinetic and thermodynamic equilibrium. The kinetics of inclusion complex formation and dissociation are reported to be fast (Szejtli, 1994). Where perturbation and competitive binding techniques have been used to measure these events, the half-lives for formation/dissociation are much less than one second, and occur at rates very close to diffusion controlled limits, with the complexes being continually formed and dissociated.

In this study, we focused on determination of the rate of prodrug 7/β-CyD complex dissociation in a model system. The method involved measurement of the rate of partitioning of the prodrug 7 from an aqueous phase containing a prodrug 7/β-CyD complex into a water insoluble 1-octanol layer and was analyzed by HPLC. The experimental data are shown in the Table 4.6.1. Figure 4.6.1 (a) and (b) shows the stability of the inclusion complex of prodrug 7 with β-CyD in aqueous solution of 1.2 mM and 6.2 mM complex, respectively. The half-life for the complex at a concentration of 1.2 mM was found to be 40 min, while at high concentration (6.2 mM) the half-life was 65 min. These data indicate that dilution is a major factor for complex dissociation as has previously been reported by Connors (1997). This factor is an extremely important consideration when designing β-CyD inclusion complex formulations for parenteral drug delivery.

Table 4.6.1. Rate of release of the prodrug 7 (1.2 mM) from the aqueous Phase (complex form) into the 1-octanol phase.

Time (min)	0	15	45	70	120	180
% prodrug 7 in water phase	100	76	58	42	21	9
S. D. (n = 3)	0	2.51	6.8	3.51	4.16	1.52

Table 4.6.2. Release of the prodrug 7 (6.2 mM) from the aqueous (complex form) into the 1-octanol phase.

Time (min)	0	20	60	90	120	150	180	210	240	270
% prodrug 7 in water phase	100	87	53	40	29	21	15	13	12	11
S. D. (n = 3)	0.0	0.0	3.1	3.6	3.1	1.7	1.6	0.6	0.0	0.6

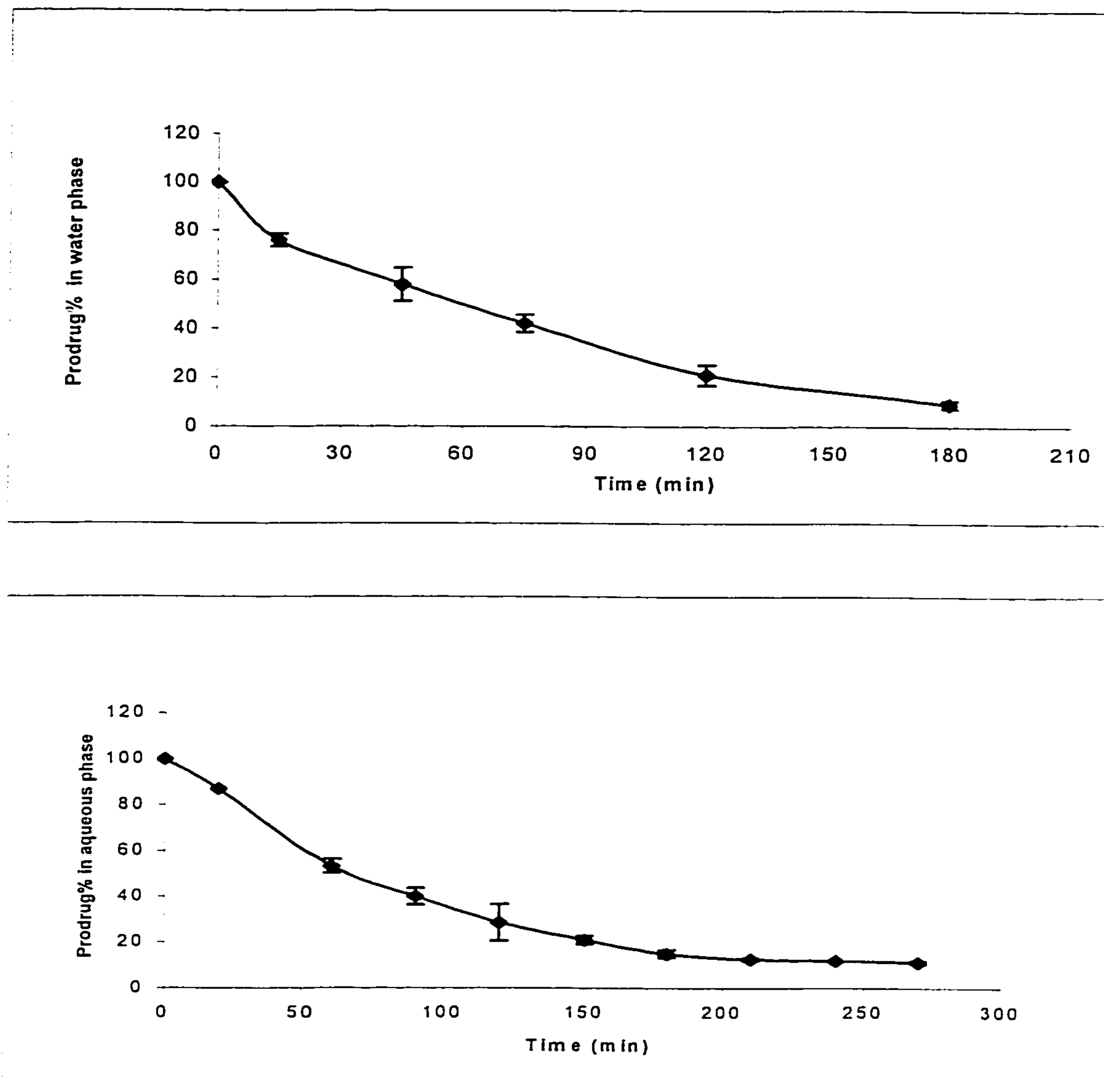


Figure 4.6.1. Diffusion of the prodrug 7 from the inclusion complex (1.2 mM) in aqueous solution into 1-octanol phase (top) and diffusion of prodrug 7 from the inclusion complex (6.2 mM) in aqueous solution into 1-octanol phase (bottom).

4.7. Stability of the prodrug 7/β-CyD inclusion complex to cholesterol challenge

Cyclodextrins are known to form inclusion complexes with many lipophilic molecules due to the hydrophobic nature of the cavity (Uekama and Otagiri, 1986). This implies that cyclodextrin can form a complex with various endogenous lipids, therefore changing the distribution of these lipids (Carpenter and Pettifor, 1987). It has been reported that cholesterol forms a complex with β-CyD *in vivo* with an association constant of 1.7×10^4 M^{-1} (Frijlink, 1991). As a consequence, the drug/β-CyD complex can quickly be dissociated *in vivo* because cholesterol effectively competes with β-CyD for drug binding. This is highly undesirable, as the cholesterol/β-CyD complex has been found to crystallize in the kidney and cause kidney damage. In fact this is the major drawback of using CyDs for parenteral drug delivery. In this study, we chose to study the stability of the complex at two different the cholesterol concentrations: 6.2 mM and 62 mM (in humans the total serum cholesterol concentrations is 5.2–6.2 mM) while the prodrug 7/β-CyD inclusion complex concentration was maintained at 6.2 mM. The experiment data obtained are shown in Table 4.7.1 and Figure 4.7.1. The data show that even at a cholesterol concentration 10 times higher than the complex, cholesterol competes poorly for binding to CyD compared to the prodrug. Presumably, this is the result of the higher binding constant between β-CyD and the prodrug than between cholesterol and β-CyD.

Table 4.7.1. The stability of the prodrug 7/β-CyD inclusion complex to cholesterol Challenge.

Time (min)	% prodrug 7 inclusion complex in aqueous phase (6.2 mM)[a]	% prodrug 7 inclusion complex in aqueous phase (6.2/6.2 mM)[b]	% prodrug 7 inclusion complex in aqueous phase (6.2/62 mM)	Average of 3	S. D. (n = 3)
0	100	100	100	100	0.0
20	87	79	83	83	4.0
60	53	70	69	61	9.0
90	40	45	53	46	7.0
120	29	29	37	32	4.8
150	21	21	19	20	1.2
180	15	16	14	15	0.7
210	13	13	12	13	0. 5
240	12	12	12	11	0.2
270	11	12	11	11	0.3

a = 6.2 mM prodrug 7 inclusion complex.

B = 6.2 mM prodrug 7 inclusion complex and 6.2 mM cholesterol.

S.D. is the standard deviation.

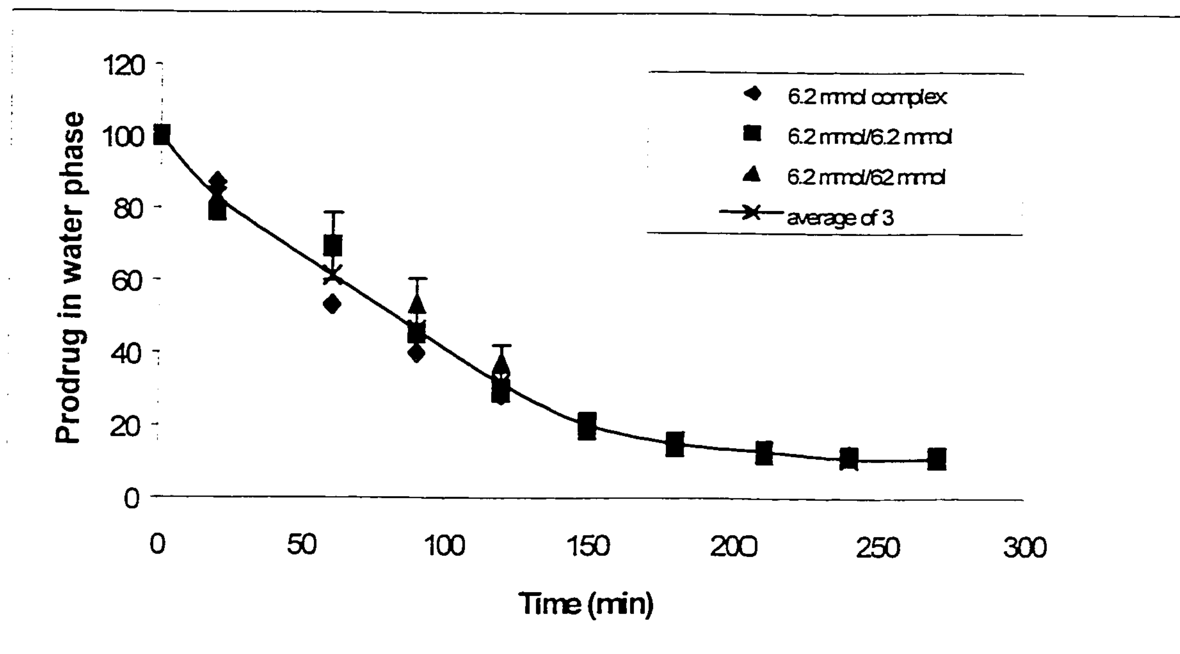


Figure 4.7.1. The stability of the prodrug 7/β-CyD inclusion complex to cholesterol challenge.

4.8. The stability of the prodrug 7/β-CyD inclusion complex in plasma

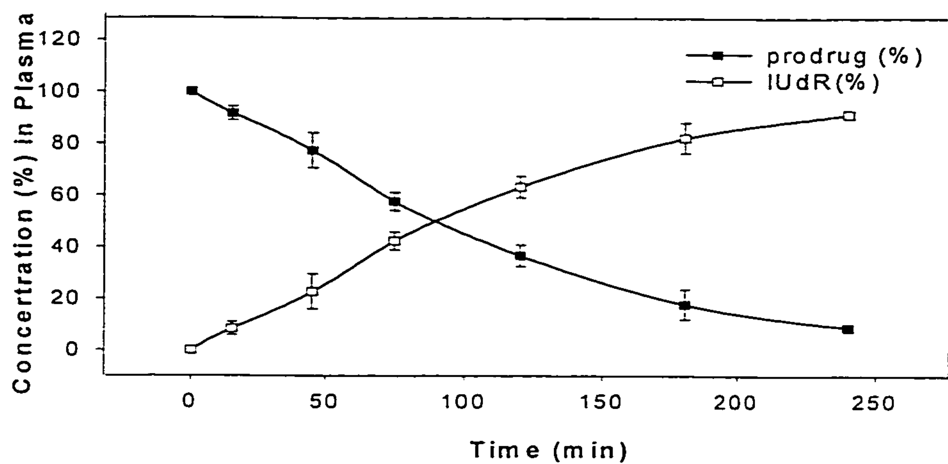
The routine use of the radiohalogenated nucleoside 5-iodo-2'-deoxyuridine (IudR) *in vivo*, is limited due to its extremely short biologic half-life in blood (the serum half-life of IudR in the human is < 5 minutes (Klecker and Kinsella, 1985). In order to address this problem, we have been exploring an IudR prodrug formulation. A prodrug is a pharmacologically inactive compound, which is converted to an active drug by metabolic biotransformation. The prodrug approach is typically used to solve pharmacokinetic problems such as retention time in plasma or drug delivery problems. In the present case, we wanted to increase the stability of IudR in plasma. Prodrug 7 was designed to form a high affinity inclusion complex with β-CyD. The transformation of prodrug **7** to the active form after administration proceeds in two steps: first the prodrug **7** must disassociate from the host; and second, hydrolysis of the prodrug is required to provide the active form. Serum esterases are enzymes, which can cleave the prodrug and then release IudR. The experimental data in Table 4.8.1, Table 4.8.2 and Figure 4.8.1. show the stability of the of prodrug 7/β-CyD inclusion complex in plasma. The results obtained show that for a concentration of 1.2 mM prodrug **7**, approximately 60 % prodrug remains after 80 min, while at 6.2 mM inclusion complex, even after 5 h, 60% prodrug still remains. This indicates that β-CyD has stabilized the prodrug and delayed prodrug hydrolysis to IudR.

Table 4.8.1. Hydrolysis of the prodrug 7/β-CyD (1 mM) in plasma.

Time (min)	0	15	45	75	120	180	240
% prodrug **7** in plasma	100	91.7	77.3	57.7	36.7	17.7	8.7
% IUdR in plasma	0	8.3	22.7	42.3	63.3	82.3	91.3
S. D. (n = 3)	0	2.51	6.80	3.51	4.16	5.85	1.52

Table 4.8.2. Hydrolysis of the prodrug 7/β-CyD (6.2 mM) in plasma.

Time (h)	0	1	2	3	4	5	6
% Prodrug **7** in plasma	100	92.2	87.1	79.2	70.1	60.1	52.1
% IUdR in plasma	0	7.8	12.9	20.8	29.9	39.9	47.9
S. D. (n = 3)	0	0.91	0.39	1.08	0.7	1.3	3.6



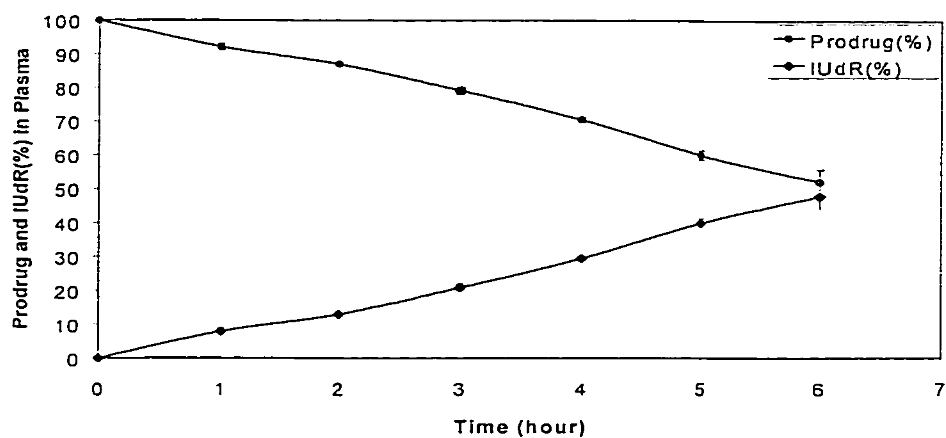


Figure 4.8.1 Stability of the prodrug 7/β-CyD inclusion complex in plasma. (a)1 mM and (b) 6.2 mM.

4.9. Diffusion of prodrug 7 from the inclusion complex in plasma into 1-octanol

As was mentioned in the section 4.7.1, β-CyD can form inclusion complexes with many drugs. These complexes have different properties than the corresponding free drugs. The ability of β-CyD to behave as a host has been used in the field of pharmaceutics to improve the chemical stability of drugs (Szejtli, 1982). We have demonstrated the stability of the prodrug 7 inclusion complex in plasma (section 4.8.1.). We also wish to know if the relative rate of the transfer of the prodrug 7 from the inclusion complex in blood to biological membranes is altered by plasma components. As 1-octanol can be viewed as an artificial biological membrane (King, 1994), an experiment to determine transfer of prodrug from plasma to 1-octanol was designed. Experimental data (Table 4.9.1 and Figure 4.9.1.) show movement of prodrug 7 from the inclusion complex in plasma, into 1-octanol. The results after 4.5 h, show 50 % transfer of prodrug 7 into the 1-octanol phase as shown in section 4.8., 60 % of the prodrug remained in the plasma after 5 h and the other 40 % prodrug was hydrolyzed to IUdR. The prodrug is hydrophobic and is therefore expected to be dissolved in the 1-octanol phase, while IUdR is hydrophilic and remains mainly in the plasma.

Table 4.9.1. Transfer of the prodrug 7 from the inclusion complex into 1-octanol.

Time (min)	Prodrug 7 (%) in 1-octanol	S. D. (n = 3)
0	0	0
20	14	1.7
60	26	3.1
90	33	2.1
120	38	1.7
150	41	3.2
180	45	2.0
210	48	2.1
240	50	2.9
270	52	2.0

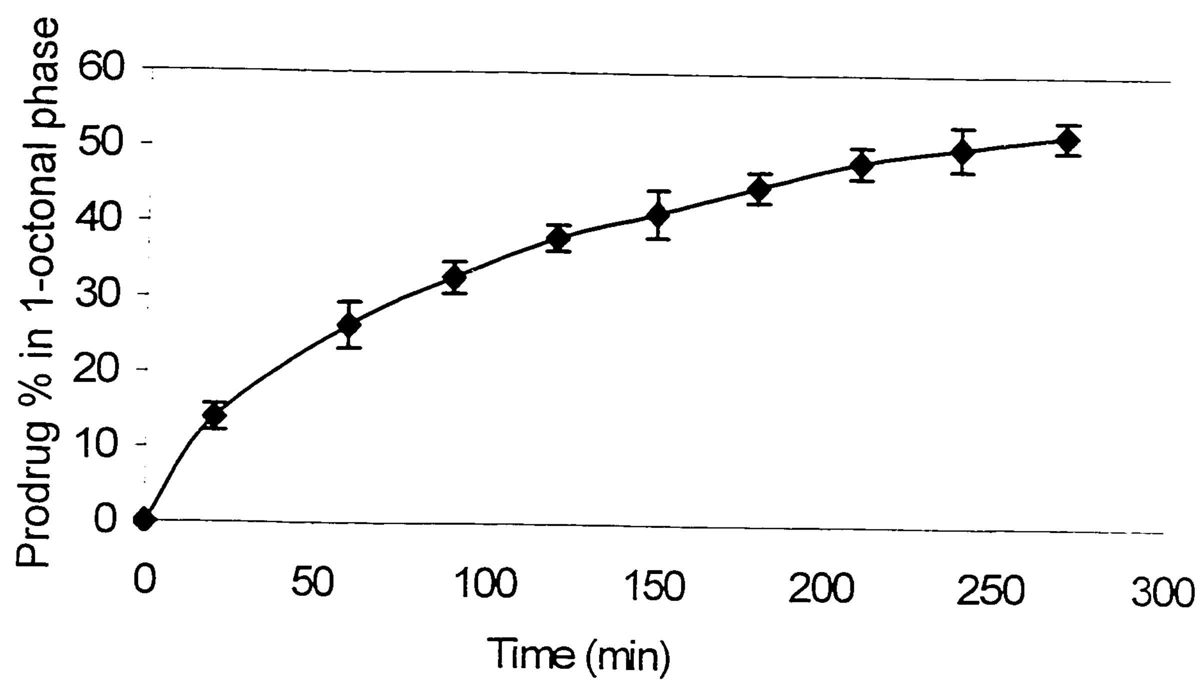


Figure 4.9.1. Transport of prodrug 7 from the inclusion complex into 1-octanol.

5. Summary and conclusions

The objective of this work was to prepare a β-cyclodextrin-IUdR prodrug complex as the non-radioactive prototype, and to completely characterize this prodrug complex. Knowledge gained with the cold formation can hopefully be applied to the IUdR radiopharmaceutical. This project explores the possibility of utilizing a host-guest delivery strategy for delivery of radiopharmaceuticals for the treatment of cancers. Techniques employed to characterize the complex included determination of the Log P of the prodrug 7 phase solubility, DSC, X-ray diffractometry, ^{1}H NMR and CE. These techniques demonstrated prodrug 7 with β-CyD formed an inclusion complex. The *in vitro* stability of prodrug 7/β-CyD inclusion complex in aqueous solution, prodrug 7/β-CyD inclusion complex to cholesterol and prodrug 7/β-CyD inclusion complex in plasma were also determined. From the investigations described above, the following results were obtained:

1). Synthesis 5-Iodo-5'-adamantoyl-2'-deoxyuridine was completed.

2). Partition coefficient (Log P) of prodrug 7 was found to be 1.91 in water and 1.97 in phosphate buffer.

3). The solubility of Prodrug 7 was increased by forming an inclusion complex with β-CyD. The solubility of prodrug 7 increased 317 times (1:1 molar ratio) in aqueous β-CyD.

4). DSC was used to confirm the formation of an inclusion complex. The DSC of the free prodrug 7 showed one exothermic peak at 208 ^{0}C andβ-CyD had a very broad transition range between 70 – 180 ^{0}C and a small endothermic peak at 220 ^{0}C, while the DSC of the complex gave no indication of free prodrug 7 and β-CyD.

5). X-ray powder diffraction studies showed different patterns for the prodrug/β-CyD physical mixture and the inclusion complex.

6). ^{1}H NMR confirmed inclusion complex formation and showed the β-CyD

(H3, H5) protons shifted change upfield upon the inclusion complex.

7). Prodrug/β-CyD inclusion complex binding constant, as measured by HPLC, was found to be 1.49 x 10^5 M^{-1}.

8). The stability of prodrug 7/β-CyD of the inclusion complex to cholesterol challenge showed that even at 10 times higher concentration of the cholesterol, the complex remains intact.

9). In the plasma inclusion complex (1.2 mM), approximately 60 % of the prodrug remains intact after 70 min, while at 6.2 mM, even after 5 h, 60 % of the prodrug still remains.

The prodrug 7/β-CyD complex has been characterized and preliminary results indicated that is successful completion of these goals. Future work will involve choosing a more realistic biological model. The project is now ready for radiolabelling prodrug 7/β-CyD complex and evaluation of the biodistribution of radiolabels.

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