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University of Alberta

Solubilization and the Pharmacokinetics of Flutamide with 2-Hydroxypropyl-β Cyclodextrin Preparations

by

Zhong Zuo 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement for the degree of Doctor of Philosophy

in

Pharmaceutical Sciences

Faculty of Pharmacy and Pharmaceutical Sciences

Edmonton, Alberta

Fall 1998

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UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the faculty of Gradate Studies and Research for acceptance, a thesis entitled ***SOLUBILIZATION AND THE PHARMACOKINETICS OF FLUTAMIDE WITH 2-HYDROXYPROPYL-β-CYCLODEXTRIN PREPARATION*** submitted by ***ZHONG ZUO*** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in Pharmaceutical Sciences

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Abstract

Flutamide is widely used for its antiandrogen effects in prostrate cancer chemotherapy. It is reported to act as a prodrug that is rapidly metabolized to 2-hydroxyflutamide (2-HY-FLT), which functionally inhibits the binding of testosterone and dihydrotestosterone (DHT) to nuclear receptors. Due to the low affinity of 2-HY-FLT for the androgen receptor, the blood concentration of 2-HY-FLT must be much higher than DHT concentration in order to obtain the total blockage of androgen receptor. Therefore, the bioavailability of flutamide must be increased so that high blood levels can be obtained from moderate doses. Very limited water solubility and poor wettability properties of flutamide contribute to its low absorption from commercially available tablets, and preclude intravenous (*i.v.*) loading.

Several cyclodextrins were used in this study to increase the water solubility of flutamide. The solubility of flutamide was increased the most by HPßCD (170 times in 50% (w/v) aqueous HPßCD). The HPßCD-flutamide complex was characterized by an A_L isotherm type. The thermodynamic parameters ($\Delta G°$ at 298 K = -3.48 kcal/mole, $\Delta H°$=2.85 kcal/mole, $\Delta S°$ at 298 K = 21.24 cal/K.mole) of the complex were determined. Differential scanning calorimetry (DSC) was used to confirm the formation of inclusion complexes. ^{19}F NMR and ^{1}H NMR were also used to estimate the stoichiometry of the complexes.

Sprague-Dawley rats were orally dosed with either the suspension or the HPβCD flutamide formulation. Formulation of flutamide with HPβCD produced a

significant increase in the AUC and $C_{max,}$ and resulted in a shorter T_{max} relative to the suspension. From the drug release data for the two dosage forms, it was found that flutamide was released 80 times faster from the HPβCD formulation than from the suspension. The improved bioavailability may be attributed to an increase in the dissolution rate of the HPβCD-flutamide complex. These results also demonstrate the potential to formulate an aqueous parenteral dosage form for flutamide through the use of HPβCD.

The Caco-2 cell monolayer model was used to rapidly assess the membrane permeation and metabolism of flutamide, to elucidate pathways of flutamide transport, and to assess formulation strategies. Results from these *in vitro* experiments indicated that the mechanism of flutamide transport is transcellular transport, and that flutamide release from its HPβCD complex is dependant more on the dilution effect than on its change in permeation.

This thesis is dedicated with all my love

to my

Son: Andy

Husband: Kebin

Father: Caijie Zuo

Mother: Late Jizhi Tan

Acknowledgements:

I wish to express sincerely thanks to my supervisor Dr. L. I Wiebe for his kind support over the course of this research project. Sincere thanks are also extended to Dr. G. Kwon, Dr. Y. K. Tam and Dr. J. Diakur for their guidance and critical comments at various stages of this project. Appreciation is also extended to Dr. B. Stevenson for his guidance in the cell culture work.

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Glossary of Abbreviations

αCD	alpha-cylodextrin
AUC	area under the plasma concentration-time curve
βCD	beta-cyclodextrin
CAB	combined androgen blockade
CD	cyclodextrin
CL_t	total clearance
CM-β-CD	carboxymethyl-β-cyclodextrin
CME-β-CD	carboxymethyl-ethyl-β-cyclodextrin
C_{max}	peak concentration
CPA	cyproterone acetate
DE-β-CD	diethyl-β-cyclodextrin
DHT	dihydrotestosterone
DM-β-CD	dimethyl-β-cyclodextrin
DMEM	Dulbecco's modified eagle's medium
DSC	differential scanning calorimetry
DTA	differential thermal analysis
FBS	fetal bovine serum
FLT	flutamide
γCD	gamma-cyclodextrin
G_1-β-CD	glucosyl-β-cyclodextrin

G_2-β-CD	maltosyl-β-cyclodextrin
g	gram
h	hour
HE-β-CD	hydroxyethyl-β-cyclodextrin
HPβCD	hydroxypropyl-β-cyclodextrin
HRE	hormone response element
HPLC	high performance liquid chromatography
2-HY-FLT	2-hydroxyflutamide
i.v.	intravenous
kg	kilogram
L	liter
LH	lutenizing hormone
LHRH	lutenizing hormone releasing hormone
MAB	maximal androgen blockade
M-β-CD	methyl-β-cyclodextrin
μg	microgram
μL	microliter
M	molar
mg	milligram
min	minute
mL	milliliter
mM	millimolar

n	number of observation
ng	nanogram
°C	degree celsius
P_{eff}	permeability coefficient
PEG	polyethyleneglycol
pH	negative logarithm (base 10) of the hydrogen ion concentration
po	per os
RM-β-CD	random methyl-β-cyclodextrin
rpm	revolutions per minute
SBE-β-CD	sulfobutylether-β-cyclodextrin
STDEV	standard deviation
TEER	transepithelial electrical resistance
TG	thermogravimetry
$T_{1/2}$	half-life
TLC	thin layer chromatography
T_{max}	time to maximal plasma concentration
UV	ultraviolet
Vd	volume of distribution
vs	versus

1. Introduction

1.1 Flutamide

1.1.1. Prostate cancer therapy

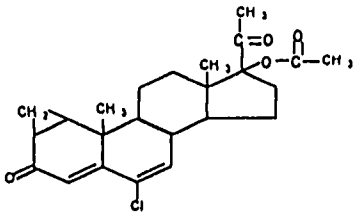
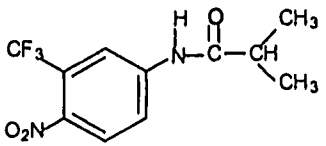
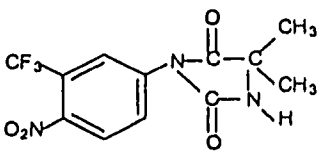
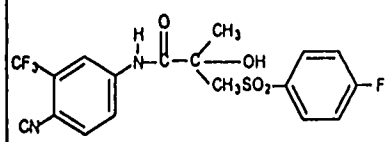
Prostate cancer has become one of the most common malignancies in the male population worldwide, and in many countries of the western world it now represents the most frequently, newly diagnosed cancer in men. It has been known since 1941 that prostate cancer is androgen dependent (Denis 1995). Since the pioneering work by Huggins and Hodges in 1941, the treatment of metastatic prostate cancer has been hormonal. Bilateral orchidectomy (surgical or medical) is still generally accepted as the "gold standard". Combined androgen blockade (CAB), where surgical or medical castration is combined with a steroidal or nonsteroidal antiandrogen, blocks both testicular and adrenal androgens. The steroidal antiandrogens, e.g. cyproterone acetate, which were first used in CAB have the disadvantage of progestational activity and undesirable side effects such as thromboembolism. Nonsteroidal antiandrogens, also termed 'pure' antiandrogens, lack this progestational activity and have much improved side effect profiles compared with steroidal antiandrogens. The nonsteroidal antiandrogens include bicalutamide (Casodex™, Zeneca Limited), flutamide (Eulexin™, Schering-Plough International) and nilutamide (Anandron™, Roussel) (Debruyne 1996). Their structures and properties are shown in Table 1.1.1.

There has been an unresolved controversy surrounding the benefits of maximal androgen blockage (MAB) as a valid approach for the treatment of non-curative prostate cancer since it was first proposed in 1945. Studies of MAB using medical castration (lutenizing hormone releasing hormone (LHRH) analogue plus antiandrogen) *vs.* LHRH analogues alone are inconclusive when viewed collectively, although the largest study revealed objective benefits for MAB. The remaining studies have insufficient power to show the expected effect size. MAB studies using surgical castration plus antiandrogens *vs.* surgical castration alone also gave inconsistent results, although a meta-analysis is in favor of MAB on objective criteria of response. Among the MAB trials using LHRH analogue *vs.* surgical castration alone, one is positive and the remaining two are neutral for MAB. No study to date have shown MAB to be worse than either medical or surgical castration alone. An overall meta-analysis has shown a trend for benefit with MAB, but this trend is not statistically significant. The existing data have strongly suggested that there may be a clear benefit for certain subgroups of patients (including those with minimal disease), however, the number of patients studied have been too small to allow valid conclusions. In the meantime one of the drawbacks to current MAB regimens is the exchange of modest clinical advantages for the side effects of nilutamide and flutamide. Given that the disease is noncurative, improved quality of life is the main goal of therapy, and excellent tolerability of treatment is fundamental to this. In a comparative trial, bicalutamide (Casodex) was shown to be more effective than flutamide (each in

combination with an LHRH analogue) in terms of time to treatment failure and produced a significantly lower incidence of diarrhea. In conclusion, the evidence supports early use adequate hormonal treatment, and this should mean either medical or surgical castration, ideally augmented by an antiandrogen. Tolerability of the antiandrogen is a key consideration in gaining an improvement in quality of life with MAB (Denis 1995).

Any potential benefit for MAB combinations should be weighed against the "price" of treatment in terms of the toxicity of treatment. For example, the steroidal antiandrogen, cyproterone acetate (CPA), has progestogenic and antigonadrotropic activity as well as an antiandrogen action, however, is associated with alterations in liver function (Barradell and Faulds 1994). In addition, the older nonsteroidal antiandrogens are often associated with side effects that could reduce the quality of life of patients undergoing treatment for prostate cancer. Flutamide use is associated with diarrhea (Delaere 1991; Macfarlane 1985; Sogani 1984) and may pose serious hepatotoxicity (Wysowski 1993), while nilutamide is associated with alcohol intolerance, defective light-dark adaptation and interstitial pneumonitis (Decensi *et al.* 1991). Clearly, there is room for improvement in the tolerability of antiandrogen therapy.

Table 1.1.1. Structures and properties of some anti-androgens (modified from Newling, 1996)

Name	Type and Characteristics	Structure	Clinical Properties	Disadvantage	Half-life (h)
Cyproterone acetate	Steroidal LH decreased Testosterone decreased		Effective as monotherapy Additional benefit in combination therapy × 3 daily dosage Few hot flushes	Decreased potency Decreased libido Gynaeocomastia Hepatic toxicity	8
Flutamide (Eulexin, Drogenil)	Non-steroidal LH increased		Effective monotherapy Effective in combination × 3 daily dosage is necessary. Potency spared libido unaffected	Nausea/vomiting Diarrhea Hepatic dysfunction Gynaecomastia ++ Lethargy	8
Nilutamide (Anandrone)	Non-steroidal LH increased Testosterone increased		Monotherapy not recommended Effective in combination therapy few clinical studies × 1 daily dosage potency spared libido unaffected	Nausea/vomiting Vesical problems Alcohol intolerance Rare pulmonary fibrosis Gynaecomastia	40
Bicalutamide (Casodex)	Non-steroidal LH increased Testosterone increased		Monotherapy not recommended Effective in combination therapy × 1 daily dosage potency spared libido unaffected	Gynaecomastia (hot flushes)	48

1.1.2. General properties of flutamide

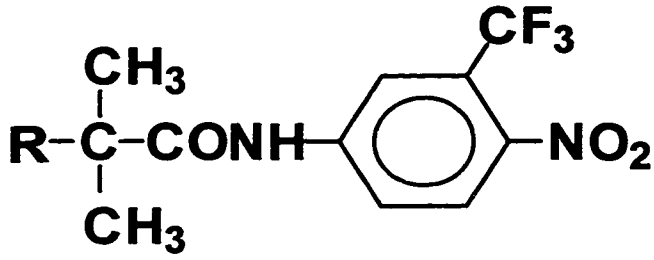


Figure 1.1.2. Structure of flutamide (R=H) and 2-hydroxyflutamide (R=OH).

Flutamide (FLT), α, α, α-trifluoro-2-methyl-4'-nitro-m-propionotoluidide, is a non-steroidal fluorine-containing nitrophenyl propanamide (Figure 1.1.2). It was first synthesized by Baker *et al.* in 1967. Flutamide has been marketed since February, 1989 in United States for treatment of patients with prostate cancer (stage D_2) (Wysowski and Fourcroy 1996). Since 1989, this antiandrogen has been available for managing metastatic carcinoma of the prostate, in combination with either a luteinizing hormone-releasing hormone analogue or orchiectomy (Brogden and Clissold 1989). Flutamide is used increasingly as part of total androgen ablation therapy, and in neoadjuvant treatment before radical prostatectomy (deKernion 1996). Detailed animal and *in vitro* studies have shown that flutamide, unlike the less specific progestin derivatives, is a potent antiandrogen that exerts no androgenic, esterogenic, progestational, glucocorticoid, or other hormonal or antihormonal activity. After oral administration, flutamide is well absorbed and displays high protein binding (94 to 96%) properties (Radwanski *et al.* 1989). At least 6 metabolites have been identified; the major one found in plasma is a biologically active alpha-hydroxylated

derivative (2-hydroxyflutamide). Flutamide (EUFLEX®, Drogenil®) is commercially available in two oral dosage forms, capsules and tablets, both with the same adult dose. Flutamide capsules are not commercially available in Canada and flutamide tablets are not commercially available in the U.S. Of the non-steroidal anti-androgens, flutamide is the only one presently recommended for monotherapy.

The currently recommended dose of flutamide is 250 mg p.o. given three times daily. Higher doses do not appear to produce a better therapeutic response and may be associated with a higher incidence and degree of gynecomastia (Airhart 1978). A bioequivalent sustained-release dosage form has been investigated in an effort to enhance patients' compliance by lowering the number of tablets to be taken daily and reduce the incidence of local side effects such as nausea and diarrhea (Asade 1991).

1.1.3. Mechanism of flutamide action

Androgens are produced by the testis, ovary, and adrenal glands. Testosterone, the most important androgen in males, is produced by the Leydig cells of the testis. It stimulates virilization and is an important spermatogenic hormone. Within the ovary, testosterone and androstenedione are precursor steroids for estradiol production. In both sexes, androgens stimulate body hair growth, positive nitrogen balance, bone growth, muscle development and erythropoiesis. The mechanism of action of testosterone at its target organs is similar to that of other steroid hormones. The major use of androgens in clinical medicine is for replacement therapy in men whose

production of testosterone is impaired, which is a relatively common condition. Testosterone synthesis inhibitors and antiandrogens are used to limit the effects of androgens in patients with androgen-dependent disorders, such as prostatic cancer, hirsutism and precocious puberty.

Circulating endogenous testosterone or exogenous testosterone derivatives are transported to the target tissues, where testosterone or the exogenous androgen derivatives enter the cells. Circulating testosterone is bound tightly to a serum glycoprotein of hepatic origin, called sex hormone binding globulin, and weakly to albumin. Together the free and albumin bound testosterone, which account for approximately 50% of the testosterone found in adult male serum, can enter target tissues. Once the androgens enter the target tissue cell, they may be enzymatically converted to metabolites which display greater or less androgen activity, or they may interact directly with androgen receptors. When testosterone, the prototype, as well as endogenous androgens enter target tissue such as the prostate gland, testosterone can be metabolized to 5α-dihydrotestosterone, but in other target tissues such as muscle and kidney, testosterone remains unchanged. Dihydrotestosterone binds with slightly higher affinity to the androgen receptor than does testosterone. Intracellular receptor binding of androgens and the postreceptor events are similar to those of other steroid hormones. Testosterone or dihydrotestosterone binds to a hormone-binding site near the carboxy terminus of the receptor, thereby activating the receptor complex so it can bind to a nuclear DNA acceptor site, known as the hormone response element (HRE).

Binding of the receptor to DNA-binding is followed by the transcription of mRNAs for tissue-specific proteins, which constitutes the hormonal response (Figure 1.1.3. from Wingard *et al.* 1991).

The mechanism of action of antiandrogens is to block the synthesis of endogenous testosterone. Flutamide appears to exert its antiandrogenic effects on male secondary sex structures by inhibition of androgen uptake and/or inhibition of nuclear binding of androgens in target tissue where it appears to form inactive complexes with nuclear androgen receptors. In addition, flutamide reduces the rate of DNA synthesis in the prostate of rats to a greater degree than other steroidal antiandrogens (Neri 1989).

The non-steroidal antiandrogens block the attachment of androgens to receptors, not only in the prostate gland, but also in the hypothalamic pituitary axis. This blocks the negative feedback effect of the serum testosterone level, leading to an increased production of LHRH, Luteinizing Hormone (LH) and subsequently, testosterone. On the other hand, the steroidal anti-androgens, because of their progestogen-like structure, decrease the production of LH by the pituitary and therefore have an additional effect in diminishing the production and activity of testosterone. Because of their failure to block the production of LH by the pituitary, the non-steroidal anti-androgens may adversely influence the development of prostate cancer. A higher dose of flutamide is expected to be required to counteract such elevated levels of serum androgens in intact male rats (Simard *et al.* 1997).

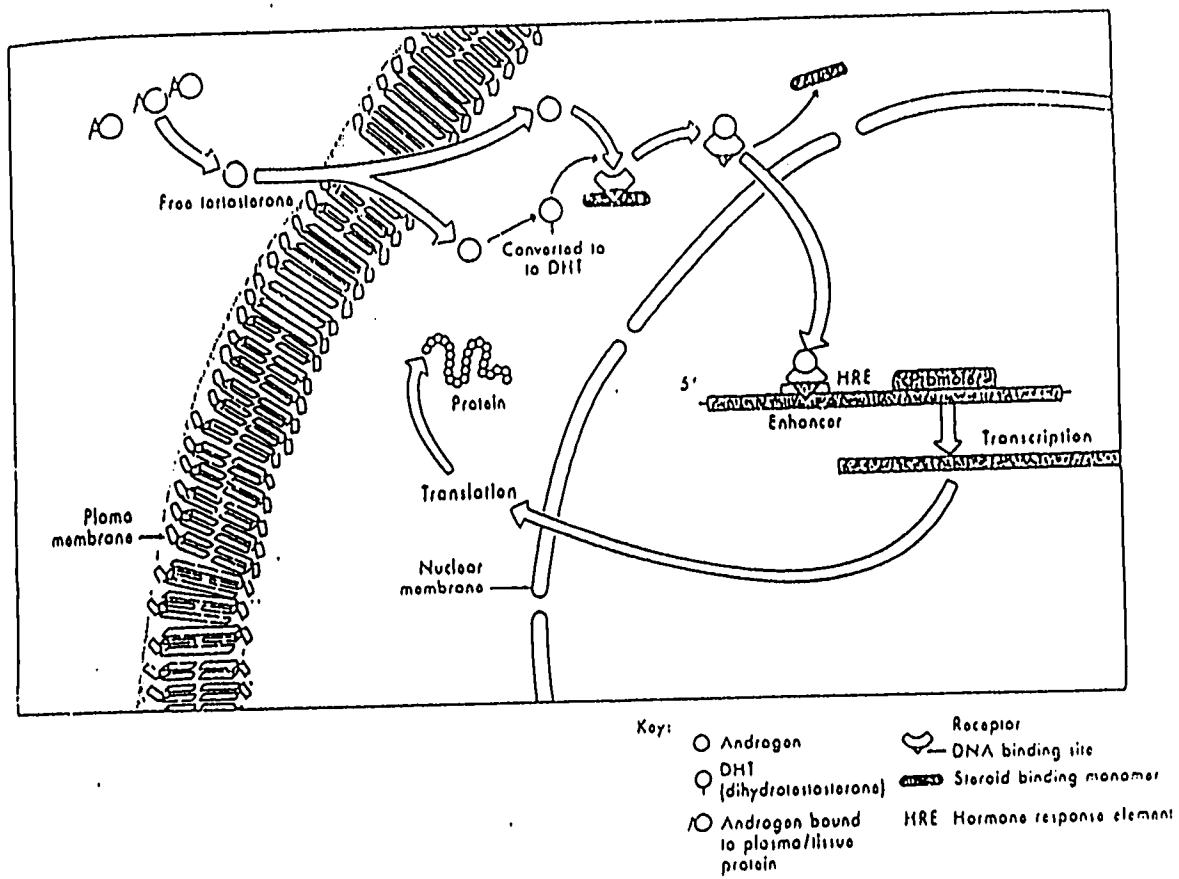


Figure 1.1.3. Schematic diagram of androgen action at target cells.

The ideal antiandrogen should act exclusively as an inhibitor of androgen action at the receptor level and be devoid of any other hormonal or activity. Among all of the classes of compounds, the only antagonist that meets these criteria to date is the non-steroidal antiandrogen flutamide (Marchetti and Labrie 1988; Neri 1972; Poyet and Labrie 1985).

The advantage of flutamide over the other antiandrogens is that sexual potency is preserved and in combination with an "LHRH agonist", it is the first line agent for previously untreated patients with prostate cancer. Libido and sexual potency are maintained in 80-100% of patients treated with the flutamide alone and thus they are particularly useful for patients who wish to remain sexually active (Lund and Rasmussen 1988).

The side effects of flutamide include cardiovascular, CNS (headache, dizziness), endocrine/metabolic, gynecomastia and gastrointestinal (nausea, vomiting, increased appetite) symptoms. In all of the studies of monotherapy using flutamide, gastrointestinal and hepatic disturbances as well as gynaecomastia have been reported. Diarrhea sufficiently debilitating to cause withdrawal of treatment occurs in 5-8% of patients, and nausea and anorexia in 10-12% (Newling 1996). The hepatotoxicity has been discussed and reviewed by Gomez (1992), and by Wysowski and Fourcroy (1996). These authors estimate the incidence of flutamide hepatotoxicity to be in the range of 1 to 10%. Serial blood aminotransferase levels should be monitored during the first few months of flutamide treatment (Wysowski and Fourcroy 1996).

Flutamide does not appreciably influence the conversion of testosterone to dihydrotestosterone or to other metabolic products. Its hydroxylated metabolite, 2-hydroxyflutamide (2-HY-FLT), is active and can reach much higher plasma concentrations than the parent drug. The affinity constant of androgens, testosterone and dihydrotestosterone, for the androgen receptor is approximately 1.27 nM and 0.2 nM, respectively (Simard *et al.* 1986). However, a much lower affinity (0.134 μM & 3.4 μM) is observed for 2-HY-FLT and FLT binding, respectively. Approximately 50% inhibition of 1 nM [^{3}H] testosterone uptake achieved with 50 nM 2-HY-FLT and complete inhibition is obtained at a 5 μM. Knowledge of the relative affinity of dihydrotestosterone, testosterone, and 2-HY-FLT indicates that higher concentration of antiandrogen is required in order to efficiently prevent the access of androgens to the receptor. In order to have androgen receptors occupied by 2-HY-FLT instead of dihydrotestosterone in primary prostatic cells as well as in metastatic cells, a concentration of the antiandrogen 500-1000-fold higher than the concentration of the androgen has to be reached in the circulation (Belanger *et al.* 1988). Such calculations show that administration of the antiandrogen alone is clearly insufficient and leaves an important fraction of androgens bound to the receptor. Consequently, complete blockage of testicular androgens is required in order to facilitate a more complete interaction of 2-HY-FLT with the androgen receptor and thus permit its optimal action on androgen-dependent processes, especially growth of prostatic carcinoma.

1.1.4. Metabolism and pharmacokinetics of flutamide

After oral administration, flutamide is absorbed from the GI tract and is mainly metabolized to 2-HY-FLT during first passage through the liver. Hydroxylation at this position is an uncommon metabolic process since hydroxylation normally occurs at the primary carbon atom or on the aromatic ring. The final step in the elimination of flutamide is glucuronidation after hydroxylation of the aromatic ring. The metabolism of flutamide in man is shown in Figure 1.1.4. (Katchen and Buxbaum 1975).

The pharmacokinetic parameters for flutamide in rat and man are listed in Table 1.1.4. Plasma half-life of both flutamide and 2-HY-FLT in man is 5-6 h. Excretion of intact drug and metabolites is via the kidney with approximately 28% of the dose is excreted in the urine within 24 h. During long-term administration of the standard dose of 250 mg every 8 h, steady-state levels of 2-HY-FLT are maintained up to at least 18 months, thus indicating minimal changes in the metabolism of the drug during this period (Sogani *et al.* 1984). In male patients, flutamide is reported to have 48.5% of the dose excreted in 0-72 h urine and 4.2% in 0-72 h feces (Katchen and Buxbaum, 1975).

The minimum effective antiandrogen dose is 5 mg/kg body weight orally for rats (Neri 1989). A recent study of flutamide in rats confirmed that the drug is toxic to rat hepatocytes as a result of the cytochrome P450 mediated formation of metabolites (Fau 1994). Contrary to nilutamide, flutamide was not reduced detectably to a nitro anion free radical by rat liver microsomes (NADPH-cytochrome P450 reductase).

Instead, flutamide was oxidatively metabolized by microsomal cytochrome P-450, particularly cytochrome P-450 of the 3A and 1A subfamilies, into chemically reactive metabolites responsible for covalent binding to microsomal proteins (Berson 1992).

In conclusion, a primary concern is that a partial blockage of the androgen receptor by the antiandrogen can induce growth of tumor cells (Labrie *et al.* 1985). In view of the relatively low affinity of FLT and 2-HY-FLT for the androgen receptor, it is extremely important that a relatively high level of FLT and 2-HY-FLT be maintained in the plasma.

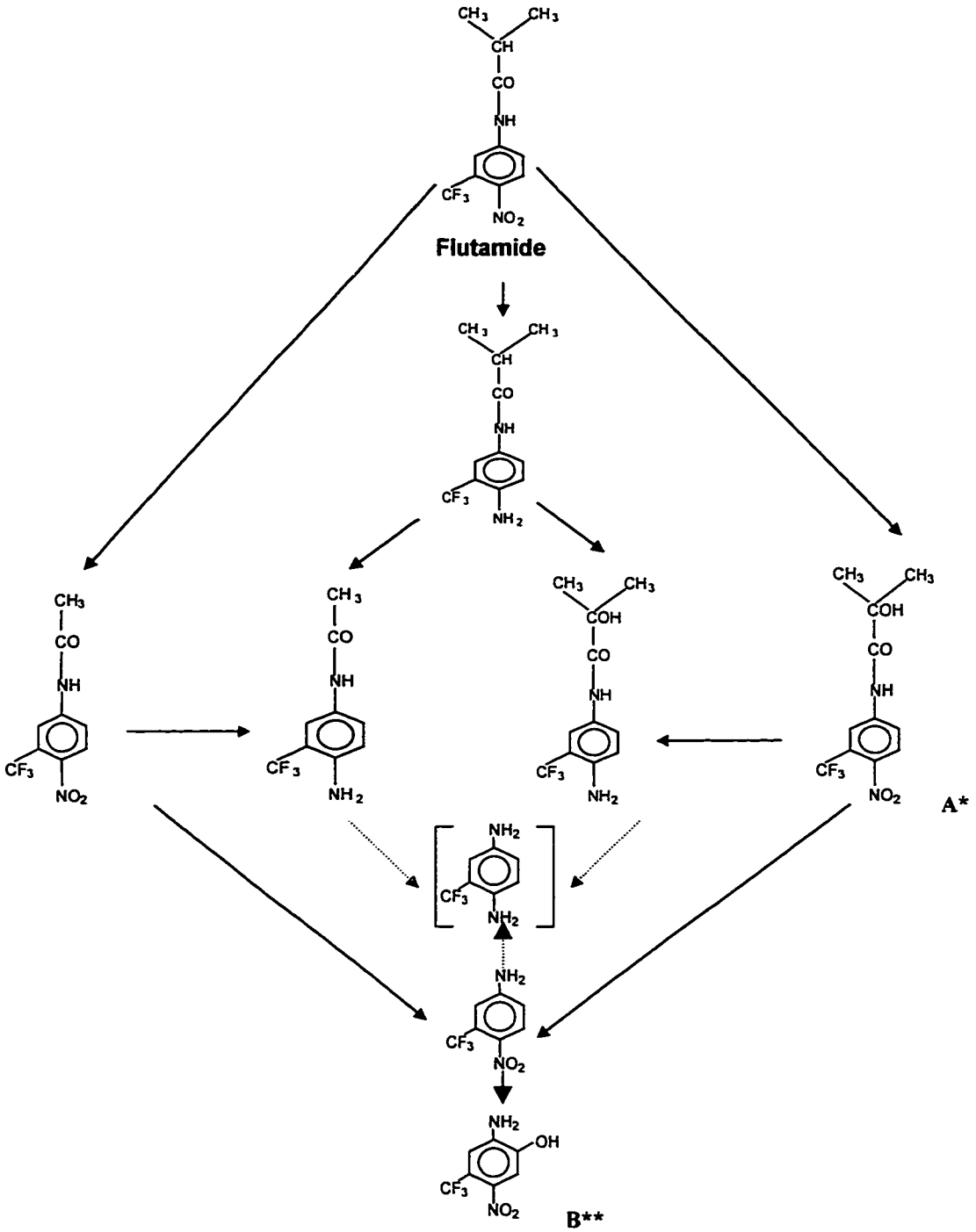


Figure 1.1.4. Flutamide metabolism in men (Katchen and Buxbaum 1975). A* is the major plasma metabolite, B is the major urine metabolite.**

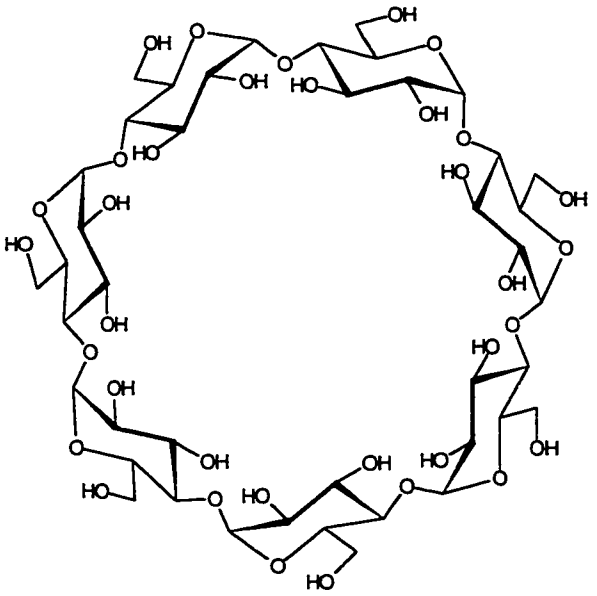
Table 1.1.4. Summary of flutamide pharmacokinetics

DOSE	SPECIES	FLUTAMIDE	2-HY-FLT	ANALYSIS METHOD	REFERENCE
250mg	Human	T_{max} = 1.9 h C_{max} = 25.3 ng/mL AUC = 63 ng.h/mL t1/2 (α) = 0.8 h t1/2 (β) =7.8 h	T_{max} = 2.7 h C_{max} = 894 ng/mL AUC = 3645 ng.h/mL t1/2(α) = 1.7 h t1/2 (β) =8.1 h	GLC ; Detection limit is 2 ng/mL	Radwanski *et al.* 1989
200mg	Human	48.5% dose excreted in 0-72 h urine; 4.2% dose in 0-72 h feces		Radioactive	Katchen and Buebaum 1975
50mg /day	Rat	T_{max} = 4 ~6 h 5% of dose recovered in feces	T_{max} = 4~ 6h	Radioactive	Neri 1989
250mg	Human	T_{max} = 2 h C_{max} = 20 ng/mL	C_{max} = 1300 ng/mL AUC = 11400 ng.h/mL. t1/2 (β) = 4.3~ 6.6 h C_{ss} = 940 ng/mL	GC; Detection limit is 10 ng/mL	Schulz 1988
500mg	Human	T_{max} = 2.3±1.5 h C_{max} = 100±60 ng/mL	T_{max} = 3.3±1.2 h t1/2 (β) = 8.5~21.9 h C_{max} = 2400±700 ng/mL AUC = 24300 ng.h/mL	GC; Detection limit is 10 ng/mL	Schulz 1988

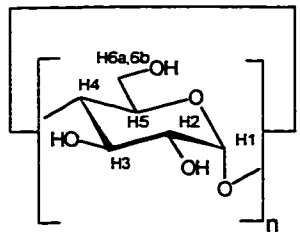
1.2. Cyclodextrins

1.2.1. General description of cyclodextrins

Cyclodextrins (CDs) are cyclic oligosaccharides composed of α(1-4) linked glycopyranose units of six, seven or eight glucose residues, designated α, β and γ, respectively (Figure 1.2.1.). Cyclodextrins have an overall geometric shape of a torus or truncated cone. The two secondary hydroxyl groups at carbons 2 and 3 are on one face of the cone and a primary hydroxyl group at carbon 6 is on the opposite face. Thus, the external surface of cyclodextrins is hydrophilic while the interior is relatively hydrophobic and includes the H_3 and H_5 hydrogens and ether-like oxygens (McCormack and Gregoriadis, 1994).



β-Cyclodextrin



n=6 α-Cyclodextrin
n=7 β-Cyclodextrin
n=8 γ-Cyclodextrin

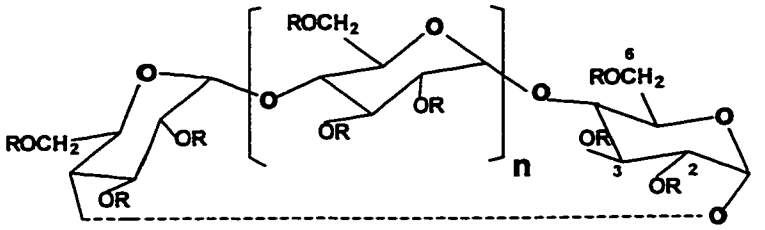
Figure 1.2.1. Structures of α, β and γ-cyclodextrin.

1.2.2. Derivatives of cyclodextrin

Although βCD has a large potential for application in drug formulation, its use is limited by its poor aqueous solubility (1.8 g/100 mL at 298 K). Recently, chemically modified cyclodextrins have received considerable attention because their physiochemical and inclusion properties are different from those of the natural cyclodextrins. Since the low solubility of βCD is mainly due to intra-molecular hydrogen bonding, various modifications have been performed incorporating alkyl and aromatic substituents at the primary and secondary hydroxyl groups. Muller *et al.* (1986) studied the influence of the average degree of substitution on complexing ability and surface activity. They found that a low degree of substitution is preferable, since these derivatives show the best complexing properties and, at the same time, low surface activities. Partial alkylation of the hydroxyl groups of βCD results in enhanced water solubility, and such derivatives have been found to be very effective in binding guest molecules. Unfortunately, some cyclodextrins are nephrotoxic, precluding their application to parenteral formulations (Frank 1976). One potentially useful class of these cyclic starches, the hydroxyalkylated-β-cyclodextrins, appears to lack the toxic properties of unsubstituted or alkylated cyclodextrins (Brewser *et al.* 1989). Compared to the methylated βCD derivatives, hydroxypropyl-βCD and hydroxypropylmethyl-βCD, show reduced hemolytic activity. 2-Hydroxypropyl-βCD is obtained by treating a base-solubilized solution of βCD with propylene oxide, resulting in an isomeric system that has an aqueous solubility well in excess of 60% (w/v) (Pitha *et al.* 1986).

U. S. Pharmacopoeia XXII already has a monograph for βCD. The monograph for HPβCD is in preparation for the U.S. Pharmacopoeia, and versions have already appeared in such compendial sources as the Handbook of Pharmaceutical Excipients (Wade and Weller 1994). 2-Hydroxypropyl-βCD has been used as a parenteral (*i.v.*) excipient for drugs in both preliminary and advanced human clinical trials, and the first such formulation is expected to garner regulatory approval within the next year or so. Sulfobutyl ether βCD [$(SBE)_{7m}$-β-CD, average degree of sulfobutyl substitution is 7] has also been found to be safe when administered parenterally, with doses greater than 10 g/kg causing no toxic effects in mice (Rajewski *et al.* 1995). Antennae-bearing CDs are the other widely-used derivatives for targeted drug delivery. The receptor specific oligosaccharide branches linked to the CD will be bound selectively to the target-organ cells. Additionally, duplex homo- or heterodimer CDs are now used for photodynamic tumor therapy (Szejtli 1997). Overall, there are more than 1500 CD derivatives published and the commonly used derivatized CDs are summarized in Table 1.2.2 (modified from Stella and Rajewski 1997). Recently, a fluorine-substituted cyclodextrin, JD1-128 CD has been synthesized (Synthesis and drug complexation studies of βCD fluorinated on the primary face. J. Diakur, Z, Zuo and L. I. Wiebe. Manuscript submitted). Fluorinated derivatives of βCD would be expected to have useful drug carrier properties, depending on the physicochemical properties of the result of fluorine-containing derivatives.

Table 1.2.2. Commonly used derivatized CDs



Cyclodextrin	Abbreviation	R	n
Carboxymethyl-β-cyclodextrin	CM-β-CD	CH_2CO_2H or H	5
Carboxymethyl-ethyl-β-cyclodextrin	CME-β-CD	CH_2CO_2H, CH_2CH_3 or H	5
diethyl-β-cyclodextrin	DE-β-CD	CH_2CH_3 or H	5
Dimethyl-β-cyclodextrin	DM-β-CD	CH_3 or H	5
methyl-β-cyclodextrin	M-β-CD	CH_3 or H	5
random methyl-β-cyclodextrin	RM-β-CD	CH_3 or H	5
glucosyl-β-cyclodextrin	G_1-β-CD	glucosyl or H	5
Maltosyl-β-cyclodextrin	G_2-β-CD	maltosyl or H	5
Hydroxyethyl-β-cyclodextrin	HE-β-CD	CH_2CH_2OH or H,	5
Hydroxypropyl-β-cyclodextrin	HPβCD	$CH_2CHOHCH_3$ or H,	5
Sulfobutylether-β-cyclodextrin	SBE-β-CD	$(CH_2)_4SO_3Na$ or H	5

Note: derivatives may have different degrees of substitution on the 2, 3 and 6 positions.

1.2.3. Review and applications of CDs

The cyclodextrin story began over a century ago. This class of carbohydrates was first isolated by Villiers in 1891 (Lasagna 1993) and in 1903, α, β and γ CDs were identified by Schardinger. Between 1911 and 1935, a leading researcher Pringsheim showed that these sugars formed stable aqueous complexes with many other chemicals (Straighten 1990). The first patent on drug/complexes was issued to Freudenberg in 1953. Since the publication of an alleged high toxicity of orally administered βCD appeared in 1957 (Szejtli 1997), the development of CDs for pharmaceutical application came to a stand still. The industrial interest began again in the 1970s. By the mid 1970's, each CD had been structurally and chemically characterized. From then on, the complexing properties of CD have been extensively investigated. It has been estimated that by 1993, over 2400 publications on medical aspects of CDs had appeared. To date, there are around 513 CDs related patents in U.S.A. alone, which cover applications for foods, cosmetics, medicines and others. CDs are produced in thousand ton quantities, while CD-derivatives are produced at the hundred ton level. Industrially available CDs include : Dimethyl-βCD (DIMEB), Random methyl-βCD (RAMEM), HPβCD, SBE-βCD, acetyl-γCD, chlorotrianzinyl-βCD, and glucosyl & maltosyl βCD. At the same time, the CD-utilizing technologies are continuously increasing.

1.2.4. Metabolism and pharmacokinetics of CDs:

Andersen *et al.* (1963) fed ^{14}C-labeled potato starch, α and βCD to rats and measured the radioactivity of exhaled carbon dioxide as a function of time. They also determined the radioactivity in urine, in the contents of the intestines and in various organs. These authors concluded that cyclodextrins are not metabolized as rapidly as starch. Since the cyclic nature of CDs renders them less susceptible to attack by enzymes such as α-1,4-glucanohydrase which hydrolyze terminal sugars. This conclusion was verified by Szejtli (1980), who fed βCD and glucose, both uniformly labeled with ^{14}C, to rats and measured the radioactivity levels of the blood. These studies suggested that rats metabolize cyclodextrins, but they could not exclude that the first step of this metabolism is performed by the intestinal flora.

The pharmacokinetic behavior of ^{14}C-βCD administered orally to rats was described by Gerloczy *et al.* (1985). These authors investigated the absorption, distribution, excretion and metabolism of orally administered ^{14}C-βCD in rats. No specific accumulation in the various organs was observed after ^{14}C-βCD treatment. It was assumed that βCD is metabolized in rats more slowly but qualitatively similarly to glucose, therefore *p.o.* administered βCD is unlikely to induce toxic symptoms. It can not be excluded that a very small amount of βCD is absorbed as the intact molecule. These results suggested that the rate determining step in βCD absorption is the enzymatic hydrolysis of βCD to linear dextrins, which thereafter are rapidly hydrolyzed to maltose and glucose. In this case, βCD would not be toxic, as already

proven by 6-month chronic toxicity studies in rats and dogs. However, radioactivity measurements alone do not reveal whether the intact βCD molecule enters the blood or whether only some of the metabolites formed by enzymatic hydrolysis of βCD are absorbed. These investigations did not differentiate between the parent cyclodextrin and metabolites formed by metabolism of cyclodextrins by microorganisms in the colon. Uekama (1979) conducted *in situ* absorption experiments which proved that a small amount of βCD is absorbed from the gastrointestinal tract.

Nevertheless, the mechanism of gastrointestinal absorption of the complexes is still uncertain. Generally, it has been assumed that only free drug coexisting with the drug-βCD complex at equilibrium in gastrointestinal fluids is absorbed upon oral administration of the complex. Koizumi *et al.* (1985) did an *in vitro* absorption experiment using rat everted intestinal sacs. βCD was detected in serosal fluid, and hence it was suggested that βCD may be absorbed from the gastrointestine in the form of a complex or in the intact form. Although the rate of appearance of βCD in mesenteric blood, *in situ*, is slower than that of phenobarbital and the cumulative amount of βCD absorbed is smaller, βCD can undoubtedly be absorbed from the rat intestinal lumen.

Oral administration: It is generally recognized that the gastrointestinal absorption of CDs in an intact form is limited due to their bulky and hydrophilic nature. CDs may act as drug carriers that deliver the drug through an aqueous milieu to the absorption site and remain in the gastrointestinal tract. Only an insignificant

amount of intact βCD has proven to be absorbed from the gastrointestinal tract in rats (Gerloczy *et al.* 1985). The fate of the parent CDs in the gastrointestinal tract depends upon its resistance to hydrolysis and enzymatic degradation. The transit time through the human colon is ~ 40 h, which is adequate time for bacterial enzymes to completely hydrolyze the parent CDs. Since modified CDs may be more resistant to the intestinal hydrolases than the parent CDs upon oral administration, their absorption is minimal and the CD carrier is primarily excreted intact in the feces.

Parenteral administration: Intravenously administered CDs disappear rapidly from systemic circulation and are excreted mainly via the kidney. The steady-state volume of distribution (V_{dss}) for βCD and HPβCD in rat, rabbits, dogs and humans corresponds well with the extracellular fluid volume of each species, suggesting that no deep compartments or storage in pools are involved. The total plasma clearances (Cl_t) for HPβCD in all the species tested are similar to that of inulin, a polysaccharide known to be rapidly distributed in extracellular fluid and then excreted at the rate of glomerular filtration. Furthermore, the renal clearance of HPβCD in humans (110-130 mL/min) is independent of the dose administered and nearly equivalent to the reported glomerular filtration rate (125 mL/min). The elimination of CDs is strongly dependent on renal function, thus nonlinear pharmacokinetics for βCD at higher doses (>200 mg/kg) in rats can be explained by the untoward effects of βCD on the kidney (Davies and Morris 1993).

1.2.5. Toxicity of CDs:

Cellular interactions: CDs are known to induce membrane shape changes in human erythrocytes and, at higher concentrations, induce lysis (Irie *et al.* 1982; Ohtani *et al.* 1989). The hemolytic activity of the parent CDs is reported to be in the order βCD > αCD > γCD (Miyazawa *et al.* 1995).

Parenteral safety: The most critical drawback of the parent CDs for parenteral use is their toxic effects on the kidneys, which concentrate CDs in the proximal convoluted tubule after glomerular filtration. The kidney is the main organ involved in clearance of CDs from the blood. Chemical modifications have been made to CDs to increase their hydrophilicity with the hope that the improved solubility would eliminate the renal toxicity. Of the CD derivatives discussed so far, HPβCD has been most extensively studied with regard to the parenteral safety in several types of animals and in humans. An overview of safety profiles of HPβCD administered parenterally to different species is listed in Table 1.2.5. (modified from Irie and Uekama 1997).

Oral safety: All toxicity studies have demonstrated that orally administered CDs are practically nontoxic, because of lack of absorption through the gastrointestinal tract. Szejtli reported in 1982 that the acute LD_{50} value for βCD is 12500 mg/kg in mice, 12000 mg/kg in rats, above 5000 mg/kg in dogs.

Table 1.2.5. Overview of safety profiles of HPβCD administered parenterally

Species	Route of Administration	Dose	Toxicity	Reference
Mouse	i. p. i. c. (intracerebral)	10 g/kg 400 μg	no mortality no necrosis at the injection site	Pitha *et al.* 1988 Pitha *et al.* 1988
Rat	i.v. (acute) i.v. (subacute) i.v. (subchronic) i.v. (subchronic) i.v. (teratogenicity and embroyotoxicity) i. m. (acute)	2 g/kg 5 g/kg daily for 7 days 200 mg/kg every second day for 90 days 50, 100, or 400 mg/kg daily for 90 days 50-400 mg/kg from day 6 to day 18 of pregnancy 5-40% (w/v) solutions	no toxicity no toxicity no toxicity no toxicity at 50 mg/kg, minimal effects at higher doses no toxicity no or minimal toxicity	Brewster *et al.* 1991 Brewster *et al.* 1991 Brewster *et al.* 1990 Coussement *et al.* 1990 Coussement *et al.* 1990 Brewster 1991
Rabbit	i.v. (teratogenicity and embroyotoxicity) i.v.(subchronic) i.m. (acute)	50-400 mg/kg from day 6 to day 18 of pregnancy 1 g/kg twice a week for 80 days or 2 g/kg daily for 20 days 1 mL of 1-10% (w/v) solutions	no toxicity reversible vacuolation of renal tubular cells no or minimal toxicity	Coussement *et al.* 1990 Irie *et al.* 1992 Yoshida *et al.* 1988
Dog	i.v. (subchronic) i.v. (subchronic)	50, 100, or 400 mg/kg daily for 90 days 700-1000 mg/kg daily for 90 days	no toxicity at 50 and 100 mg/kg, minimal effects at 400 mg/kg; reversible vacuolation of renal tubular cells;	Coussement *et al.* 1990 Strattan 1992
Monkey	i.v. (subacute) i.v. (acute)	200 mg/kg every second day for 91 days 10 g/kg	no toxicity no mortality	Brewster *et al.* 1990 Brewster *et al.*1990
Human	i.v.(acute) i.v.(acute)	infusion of 5% (w/v) solution at a rate of 470 mg/kg/day, total dose of 30g over 4 days infusion at a rate of 100 mg/min, total doses of 0.5-3 g	no adverse effects no adverse effects	Carpenter *et al.* 1987 & Carpenter *et al.* 1995 Szathmary *et al.* 1990 & Seiler *et al.* 1990

1.3. Cyclodextrin inclusion complexes

1.3.1. Definition

Inclusion complexes are entities comprising two or more molecules, in which one of the molecules, the "host" includes, totally or in part, only by physical forces, a "guest" molecule. Cyclodextrins are typical "host" molecules that may trap a great variety of "guest" molecules having the size of one or two benzene rings, or even larger guests carrying a side chain of comparable size, to form inclusion complexes.

Molecules or functional groups of molecules having molecular dimensions that match the cyclodextrin cavity, that are less hydrophilic than water, can be induced into the cyclodextrin cavity in the presence of water. In aqueous solution the slightly apolar cyclodextrin cavity is occupied by water molecules that are energetically unfavoured (polar-apolar interaction) and are therefore readily substituted by appropriate "guest molecules" that are less polar than water.

The dissolved cyclodextrin is the "host" molecule, and the "driving force" for the formation of the complex is the substitution of the high-enthalpy water molecules by an appropriate "guest" molecule. One, two or three cyclodextrin molecules may contain one or more entrapped "guest" molecules; this is the essence of the "molecular encapsulation". The driving force involved in the inclusion complex formation include van der Waals interactions (Nishijo and Nagai 1991), hydrogen bonding (Tong *et al.* 1991; Jones *et al.* 1984), hydrophobic interactions (Nishijo and Nagai 1991; Tabushi

et al. 1978), release of ring strain in the cyclodextrin molecule (Jones *et al.* 1984), and change in solvent-surface tension (Orstan and Ross 1987).

1.3.2. Methods of inclusion complex formation

As concluded by Pagington in 1987, the common procedure to prepare inclusion complexes is to stir or shake an aqueous solution of cyclodextrin (cold or warm, neutral or acidic) with the guest molecule or its solution. This may be carried out using a common solvent, different but miscible solvents, different immiscible solvents, or no solvent at all. The guest molecule is generally added to a warm cyclodextrin solution. Equilibrium is reached with intense stirring and under slow cooling in a few hours. At this point, guest molecule content cannot be increased by repetition of the process, i.e. by reheating and cooling. After having attained equilibrium, water can be removed by freeze-drying, spray-drying or by any other suitable method, or the mother liquor can be separated by filtration.

Another method for the preparation of complexes is the so-called kneading method. In this case the cyclodextrin is not dissolved; it is kneaded with a small amount of water to which the guest component has been added without a solvent. Since the cyclodextrin-water complex is energetically less favored than the inclusion complex to be formed with the guest molecule, and since the crystal lattice of the CD-drug is different from that of the cyclodextrin-water complex, the reaction goes to completion.

The most common method for preparation of drug-cyclodextrin complexes in the laboratory is to add an excess of the drug to an aqueous cyclodextrin solution. The suspension formed is then agitated for up to 1 week at room temperature, and is then filtered or centrifuged to form a clear drug-cyclodextrin complex solution. It is possible to shorten this process by formation of supersaturated solutions through sonication followed by precipitation at the desired temperature.

For preparation of solid formulations of the drug-cyclodextrin complex, water is removed from the aqueous drug-cyclodextrin complex solution by evaporation in a rotary evaporator, in a spray dryer or by lyophilisation. Frequently, the efficiency of complexation is not very high and therefore relatively large amounts of cyclodextrins must be used to complex small amounts of the drug. Vehicle additives such as sodium chloride, buffer salts, surfactants, preservatives and organic solvents may reduce the efficiency.

1.3.3. Determination of inclusion complex formation

Methods for analyzing the formed inclusion complexes include NMR, IR, circular dichroism, fluorescence spectroscopy, chromatography and X-ray diffraction, coupled with Differential Scanning Calorimetry (DSC), Differential Thermal analysis (DTA) or Thermogravimetry (TG).

In the case of liquid guest molecules, X-ray powder diffraction is the most useful method for the detection of inclusion complex formation. If the diffractogram

differs significantly from that of uncomplexed cyclodextrin, the formation of a crystal lattice of a new type, i.e. the CD-drug complex, can be established, since the liquid guest molecules produce no diffraction patterns at all (Szejtli 1982).

Complex formation may be proven in some cases by IR spectroscopy (Szejtli 1982), but in general this method is of limited use in the investigation of cyclodextrin inclusion complexes. The overwhelming parts of the characteristic bands of a cyclodextrin complex are hardly influenced by complex formation. Bands due to the included part of the guest molecule are generally shifted or their intensities are altered, but since the mass of the guest molecule does not exceed 5-15% of the mass of the complex, these alterations are usually obscured by the spectrum of the host. For similar reasons, no useful results can be obtained in the far infrared region either.

Thin-layer chromatography may also be useful for the verification of complex formation, since this process alters the R_f values considerably (Szejtli 1982). They are usually strongly diminished, provided that the complex is sufficiently stable in the solvent mixture used. The R_f value obtained with a mechanical mixture is between the R_f of the pure guest molecule and that of the complex.

Single crystal x-ray structure analysis is the best method for detecting complex formation. The precise geometrical relationships between the guest and host molecules can be established and interactions can be identified. However, the technique is too complicated for routine use. Several common laboratory methods will be discussed in the following paragraphs.

1.3.3.1. Differential scanning calorimetery

In certain cases, cyclodextrin complex formation can be confirmed by DSC. Kurozumi *et al.* (1975) have reported that freeze-dried mefenamic acid gives an endothermic peak at 232 °C; its mechanical mixture with freeze-dried βCD gives the same peak at the same temperature, but the βCD inclusion complex prepared by freeze-drying or crystallization from water does not give any peak.

The DSC curve of βCD shows that most of the 13.4% water content escapes below 100 °C, then there is no change until 270 °C. Above 270 °C the exothermic decomposition of the cyclodextrin starts, then at 300 °C a sharp endothermic process occurs, which indicates the melting of βCD accompanied by decomposition.

1.3.3.2. Changes in the optical spectra of guest molecules

The effect of cyclodextrins on the visible range absorption spectra of colored molecules was first studied by Cramer in 1951. The high electron density prevailing inside the cyclodextrin cavity mobilizes the electrons of the incorporated molecule. This is manifested in spectral changes, as well as in abnormal pH values for the color change of indicators in the presence of cyclodextrins.

Fluorescence enhancements have been reported for some molecules in the presence of cyclodextrin. Cramer *et al.* (1967) observed that 1-anilinonaphthalene-8-sulphonate, which is strongly fluorescent in organic solvents but shows only negligible fluorescence in water, becomes significantly fluorescent in aqueous CD solutions.

αCD enhances the fluorescence of the aqueous solution about 2-fold, whereas βCD has a nearly 10-fold effect.

UV spectroscopic changes (Szejtli 1982): sometimes complex formation with cyclodextrin alters the original UV absorption spectrum of the molecule; usually a bathochromic shift and/or band broadening occurs. The shift of the UV absorption maximum upon complex formation may be explained by a partial shielding of the excitable electrons in the cyclodextrin cavity. The dissociation constant of the complex can be determined from the spectroscopic changes using the Benishei-Hildebrand equation.

Changes in the circular dichroism of the guest component on addition of CD can also be used to exploit the formation of inclusion complex (Nakajima *et al.* 1984).

1.3.3.3. ^{1}H spectroscopy

The NMR investigation of the complexes of unsubstituted cyclodextrins is technically difficult due to the low solubility of the complexes in D_2O. With many complexes, sufficiently high concentration can not be achieved in water, and in organic solvents the complexes may decompose, i.e. the cyclodextrin complex of the solvent may be formed. The majority of results obtained so far refer to the complexes of guest molecules that are relatively soluble in D_2O.

Thakkar and Demarco (1971) investigated cyclodextrin inclusion complex formation by ^{1}H-NMR spectrometry. Previously only the X-ray diffraction analyses of

certain crystalline complexes had proved that the incorporated molecule was really located in the interior of cyclodextrin. The basic idea of the verification is that if the guest molecule is accommodated in the cyclodextrin cavity, the hydrogen atoms located in the interior of the cavity (C_3-H and C_5-H) will be considerably affected by the guest, whereas the hydrogen atoms on the outer surface (C_2-H, C_4-H and C_6-H) will not be affected by the inclusion complex formation. The complex formation constant can be calculated from the observed change in chemical shift values.

It has been noted that the main difficulty in the NMR investigations of βCD inclusion complexes is their low solubility in D_2O. This difficulty has been surmounted by the preparation of heptakis-(2,6-di-O-methyl)-βCD. This compound and its inclusion complexes are readily soluble in D_2O, so that analyzable spectra are attainable.

1.3.3.4. ^{19}F NMR

Fluorine-19 has been used as a chemical probe for structure elucidation by NMR for many years. The nucleus has a spin of 1/2 and has a 100% natural abundance. The ^{19}F has a relative sensitivity 0.833 that of ^{1}H, but unlike ^{1}H, there is no background signal *in vivo*. Its chemical shift is highly sensitive to changes in its environment and has a wide range (960 ppm), which allows various fluorinated compounds to be clearly distinguished in spite of signal line-broadening due to influences of the biological system. Quantitative follow-up of the evolution of any

fluorinated drug is possible with no confusion with endogenous biological compounds, because of the absence of fluorine in the organisms. Heterogeneous biological samples such as blood can be directly analyzed with no need for separation, extraction, or derivatization. Moreover, the NMR method can be noninvasive and nondestructive, so it may be possible to measure biological samples more than once. Also, simultaneous identification of the fluorinated metabolites may be easy, without any differential treatment. The main limitation of NMR when applied to biological problems is its low intrinsic sensitivity compared with GC or HPLC.

^{1}H spectroscopy has proved to be a powerful tool in the study of cyclodextrin inclusion complexes, but has been hampered by the substantial number of resonances arising from both the cyclodextrin and the guest molecules. The use of selectively fluorinated substrates and ^{19}F NMR spectroscopy avoids such potential difficulties and can provide an opportunity to determine the stoichiometry of the inclusion complexes formed.

Several fluorinated drugs have already been incorporated into CDs, ^{19}F NMR has been used to study the complexation of fluorine-containing drugs with cyclodextrin (Brereton *et al.* 1984; Pisaniello *et al.;* 1985, Lincoln *et al.* 1987). These studies are based on the inclusion-induced ^{19}F chemical shift changes of the fluorine-containing drugs.

MacNicol (1975) reported the use of cyclodextrins as shift reagents in NMR spectroscopy. MacNicol and Rycroft (1977) used βCD to achieve enhanced resolution

in certain ^{19}F-NMR investigations.

Smith *et al.* (1989) utilized ^{19}F NMR to study the formation of the αCD inclusion complexes of the enantiomers of N-trifluroacetyl-4-fluorophenylalanine and N-trifluoroacetylphenyl-alanine. They plotted the concentration of αCD against the observed chemical shifts of the ^{19}F resonances of the trifluoroacetyl and 4-fluorogroups of these guest molecules. These authors pointed out that the stability constant of the inclusion complex could be derived from the observed chemical shift of the guest resonances. In principle, the direction of change of the chemical shift should reflect changes in the environment that arise on inclusion of the guest.

Fluorinated derivatives of βCD may also be studied by ^{19}F NMR. The measurement of ^{19}F NMR chemical shifts of the F atoms in the inclusion complex, under controlled conditions, will provide data on both the fluorinated drug and the carrier system, and may also enable estimation of the stoichiometry of the complexes, and unloading of drug from the complex. By forming the inclusion complexes with clinically used fluorinated drugs and fluorinated βCDs, it may be possible to follow the free drug, the inclusion complex and the free fluorinated βCDs both *in vitro* and *in vivo*.

1.3.4. Application of inclusion complexes

The advantageous effects of "molecular encapsulation" of drugs by cyclodextrins are manifested in two main fields, pharmaceutical effects and

pharmacological effects (Table 1.3.4.1.):

When preparing a drug-cyclodextrin complex, the following points should be considered:

1). The kind of molecules that are inclined to form inclusion complexes.

2). The stability of the inclusion complex in aqueous solution, under the circumstances of the manufacturing process and during storage.

3). The action of the inclusion complex under *in vivo* conditions.

In Europe and Japan, oral βCD complexes of prostaglandin and non-steroidal anti-inflammatory agents, such as piroxicam, have already been introduced to the market. In the United States, a monograph for βCD is available in the U.S.P., representing the first such citation for an excipient which is not yet available in a marketed (U.S.A.) product. A monograph for βCD is already in the Japanese Pharmacopoeia and it will soon appear in the European Pharmacopoeia. Cyclodextrins are, therefore, proving their usefulness as tools to generate aqueous drug solutions without the use of organic cosolvents, surfactants, or lipids, as formulation adjuncts which increase dissolution rates and oral availability of solid drug complexes, and as materials used to generate safe i.v dosage forms intended to provide important pharmacokinetic information or act as potential drug products per se. A list of approved pharmaceutical products containing CDs is shown in Table 1.3.4.2. (Stella and Rajewski 1997).

In 1994, Szejtli pointed out that a limiting factor in selecting the drug for

complexation is the dose of the complex that has to be administered. A basic requirement is that the mass of a tablet should not exceed 500 mg. In most cases, the drugs to be complexed have molecular weights between 100-400, and cyclodextrins have rather larger molecular weights of around 1000, thus 100 mg of complex may contain only 5-25 mg of drug. If the single dose of a drug is less than 25 mg, a complex which contain only 5% of drug will carry the necessary dose in a 500 mg tablet. Otherwise, a powder sachet or sparkling-tablet formulation has to be considered. Similarly, the volume of an injection should be less than 2-3 mL, and the complex dosage form can only be used if the necessary amount of drug can dissolve in 2-3 mL of the CD solutions. Thus, the relationship of the required dose and the molecular weight determines the feasibility of oral or i.v. administration in a cyclodextrin complexed form.

Table 1.3.4.1. Cyclodextrin application

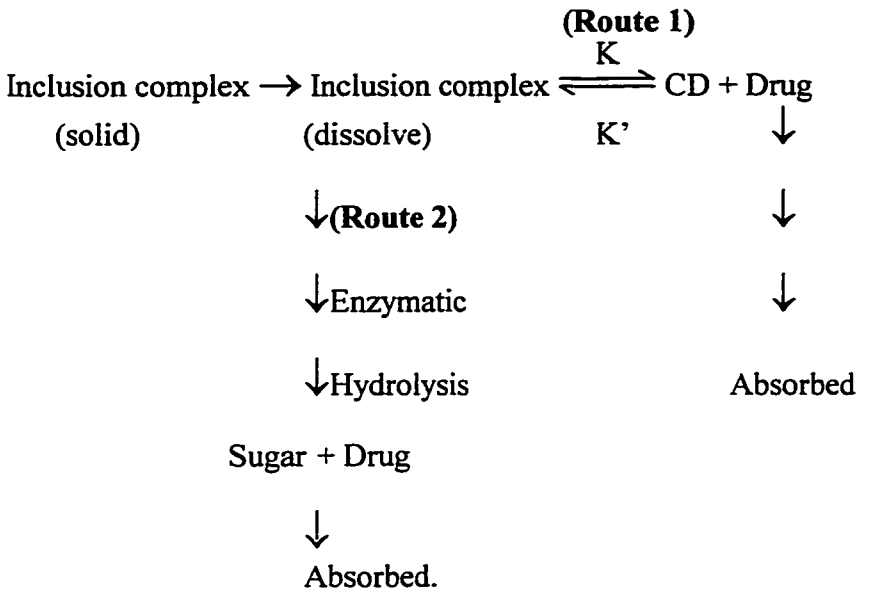
Applications	Drug	CDs	References
Pharmaceutical Application			
Improved dissolution rate and solubility	Vitamin A Progesterone Megestrol Acetate Ibuprofen Hydrocortisone Digoxin Prednisolone Digitoxin Diazepam	dimethyl-α (β, γ)-CD α,β, γ CD α,β, γ, CD, HPβCD DIMEB, RAMEB DIMEB, RAMEB βCD, DIMEB, HPβCD, HPγCD βCD, DIMEB βCD, DIMEB, HPβCD βCD, DIMEB, HPβCD	Szejtli 1994
Improved physical chemical stability	Acetylsalicyclic acid Melphalan Cholecalciferol Linoleic acid, ascaridol and chaulmoogric acid Emetine and Cephaeline	β, γ, maltosyl-, dimaltosyl-βCD and HPβCD βCD γCD and DIMEB	Loftsson *et al.* 1990 Fromming *et al.* 1972. Fromming and Szejtli 1993
Organoleptic properties	garlic oil AOK light protecting creme	βCD γCD	Szejtli 1994
Pharmacological Application			
Improved bioavailability	Fendiline	βCD	Stadler-Szoke *et al.* 1984
Reduced local irratancy	Indomethacin, Flurbiprofen Biphenylacetic acid Naproxen, Phenylbutazone Etomidate	βCD HPβCD	Szejtli 1988 Duchene 1991
Reduced hemolysis	Chlorpromazine	α, β, γ CD	Fromming and Szejtli 1993

Table 1.3.4.2. A listing of approved pharmaceutical products containing cyclodextrins (modified from Stella and Rajewski 1997)

Drug	Trade name	Formulation Type	Company	Country
PGE_1/αCD 20μg/ampoule	Prostavasin	i.a.	Ono Schwarz	Japan Germany
PGE_1/αCD 500μg/ampoule	Prostandin 500	infusion	Ono	Japan
PGE_2/βCD	Prostarmon	sublingual tablet	Ono	Japan
OP-1206/αCD	Opalmon	sublingual tablet	Ono	Japan
Piroxicam/βCD	Brexin	tablet	Chiesi Robapharm Promedica	Italy France France
	Brexi		Nycomed	Scandinavia
			Lauder	Germany
	Cycladol	suppository	Masterpharm	Italy Belgium Netherlands Switzerland
Benexate/βCD	Ulgut Lonmiel	capsule capsule	Shionogi Teikoku	Japan Japan
Iodine/βCD	Mena-Gargle	gargling solution	Kyushin	Japan
Dexamethasone Glyteer/βCD	Glymesason Ointment	Ointment	Fujinaga	Japan
Nitroglycerin/βCD	Nitropen	sublingual	Nippon Kayaku	Japan
Cefotiam hexetil HCL/αCD	Pansporin T	tablet	Takeda	Japan
ME 1207/βCD new cephalosporin	Meiact	tablet	Meiji Seika	Japan
Thyaprofeinic acid/βCD	Suramyl	tablet	Roussel-Maestrelli	Italy
Chlordiazepoxide/βCD	Transillium	tablet	Gador	Argentina
Hydrocortisone/HPβCD	Dexocort	mouth wash	Icelandic Pharm.	Iceland
Itraconazole/HPβCD	Sporanox	liquid	Janssen	Belgium
Garlic oil/βCD	Xund, Tegra Allidex Garlessence	dragees tablet	Bipharm, Hermes Pharmafontana CTD	Germany U.S.A

1.3.5. Inclusion complexes *in vivo*

Based on the above review, one possible *in vivo* process of inclusion complex drug release from the inclusion complex is shown as follows:



It was initially assumed that the *in vivo* absorption of the drug-CD complex is dependent on the enzymatic hydrolysis of the inclusion complex. Later on, it was found that only the bacteria in the colon could hydrolyze drug-CD complexes. A human study with healthy volunteers and ileostomists has shown clear evidence that βCD is poorly digested in the human small intestine but is almost completely degraded by the colonic microflora (Flourie *et al.* 1993). The transit time through the human colon is ~ 40 h, which is adequate time for bacterial enzymes to completely hydrolyze the parent CDs.

Compared with the hydrolysis of cyclodextrin, the kinetics of inclusion complex formation and dissociation between a cyclodextrin and a drug molecule is fast (Hersey *et al.* 1986; Cramer *et al.* 1967). These authors measured various perturbations using competitive binding techniques and found that the half-lives for formation/dissociation are much less than one second. Uekama *et al.* (1994) and Thompson (1997) reviewed more approaches to understanding the dilution effects and complex dissociation. Agents which compete with the drug for the cyclodextrin cavity may also influence the free fraction of drug depending on the extent of the binding constant of drug and competing agent. In summary, for weakly bound drugs, simple dissociation due to dilution is sufficient to cause rapid and complete release of the drug; the earlier pharmacokinetics time point may be perturbed for highly bound drug ($K > 10^5\ M^{-1}$). Thus, route 2 shown above is not considered the main *in vivo* process for drug release. Route 1, drug dissociation followed by absorption, is considered as the main *in vivo* process for drug release from the inclusion complex.

1.3.5.1. Oral administration

When a CD/drug complex is administered orally, the drug (guest molecule) may be absorbed from the small intestine in its free form after dissociation from the host molecule. The mechanism for liberation of the guest molecule from the complex involves several steps. Besides the simple dissociation mechanism, many kinds of hydrophobic compounds in ingested food are present in gastrointestinal secretions,

including bile salts (Miyajima *et al.* 1986, Nakanishi *et al.* 1989) that can be involved with the exchange of the guest molecule.

1.3.5.2. Intravenous administration

Frijlink *et al.* (1991) studied the effects of cyclodextrins on the disposition of drugs injected intravenously (*i.v.*) into the rat. They found that naproxen and flurbiprofen form complexes with HPβCD, with stability constants of 2207 and 12515 M^{-1}, respectively. The study of dilution with plasma on HPβCD complexes of naproxen or flurbiprofen showed that only small fractions of the drugs remained bound to the cyclodextrin in plasma *in vitro*. This may be due not only to dilution but also to the competition between albumin binding of the two high protein binding drugs and cyclodextrin binding. Plasma cholesterol could act as a competing agent as well. Consequently, it was concluded that the impact of cyclodextrin complexation on the disposition of a certain drug may depend both on the drug's relative affinity for plasma protein and on its tissue affinity.

The importance of changes in the ratio of free to complexed drug upon dilution of a sparingly water-soluble drug in a cyclodextrin complex depends on the phase-solubility behavior of the system. If the cyclodextrin complex of a drug results from a 1:1 interaction, there is a linear increase in drug solubility with increasing cyclodextrin concentration. Therefore, dilution of a true solution of the drug/cyclodextrin complex will not result in drug precipitation regardless of the extent of dilution. In other words,

precipitation of the drug may occur on dilution if there is a nonlinear relationship between drug solubility and cyclodextrin concentration.

1.4. Mechanism of inclusion complex absorption

1.4.1. Drug absorption

Drug absorption is the process of movement of a drug from the site of application into the extracellular compartments of the body. The extent of absorption is determined by the physicochemical properties of the drug and its dosage form, as well as the anatomical and physiological characteristics of the drug recipient. There are three major routes for drug administration: enteral, parenteral and topical. Because of its convenience, the oral route predictably is the most common route in use for access to the systemic circulation. The dissolution rate of solid dosage forms may be a major factor in controlling the rate and completeness of absorption, especially for sparingly soluble drugs. Intravenous injection is the most popular mode for introducing a drug directly into the systemic circulation. It has the major advantage of quick clinical response and a controlled drug concentration in the blood. Insoluble or suspended drug preparations cannot be administered intravenously as they can cause an embolism or thrombophlebitis.

Flutamide has a very low intrinsic solubility. From its physicochemical characteristics along with its previous pharmacokinetics and its androgen receptor

affinity data, it was shown that its inadequate absorption through the GI tract may not be due to its inability to permeate the intestinal epithelial membrane. In order to improve the dissolution rate, one can either enhance the solubility of the drug or its effective surface area. Micronization processes or the incorporation of surface active agents in the formulation are primary strategies to increase the effective surface area of the drug particles. Alteration of the pH of the surrounding medium is the other way (depending upon the pK_a of the compound). Eutectic mixtures, solid solution techniques, dispersion techniques, use of metastable polymorphs and micelle solubilization are also effective alternatives. Complexation with cyclodextrins is the method used in the present study (Abdou 1989).

Development programs to identify orally active drugs typically involve a lengthy process of eliminating numerous potential compounds to identify a few putative leads which have appropriate oral bioavailability characteristics. The physical and metabolic barriers presented by the intestinal mucosa greatly dictate oral bioavailability. The relative impact of the barriers for a given drug molecule depends largely on the route taken by that drug across the mucosa. As shown in Figure 1.4.1, mechanisms of drug transport across GI absorptive epithelia include (Brayden 1997): 1) transcellular passive diffusion, 2) paracellular passive diffusion, 3) carrier-mediated transcellular diffusion, 4) transcellular diffusion subject to P-glycoprotein mediated efflux. Current interest in the pharmaceutical industry is the development of a cell culture system that would mimic the intestinal mucosa.

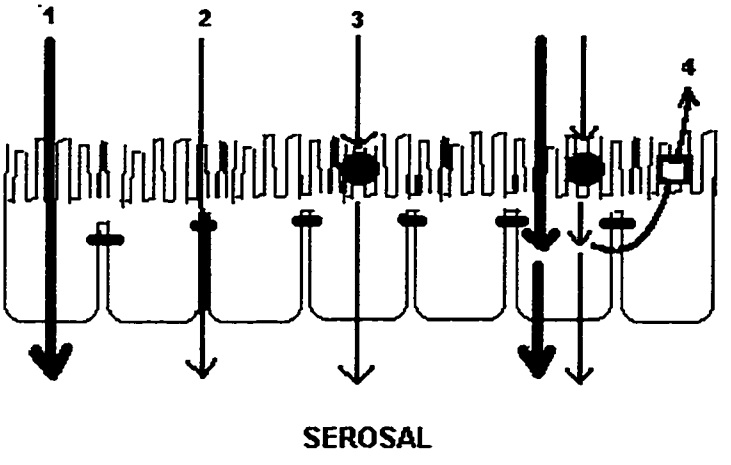


Figure 1.4.1 Drug flux transport processes in absorptive intestinal epithelia. 1. Transcellular passive; 2. Paracellular passive; 3. Carrier-mediated; 4. P-glycoprotein-mediated efflux. (Brayden 1997)

1.4.2. *In vitro* models for studying drug absorption

Because of the economical and ethical limitations of *in vivo* studies, several *in vitro* models and procedures have been introduced to assess the major factors involved in the absorption process, to predict how well the drug will be absorbed, and to determine whether the absorption is a dissolution-limited or permeability-limited process. These *in vitro* procedures may be of extreme value, especially during the screening of new drug candidates. The first class of *in vitro* methods is based on the pH partition theory which states that the relative rate and extent of absorption of the large majority of drugs can be predicted, at least qualitatively, by comparing their partition coefficients in two immisible solvents. Also, some *in vitro* procedures are

used to assess the permeability of drugs using live animal tissues, such as the "everted sac" experiment.

In vivo testing of oral absorption of the many compounds produced by drug discovery programs is very costly, time-consuming and labor intensive. A number of *in vitro* methods have been used to study the absorption of drugs through the intestinal epithelium. The majority of these systems do not have the morphological and functional properties of normal adult human epithelial layers. Moreover, they lack the viability and versatility required for quantitative measurements of transepithelial drug transport and for the examination of transport mechanisms. For example, in most transport studies using everted sacs and intestinal loops, a compound is administered to the luminal side and its rate of disappearance from this side is assumed to be equal to the absorption/uptake rate. Obviously, some compounds might bind to the cell surface or to intracellular sites after uptake. Others might undergo metabolic biotransformation in the lumen or during transepithelial transport. For these compounds, the rate of absorption obtained under the assumption that the disappearance from the lumen is equal to absorption will result in an overestimation of the true uptake/absorption rate. The vascularly perfused intestine permits the sampling of blood or lymph from the mesenteric veins and lymph ducts, and thus provides a better means of estimating the rate of absorption. It does not provide, however, additional information on the processes involved in the transmucusal transport of drug molecules. Isolated mucosal cells and brush border membrane vesicles have been used

in studies of intestinal transport at a cellular or molecular level, or both. The value of isolated mucosal cells is greatly reduced by their limited viability (30-45 min). Moreover, mucosal cell isolation procedures result in loss of cell polarity and may cause substantial cell damage. Although brush border membrane vesicles do not have the viability limitation of isolated intestinal cells, they may be relevant only in studying interactions with brush borders without providing any insight into intracellular transport. Furthermore, it is not certain to what extent the preparation of these vesicles might alter enzymes or receptors that may be involved in transepithelial transport or metabolism, or both (Hidalgo 1989).

The development of human cell culture systems has been limited by the lack of retention of anatomical and biochemical features of differentiated cells *in vivo*. In particular, the use of isolated intestinal epithelial cells has been slow to progress because these cells are difficult to culture and have limited viability. The use of human tumor cell lines has also been restricted by their lack of differentiated properties in culture. Recently, attention has turned to human adenocarcinoma cell lines that reproducibly display a number of properties characteristic of differentiated intestinal epithelial cells (Wilson *et al.* 1990). Human intestinal epithelial cell lines grown on porous filters, however, are now being used by companies to screen out and rank order the best absorbed oral candidates from families of compounds differing only by minute changes in structure, noncovalent bonding, and molecular surface characteristics. Several current human intestinal epithelial cell lines used in drug delivery research are

listed and compared in Table 1.4.2.

Table 1.4.2. Comparison of current human intestinal epithelial cell lines in pharmaceutical research (Brayden 1997)

Cell line	Origin	Phenotype
Caco-2	Colon	Spontaneously differentiated Small intestinal carriers, brush border, villous cell model, tight junctions, high resistance
TC7	Colon	Spontaneously differentiated Subclone of Caco-2 with better array of P450 metabolising enzymes, high resistance
HT29-18C_1	Colon	Induced to differentiate Villous cell absorptive model, tight junctions, small intestinal-like resistance at day 10
T84	Colon	Undifferentiated brush border Colonic crypt cell model, reduced carrier expression, secretes mucous, very high resistance.

1.4.3. Caco-2 cell monolayer model for drug absorption

The most popular commercially available human intestinal cell line for drug absorption studies is Caco-2 (Artursson 1991 and Wilson 1990). The Caco-2 cell line is the only human intestinal epithelial cell line that differentiates spontaneously to enterocyte-like cells under conventional cell culture conditions. It has the following characteristics:

- Exhibits a well-differentiated brush border on the apical surface and has tight junctions.
- Expresses typical small-intestinal microvillus hydrolases and nutrient transporters.
- Expresses sucrase-isomaltase, lactase, aminopeptidase and alkaline phosphatases, all of which are brush border membrane enzymes that are differentiation-specific markers for small intestinal enterocytes.

The Caco-2 cell line has proven to be the most popular *in vitro* model:

- To rapidly assess the cellular permeability of potential drug candidates:

  Transepithelial permeability coefficients (P_{eff}) determined *in vitro* for different molecules have been compared with *in vivo* data for oral absorption (Artusson and Karlsson 1991). For the Caco-2 cell line, a threshold P_{eff} value of 10^{-6} cm/s was necessary for maximal oral absorption. Above these threshold P_{eff} values, drugs are more than 75% absorbed, while drugs that have lower P_{eff} values are poorly absorbed in animals or

humans.

- To elucidate pathways of drug transport:

  Epithelial cells are joined by a junctional complex which is comprised of three separate structures: tight junctions, intermediate junctions and desmosomes. The integrity of these structures is dependent on Ca^{2+} ion (Madara *et al.* 1987). The Ca^{2+} dependence of the junctional complex was used to investigate the contribution of the paracellular pathway to the transepithelial transport of drugs.

- To assess formulation strategies designed to enhance membrane permeability.
- To assess potential toxic effects of drug candidates or formulation components on this biological barrier.
- For the study of presystemic drug metabolism:

  The Caco-2 monolayer contains several Phase I enzymes (CYP 1A1, CYP2A and CYP 3A) and enzymes that affect Phase II biotransformations (glutathione conjugation, sulfo-conjugation). Caco-2 cells in culture for longer periods will more closely resemble small-intestinal cells than cells in culture for short periods. The quantitative differences in enzyme expression with time in culture, together with the qualitative differences in the enzyme properties observed between the parental Caco-2 cell line and available clones, suggest that before utilizing this cultured cell system to study drug metabolism a thorough evaluation of the phase I and phase II

enzymes and isozymes should be made in comparison with the human intestine (Meunier *et. al.* 1995).

Compared to conventional drug absorption models, the Caco-2 cell line model has the advantages that include:

- Rapid evaluation of the P_{eff} for a drug.
- The opportunity to study mechanisms of drug absorption under controlled conditions.
- Rapid evaluation of methods for improving drug absorption (by use of prodrugs, absorption enhancers, or other pharmaceutical additives.)
- The opportunity to perform studies on human cells.
- The opportunity to minimize time-consuming, expensive, and sometimes controversial animal studies.

A typical Caco-2 monolayer model is shown in Figure 1.4.3. The cells differentiate after 15 to 20 days on polycarbonate filters to express many carriers of the small intestine vili such as those for dipeptides, bile acids, and vitamin B_{12}. A high transepithelial electrical resistance (TEER) of 250-600 Ω cm^2 is indicative of the expression of epithelial tight junctions. A chamber system is adapted to allow electrophysiological parameters such as electrical potential difference and TEER to be measured in parallel so that cellular integrity can be monitored. The candidates are usually added to the donor side in the form of a radiolabeled or fluorescent labeled

molecule and both the mucosal and serosal side are sampled to obtain concentration data.

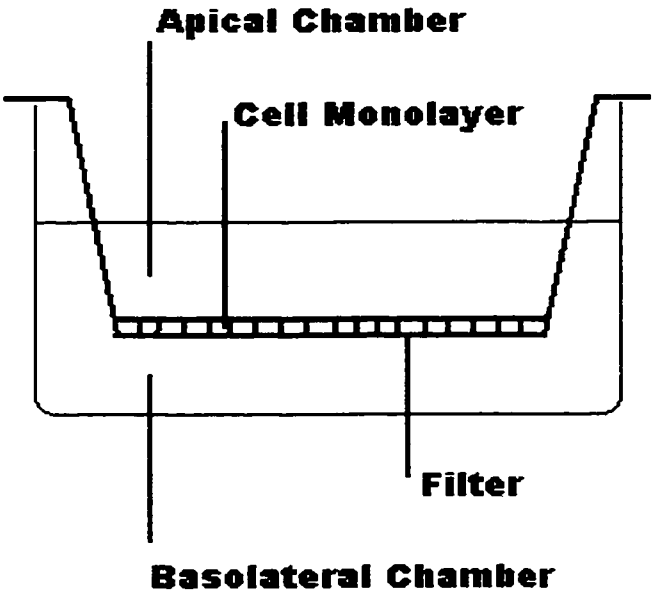


Figure 1.4.3. Diagram of apparatus used to grow Caco-2 cells.

Caco-2 cells are routinely grown in a medium comprised of Dulbecco's modified Eagle's medium (DMEM) containing 10-20% of fetal bovine serum (FBS), 1% (w/v) L-glutamine, 1% (w/v) non-essential amino acids, penicillin (100 U) / streptomycin (100 μg/mL). Routine passaging of cell stocks is carried out in 25 cm^2 or 75 cm^2 flasks. For transferring from flasks to the Transwell®, the cells are usually trypsinized with 0.25% trypsin and 0.02% EDTA and washed in cell culture medium

before seeding into the transwell insert. Plate density is usually around 6.5×10^4 cells / cm^2, depending on the kinds of Transwell assembly used. The integrity of the cell monolayer can be monitored by the use of the transport marker molecules such as [^{3}H]-D-Manitol, [^{14}C] Polyethyleneglycol 4000 and/or fluorescence markers, or by the measurement of TEER using an EVOM Epithelial Voltometer or Edohm®. The commonly used transport media in the drug transport experiment are : DMEM pH = 7.3 containing 1% non-essential amino acid (NEAA), 10 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES) buffer and 0.1% human serum albumin; Hank's balanced salts solution containing 15 mM glucose at pH = 6.7; pH 7.35 HBSS containing 25 mM glucose and 10 mM HEPES buffer; phosphate-buffered saline containing 1 mg/mL D-glucose.

Drug flux data for agents passively absorbed across Caco-2 monolayers have been correlated with *in vivo* bioavailability data (Fagerholm 1996; Rubas 1996). The correlation data show that no two labs get the same P_{eff} for the same drug. This may be due to the tendency of Caco-2 to alter its phenotype upon subculture and also to the methodology differences in the absorption experiments. It seems that the absolute value of the flux is not so important *per se* by comparison with the rank order of the individual fluxes in the hands of each laboratory as long as in-house reproducibility is achieved. The other drawback is that the correlations are more predictive for poor- and well- absorbed compounds but less accurate for those in between. Delie and Rubas found in 1997 that Caco-2 monolayers from different laboratories display very

different TEER values, which in general are much higher than values commonly reported from intestinal tissues. Therefore, transport studies of hydrophilic molecules across Caco-2 cells could potentially underestimate transport across intestinal tissue. However, a TEER value $\leq 300\ \Omega\ cm^2$ in Caco-2 monolayers appears to give similar permeability values for hydrophilic compounds across intestinal tissue regardless of the origin. In conclusion, more challenging research which would involve refinement of the model is needed so that it can more accurately reflect the *in vivo* biological barrier.

2. Experimental Hypothesis and Objectives

Based on the above information, the following hypotheses were established:

- Flutamide can be complexed with CDs, resulting in improved solubility.
- Flutamide-CD complex formation can induce ^{19}F or ^{1}H chemical shift changes of flutamide.
- Plasma drug levels in animals after oral administration of the flutamide-CD complex will be higher than without the CD.
- The low dissolution rate of flutamide is an important parameter responsible for its low bioavailability.

To test these hypotheses a number of specific experimental objectives were identified:

- To solubilize flutamide in the presence of different kinds and concentrations of CDs and to physicochemically characterize the inclusion complexes *in vitro*.
- To develop HPLC methods for the *in vitro* and *in vivo* analysis of flutamide and its main metabolite, 2-hydroxyflutamide.
- To determine the oral bioavailability of flutamide in suspension and as a CD-complex in rats.
- To develop an injectable dosage form of flutamide based on an inclusion complex with one or more CDs.
- To determine the *in vitro* transmembrane pathway of flutamide-CD inclusion complex using the Caco-2 monolayer model.

3. Experimental design

3.1. Materials

3.1.1. Chemicals, solvents and equipment

Flutamide and HPßCD (Average molar substitution = 0.8) were purchased from Sigma and Aldrich Chemical Company Inc., respectively. All other chemicals and solvents were of analytical reagent grade. HPLC grade distilled water was used throughout the study. 2-HY-FLT was gift from Schering Plough (N.J., U.S.A). Polyethylene glycol (Molecular weight = 200) and dimethylsulfoxide (DMSO) were obtained from Sigma (Mo. U.S.A). Normal saline (0.9% NaCl, Injection U.S.P) was obtained from McGaw.

A Vortex Genie™ (Scientific Industries, Inc. Bohemia, N.Y. 11716, U. S. A) was used for mixing samples. A Branson 2200 sonicator was used for sonication purpose. A Dubnoff Metabolic Shaking Incubator was used to establish the equilibrium process in the phase solubility test. Centrifugations were performed in a Eppendorf 5412 or a Becton Dickinson centrifuge depending on the size of the samples. The 0.45 μm membrane (Millipore) was fixed in a Nucleopore holder as a filtration device for phase solubility test. The Sterile Acrodisc® (0.2 μm) was used for the sterile preparation for the *i.v* injection formulation. A Spencer hemocytometer was used for the cell counting in the Caco-2 cell culture experiment. An epithelial

voltohmmeter (MILLICELL®ERS/ENDOHM-24) was used for the resistance measurement of the Caco-2 cell monolayer.

A 2 cm Silastic® tubing (0.025 in. ID ×0.047 in. OD, Dow Corning Corp.) attached to 5 cm polyethylene tubing (PE50, I.D. 0.58 mm, O.D. 0.965 mm, Becton Dickinson) was used as the rat jugular vein catheter.

3.1.2. Instruments

A PU 8740 UV/VIS Scanning Spectrophotometer (PHILIPS) was used in the phase solubility test. A differential scanning calorimeter (DSC 120, SEIKO SII, model SSC/5200) was used to confirm the formation of inclusion complex between flutamide and different CDs. A Freeze Dryer 3, Labconco, was used for the sample preparation for the DSC study. All of the ^{19}F NMR spectra were recorded on a 282.35 MHz high field NMR (Bruker AM-300) by Dr. Somayaji. The ^{1}H NMR spectra were obtained from a 500 MHz NMR (Varian VXR-500) by Dr. Diakur. The HPLC system used for plasma concentration analysis consisted of a solvent pump (Waters 501 HPLC pump), a UV detector (Waters 486 Tunable Absorbance Detector). A reverse-phase column (Radial Pak C18 10 μm) equipped with a connected guard column (μBondapak™ C18 Guard-Pak™) was used in conjunction with the HPLC apparatus.

3.1.3. Animals

Sprague Dawley male rats (350-400 g) purchased from the Health Sciences Animal Service, University of Alberta, were used for all pharmacokinetics studies. Animals were handled in accordance with guidelines of the Canadian Council on Animal Care and experimental or surgical protocols were approved by the University of Alberta Health Sciences Animal Welfare Committee (see the Appendix 1 for S.O.P).

3.1.4. Cell culture medium

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), L-glutamine, transferrin, penicillin-streptomycin, insulin and trypsin were purchased from Sigma (Mo., U.S.A.). PBS was prepared in-house (contains 140 mM NaCl, 260 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4). PBS^+ used in the transport experiment was prepared by adding 225 µl of 2 M $CaCl_2$ and 200 µl of 1M $MgCl_2$ to 500 mL of normal PBS. Transwell® inserts (24.5 mm, pore size 0.4 µm, Corning Costar) were used for the Caco-2 cell monolayer culture and transport experiment and 25 cm^2 cell culture flasks used for the normal cell culture experiment were purchased from Corning Costar. EGTA (ethylene glycol-*bis*-(β-aminoethyl ether)-*N,N,N',N'*–tetraacetic acid) was obtained from Sigma.

3.2. Methods

3.2.1. Phase Solubility tests of flutamide in aqueous βCD, JD1-128 CD and HPβCD solutions.

3.2.1.1. Determination of time to equilibrium for flutamide in HPβCD solution

Excess flutamide (≈5 mg) was added to each of two 20 mL Scintillation Vials which contained 10 mL of water and 10 mL of 5% HPβCD, respectively. After 2 min of vortex and 5 min of sonication, samples were put into the shaking water bath. Approximately 0.5 mL of the solution was withdrawn and then filtered through 0.45 μm membrane fixed in adapter at predetermined intervals from 1 to 48 h. A 0.2 mL aliquot of filtrate was diluted appropriately for assay by UV at the fixed wavelength of 300 nm (ε_{max} = 5709 $mol^{-1}cm^{-1}$). The equilibration time was then determined from the plot of flutamide concentration versus time.

3.2.1.2. Solubility of flutamide in βCD, JD1-128 CD and HPβCD

Excess flutamide was added to aqueous solutions of βCD, JD1-128 CD and HPβCD in 1.5 mL Eppendorf centrifuge tubes or 20 mL screw-capped vials. After 2 min of vortex and 5 min of water bath sonication, samples were then put in the shaking water bath at 25 °C for 3 h. Preliminary experiments showed that equilibrium was achieved within 3 h. For experiments with HPβCD, approximately 0.5 mL of the

solution was withdrawn and then filtered through 0.45 μm membrane filter. Flutamide analysis was carried out by UV spectrophotometry. Samples were prepared in methanol:water (1:1) and measured at the fixed wavelength of 300 nm (ε_{max} = 5709 $mol^{-1}cm^{-1}$). For experiments with βCD and JD1-128 CD, the samples were centrifuged and 50 μl of the supernatant was injected into HPLC for analysis. The HPLC spectrum conditions for flutamide were: Column: Radial Pak C18 10 μm; Solvent system : methanol : water : glacial acetic acid : triethylamine (61:38:1:0.02, v/v/v/v); wavelength of UV absorbance detector: 300 nm; flow rate: 1.0 mL/min. The retention time for flutamide was 11 min. The detection limit under these conditions was found to be 5 ng/mL injection of 100 μl.

3.2.2. Thermodynamic study of flutamide in aqueous HPβCD, βCD and JD1-128 CD

The phase solubility experiments were carried out under selected temperatures at 20, 25, 30, 35, 40, 45, 50 °C. For the HPβCD experiment, 0.2 mL filtrate was withdrawn from each sample at each temperature and analyzed by UV after appropriate dilution with methanol : water (1:1). For experiments with βCD and JD1-128 CD, Eppendorf microcentrifuge tubes were used for solubility determination and the supernatant from each sample was analyzed by HPLC. The solubility data at each temperature were used to calculate the binding constants for formation of the flutamide complex with ßCD, JD1-128 CD and HPßCD. Then the binding constant

data were treated according to Van't Hoff equation to obtain the further thermodynamic parameters (calculation is shown in Appendix 2).

3.2.3. Confirmation of the formation of flutamide inclusion complexes with HPβCD, βCD and JD1-128 CD by Differential Scanning Calorimetery

3.2.3.1. Preparation of solid dosage form of flutamide inclusion complexes with HPβCD, βCD, JD1-128 CD

Excess flutamide was added to aqueous 50% (w/v) HPßCD, 1% (w/v) ßCD or JD1-128 CD. After vortex mixing, sonication and equilibration in a water bath for 3 h, water in the filtrate was removed by lyophilization.

3.2.3.2. DSC

Free flutamide; the cyclodextrins (HPßCD, βCD or JD1-128 CD); the physical mixture of flutamide and the cyclodextrins and the inclusion complexes (flutamide and the cyclodextrins) were sealed singly in aluminum pans and measured, with a sealed empty aluminum pan as reference. The transition temperature for each sample was obtained from the thermogram. The temperature program was run with a linear ramp of 10 °C/min from 60 to 90 °C and 5 °C/min from 90 °C to 300 °C. The cell was purged with N_2 at a flow rate of 50 mL/min during the experiment.

3.2.4. Further proof of the formation of flutamide inclusion complexes with HPßCD and JD1-128 CD by ^{19}F NMR

All ^{19}F NMR spectra were recorded at 282.35 MHz. Solutions of 0.5 mM of flutamide with HPßCD or 0.1 mM of flutamide with JD1-128 CD were prepared in relative amount of internal standard (0.2 M KF) and D_2O. The ^{19}F NMR chemical shifts were compared with the control sample which only contained flutamide in D_2O.

3.2.5. More evidence of inclusion complex formation by ^{1}H NMR

A 500 MHz ^{1}H NMR was used in this study. Excess flutamide was added in D_2O or 1% (w/v) HPßCD solution in D_2O, after equilibration by 2 min vortex mixing, 5 min sonication and shaking in water bath for 3 h, the filtrate was analyzed by ^{1}H NMR. ^{1}H NMR of flutamide in D_2O and 1% (w/v) HPßCD in D_2O were also performed under the same condition as comparison.

3.2.6. Drug release from different dosage forms

3.2.6.1. Preparation of flutamide HPβCD inclusion complex

Inclusion complex dosage forms of flutamide were prepared by adding excess flutamide to a solution of HPβCD in water (50% w/v). After 2 min of vortex mixing, 5 min of sonication, and shaking in water bath under room temperature for 3 h, the sample was filtered and the filtrate was used for further study. Water in the filtrate was

removed by lyophilization.

3.2.6.2. Preparation of flutamide suspension dosage form

For the suspension dosage form, flutamide was suspended in an aqueous vehicle composed of 0.9% NaCl qs, 0.5% carboxymethyl cellulose, 0.4% polysorbate-80, and 0.9% benzyl alcohol (modified from Neri and Peets 1975) to prepare a 24.49 mg/mL of flutamide suspension.

3.2.6.3. *In vitro* dissolution studies

The dissolution of two dosage forms as a powder (<100 mesh) were studied in simulated gastric fluid. Flutamide powder (44.3 mg) or flutamide-HPBCD complex powder (containing the equivalent of 44.3 mg flutamide) were dispersed in 900 mL of dissolution medium (simulated gastric fluid, U.S.P.) previously equilibrated to 37±0.5 °C. The medium was stirred immediately at a rate of 50 r.p.m. Aliquots (1 mL) were withdrawn at 0.5, 1.5, 9, 24, 34, 64, 90, 120, 150 and 180 min for analysis. The cumulative dilution caused by sampling was corrected by immediately replacing 1 mL of the original medium after each sampling. Sample aliquots were centrifuged and analyzed by HPLC.

3.2.7. Pharmacokinetics of the flutamide-HPβCD inclusion complex

3.2.7.1. HPLC analysis for flutamide and 2-hydroxy-flutamide

The ideal HPLC conditions obtained for both flutamide and 2-hydroxyflutamide were as follows: Injection volume: 50 μl; mobile phase is: methanol : water : glacial acetic acid : triethylamine (61:38:1:0.02, v/v/v/v); wavelength of UV absorbance detector: 300 nm; flow rate: controlled at 1.0 mL/min. The recoveries of the extraction step were determined by comparing the peak area of spiked samples with those obtained for an equivalent amount (dissolved in methanol) directly injected into HPLC. The recovery rate was found to be 90.9 ± 0.5% at 1 μg/mL. The inter- and intra- assay variability was determined to be less than 10.0% for both.

3.2.7.2. Oral administration

3.2.7.2.1. Preparation of the inclusion complex form of flutamide

Excess flutamide was added to aqueous solutions containing 50% (w/v) of HPβCD. After 2 min of vortex mixing and 5 min of sonication in a water bath, samples were shaken in a water bath at 25 °C for 3 h. Aliquots were withdrawn and filtered through a 0.45 μm membrane fixed in a holder. Part of the filtrate was diluted appropriately for HPLC analysis to determine the final concentration and the rest of

the filtrate was used as the final HPβCD dosage form for the rat oral administration.

3.2.7.2.2. Preparation of the suspension dosage form of flutamide

Flutamide was suspended in an aqueous vehicle composed of 0.9% NaCl qs, 0.5% carboxymethyl cellulose, 0.4% polysorbate-80, and 0.9% benzyl alcohol to have a final concentration of 24.49 mg/mL (modified from Neri and Peets 1975).

3.2.7.2.3. Pharmacokinetics of flutamide after oral administration of suspension and HPβCD complex dosage forms

Sprague Dawley male rats (350-400 g) were used for the pharmacokinetic studies. The animals were fasted overnight before the experiment. Groups of 3 rats were given dosage forms containing flutamide (15 mg/kg per rat). Blood (0.2 mL) was withdrawn through a catheter inserted into the jugular vein at: 0.17, 0.33, 0.5, 0.67, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 h following dosing for flutamide concentration analysis; and at 0.5, 1, 2, 4, 6, 8, 10, 15, 20 and 25 h after dosing for 2-HY-FLT analysis. Collected plasma (100 μl) containing 25 μl of 2-hydroxy-5-nitro-benzaldehyde (as internal standard) was treated with 1 mL of methanol, followed by vortexing for 30 sec and storage at 4 °C for 1 h to deproteinize the plasma. After centrifugation (2500 rpm for 20 min), the supernatant was withdrawn and evaporated at room temperature under a stream of dry N_2. The residue was redissolved in 1 mL of H_2O : ethyl ether (1:10, v/v) and the organic layer was separated and evaporated. This

final residue was redissolved in 100 µl of methanol for HPLC analysis.

3.2.7.3. *I.V.* administration

3.2.7.3.1. Preparation of the flutamide inclusion complex dosage form

Excess flutamide was added to an aqueous solution of 30 % (w/v) HPβCD. After 2 min of vortex mixing and 5 min of sonication in a water bath, samples were shaken in a water bath at 25 °C for 3 h. The sample was filtered through a 0.45 µm membrane fixed in a holder. The filtrate was diluted appropriately for HPLC analysis. It was found that the complex contained 1.97 mg of flutamide per mL.

3.2.7.3.2. Preparation of the cosolvent dosage form of flutamide

Co-solvent dosage forms were prepared by dissolving flutamide in 0.9 % NaCl : ethanol : PEG200 (2:1:3). Both dosage forms were filtered through a 0.22 µm filter for sterilization before they were used for *i.v.* injection.

3.2.7.3.3. Jugular vein catheter wash-out experiment

The calculated dose of each formulation was injected through a 2 cm Silastic® tube catheter attached to a 5 cm polyethylene tube. This assembly was washed with either 1) saline (0.1 mL × 3) or 2) rat blood (0.1 mL × 3) followed by saline (0.1 mL ×3). In either case, the assembly received a final rinse with methanol (0.1 mL ×3).

Every wash out fluid was collected and analyzed by HPLC.

3.2.7.3.4. Pharmacokinetics of the *i.v.* dosage forms of flutamide

Sprague Dawley male rats (350-400 g) were used in the experiment. The jugular vein canulation operation was performed 48 h ahead of the experiment. Groups of 3 rats were given one of the 2 dosage forms of flutamide *i.v.*, at a flutamide dose of 1.60 mg/kg . Around 0.3 mL of each dose was given through the jugular vein catheter. The dose was followed by 3 times 0.1 mL of blank rat blood and 3 times 0.1 mL of normal saline (with 100 unit/mL Heparin) wash Blood was withdrawn through the dosing catheter at 0.08, 0.17, 0.33, 0.5, 0.67, 1, 1.5, 2, 2.5, 3 and 3.5 h after dosing. Collected plasma (100 μl) containing 25 μl of 2-hydroxy-5-nitro-benzaldehyde (as internal standard) was treated with 1 mL of methanol, followed by vortexing for 30 sec and storage under 4 °C for 1 h to deproteinize the plasma. After centrifugation (2500 rpm for 20 min), the supernatant was withdrawn and evaporated at room temperature under a stream of dry N_2. The residue was redissolved in 1 mL of H_2O: ethyl ether (1:10, v/v), the organic layer was separated and evaporated, and the residue was redissolved in 100 μl of methanol for HPLC analysis.

3.2.7.4. Pharmacokinetic and statistical analysis

Drug plasma profiles were analyzed with "Winnolin" (version 1.1) using a non-compartmental model. Each AUC was obtained from the program by a log-linear

trapezoid method. Significance of differences between experiments was calculated by Student's t test in Sigma Plot using two tailed distributions and either a two-sample equal variance (homoscedastic) or a two-sample unequal variance (heteroscedastic) method. In all cases, statistical significance was determined at the 95 % confidence level ($p<0.05$).

3.2.8. Mechanism of flutamide HPβCD inclusion complex absorption

3.2.8.1. Culture of Caco-2 cells in flasks

Caco-2 cells were maintained at 37°C in Dulbecco's Modified Eagle's Medium, containing 20% (v/v) fetal bovine serum, 0.058% (w/v) Glutamine, 0.001% (w/v) Transferrin, 1% (v/v) P/S and 50 unit/100 mL Insulin, in an atmosphere of 5% CO_2 and 90 % relative humidity. Cells grown in 25 cm^2 flasks and were passaged every 5 days at a split ratio of 1:5 and confluence was reached within 6-7 days after passage.

3.2.8.2.Growth of Caco-2 monolayer in transwell inserts

Trypsinized Caco-2 cells from flasks were resuspended in 5 mL of culture medium. The suspension (50 µl) and 2% trypan blue (50 µl) were mixed in a Eppendorf microcentrifuge tube. Cell suspension was immediately transferred to the edge of the hemocytometer chamber and cells were counted in 25 smaller squares of each chamber. Total counts from 2 chambers were summed and the cell density was

calculated based on the formula $C = n \times 10^4$ (volume of each chamber is 10^{-4} mL), c = cells/mL, n= number of cells counted.

For the drug transport studies, 1.4×10^6 cells were seeded in each Transwell® insert (24.5 mm of diameter, 4.71 cm^2, pore size 0.4 μm, Corning Costar). The medium was changed daily in the Transwell®.

3.2.8.3. TEER measurement of caco-2 monolayer

The integrity and permeability of cell monolayer was determined by electrical resistance measurements (MILLICELL®ERS, ENDOHM-24). PBS^+ (1 mL) was added to the apical chamber of the transwell and 4 mL of PBS^+ were added to the measurement chamber, respectively. Air bubbles were removed from the chamber by moving the electric probe and the chamber was allowed to equilibrate at 25 °C for 15 min. Transepithelial electrical resistance values obtained in the absence of cells were considered as background.

3.2.8.4. Transport experiment of flutamide-HPβCD inclusion complex

After 4 or 5 days of the cells' seeding on the transwell, the transport experiment was initiated by using the cell culture medium or with PBS^+ (pH=7.38). Different flutamide-HPβCD inclusion complex solutions were added to 1.5 mL of PBS^+ or medium in the apical surface and incubated at 37 °C in an atmosphere of 95 % humidity. The apical to basolateral transport was continuously followed by monitoring

the appearance of flutamide in 2.6 mL of either medium or PBS^+ in the basal receiving side. To maintain sink conditions, the Transwell® with the cell monolayer containing the flutamide solution was moved to a fresh PBS^+ or medium in the next well of a 6-well plate after certain period of time. In order to find the ideal experimental conditions, the transport experiment was executed in either medium or PBS with different doses of flutamide prepared in PBS.

Samples from PBS were injected directly into the HPLC for analysis of flutamide concentration. Samples from medium were extracted with methanol and 2-hydroxy-flutamide was used as internal standard as described in previous pharmacokinetics work (section 3.2.7.2 and 3.2.7.3).

3.2.8.5. Data analysis

The effective Permeability coefficient (P_{eff}) was calculated using the following equation (Schoenwald and Huang, 1983):

$$P_{eff} = \frac{V}{A^* Co} \times \frac{dc}{dt} \quad (cm/s)$$

dc/dt: the flux across the monolayer (mM/s), is the initial slope of a plot of the cumulative receiver concentration versus time.

V: the volume of the receiver chamber (mL),

A: the surface area of the monolayer (cm^2); which is 4.7 cm^2 for the transwell insert in this experiment.

Co: initial concentration (mM) in donor compartment.

P_{eff} may also calculated as follows by rearranging the above equation:

$$P_{eff} = \frac{V}{A} \times \frac{\Delta \text{Amt}\%}{60 * \Delta t} \quad \text{(cm/s)}$$

Δ Amt%/Δt: is the initial slope of a plot of the cumulative percentage of amount transported versus time (min^{-1}).

V: the volume of the apical chamber (mL),

A: the surface area of the monolayer (cm^2); which is 4.7 cm^2 for the transwell insert in this experiment.

3.2.8.6. Flutamide transport pathway study

The Caco-2 cell monolayer was pretreated for 45 min with low calcium medium containing 2.5 mM EGTA. Transport of free flutamide in PBS^+ was determined and compared with a similar study but without any pretreatment. The integrity of the monolayers was checked at the end of each experiment.

4. Results and Discussion

4.1. Solubility of flutamide at different concentrations of ßCD, JD1-128 CD and HPßCD

Selected properties of the inclusion complexes formed between cyclodextrins and drugs can be determined by examining the effect of cyclodextrin concentration on drug solubility. The slope and intercept of the resulting phase-solubility profile can provide information on both the type of complex formed (stoichiometry) and its solution stability.

According to the definitions provided by Higuchi and Connors (1965), the two main types of solubility profiles are A and B (Figure 4.1.1). "A" type curves indicate the formation of soluble inclusion complexes while "B" type relationships indicate the formation of complexes with limited solubility. Each type is further subdivided. If a plot of cyclodextrin concentration versus the concentration of drug solubilized is linear, an A_L type is obtained. Ap systems generally reflect high order complexation at higher cyclodextrin concentrations meaning that more than one cyclodextrin molecule is complexing with each guest molecule. The origin of the type An diagram is uncertain. It may be associated with an alteration in the effective nature of the solvent in the presence of large concentrations of solubilizer, thus leading to a change in the complex formation constant. Another possibility is self-association of drug at higher concentrations, this might affect the apparent degree of complexation. If a complex of

a drug and cyclodextrin is not soluble, a B_I type curve is generated and complexes of limited solubility give B_S type relationships. For A and Bs type systems, the initial linear portion of the curve can be used in examining the efficiency of complexation. The steeper the slope, the better is the complexation.

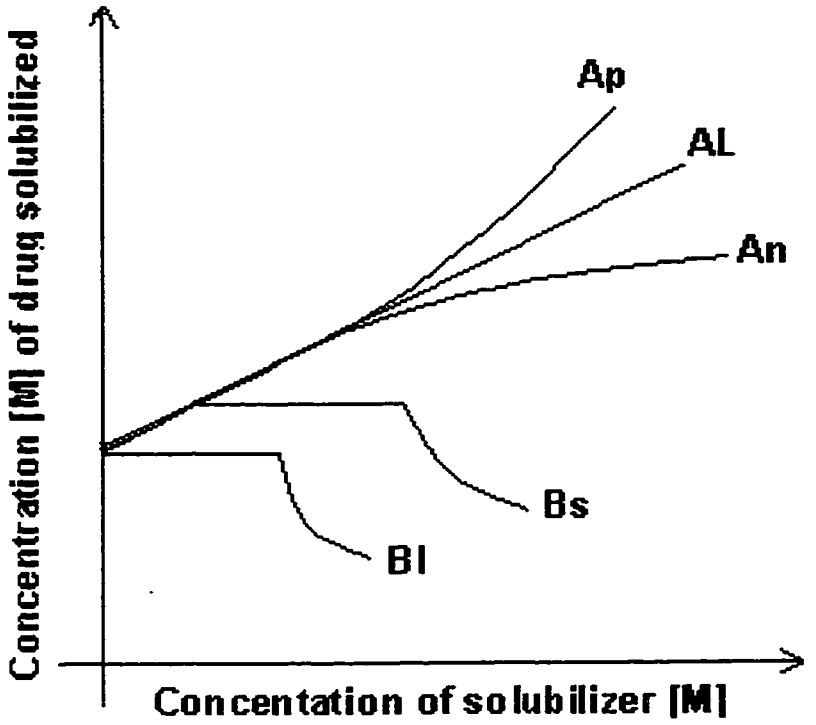


Figure 4.1.1. Model Phase-solubility relationships

Knowledge of the stoichiometry and equilibrium constants for complex formation will permit a quantitative description of the solubility behavior. These quantities can often be evaluated from the phase diagram. In most instances the complexity of the chemical system precludes a complete description of the equilibria, but even in such cases it may be possible to calculate apparent stability constants on the basis of an assumed stoichiometry. For type A phase diagrams, the problem is to determine the stoichiometry of the complex or complexes responsible for the increase in concentration of S (S represents the drug), or, in other words, to evaluate *m* and *n* in the general formula S*mLn* (L represents solubilizer). First, we may note that if the slope of a type A_L line is greater than unity, then at lease one species much be present in which *m* is greater than one, it is obviously impossible for one mole of L to take more than one mole of S in to solution if the complex is of the 1:1 type. On the other hand, a slope of less than unity with type A_L diagram does not necessarily mean that only a 1:1 complex is formed (though is the absence of additional information this assumption is usually made). At low concentration most drug-cyclodextrin complexes are of 1:1 stoichiometry. Even complexes which are of higher order stoichiometry at high concentration and/or drug concentration form 1:1 complexes at lower concentration. (Loftsson and Brewster 1996).

From the solubility equilibrium study (Figure 4.1.2.), 3 h was selected as the appropriate equilibration time. As shown in Figure 4.1.3, 4.1.4, and 4.1.5, the solubility of flutamide increased in a linear fashion as a function of ßCD, JD1-128 or

HPßCD concentration. The solubility limit of the complex appears not to have been reached within the range of concentrations of CDs used in this study. The isotherm is type A_L, which means that there is a linear increase in solubility with unchanged stoichiometry, and indicates a 1:1 (CD : flutamide) binding ratio (Cho *et al.* 1995).

In the HPßCD system, the complexes are highly water-soluble since no precipitation was observed even at HPßCD concentrations as high as 0.35 M, at room temperature. The solubility of flutamide increased almost 170 times in 0.35 M HPßCD (Figure 4.1.6). This increase in solubility may lead to the enhanced bioavailability of flutamide from the HPßCD dosage form. The difference of flutamide aqueous solubility from different CDs (ranges from 82 μM to 162 μM) is due to the analytical method and experimental method variation. In the HPßCD phase solubility study, samples were all in 20 cc vials and UV was used for sample analysis. But in ßCD and JD1-128 CD phase solubility studies, samples were prepared in 1 cc Eppendorf tubes (due to the limitation of CDs available) and HPLC was the analytical method. The difference of compound binding to different containers may also attribute to the above error.

Based on a 1:1 complex model, the stability constant K_s at 298 K was found to be 356 M^{-1} for the flutamide-HPßCD inclusion complex, 334 M^{-1} for flutamide-JD1-128 CD inclusion complex and 164 M^{-1} for the flutamide-ßCD inclusion complex. K_s was estimated using equation: K_s (1:1) = Slope / So (1-Slope) (see Appendix 3 for equation derivation), where So refers to the solubility of the drug in the absence of CD

(Brewster *et al.*, 1989). For HPßCD, K_s (1:1) = 0.054/ (0.00016*(1-0.054)) = 356 M^{-1} (from Figure 4.1.5).

All of the K_s calculated above are much less than 10^5 M^{-1}, which means a very weak stability constants exist between flutamide and each of the 3 CDs. Even though the K_s of HPßCD and JD1-128 CD are little bit higher than ßCD, the differences among them are not statistically significant.

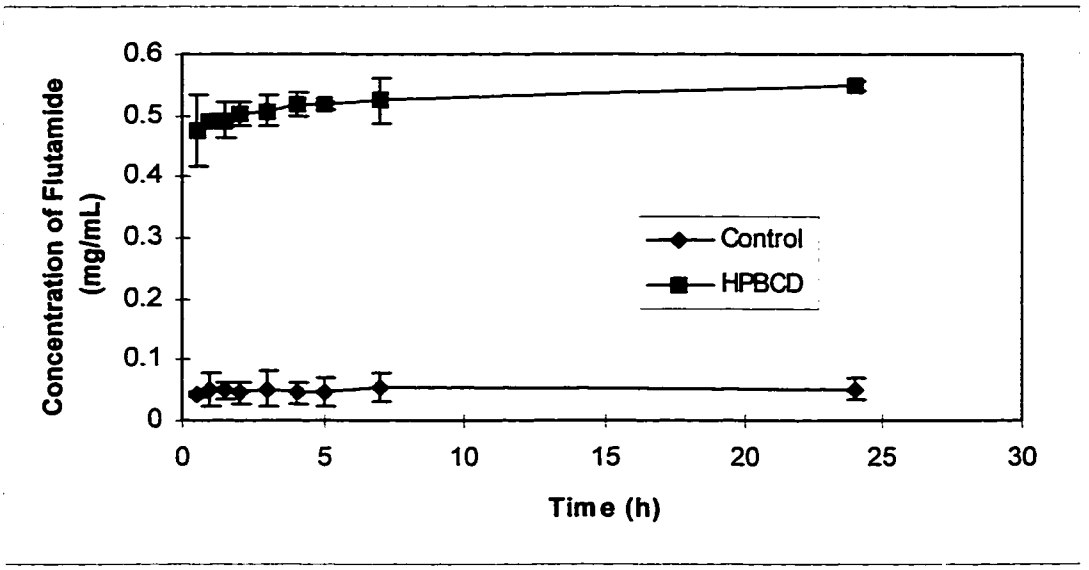


Figure 4.1.2. Equilibration of flutamide in 5% HPβCD at room temperature (n=3).

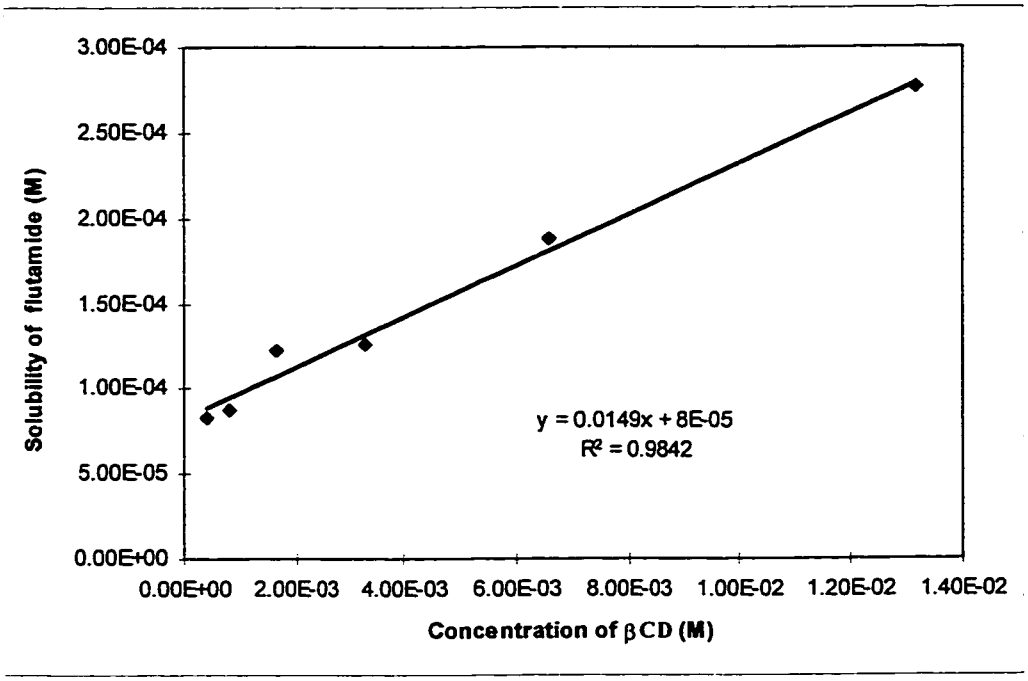


Figure 4.1.3. The phase solubility study of flutamide in βCD (n=2).

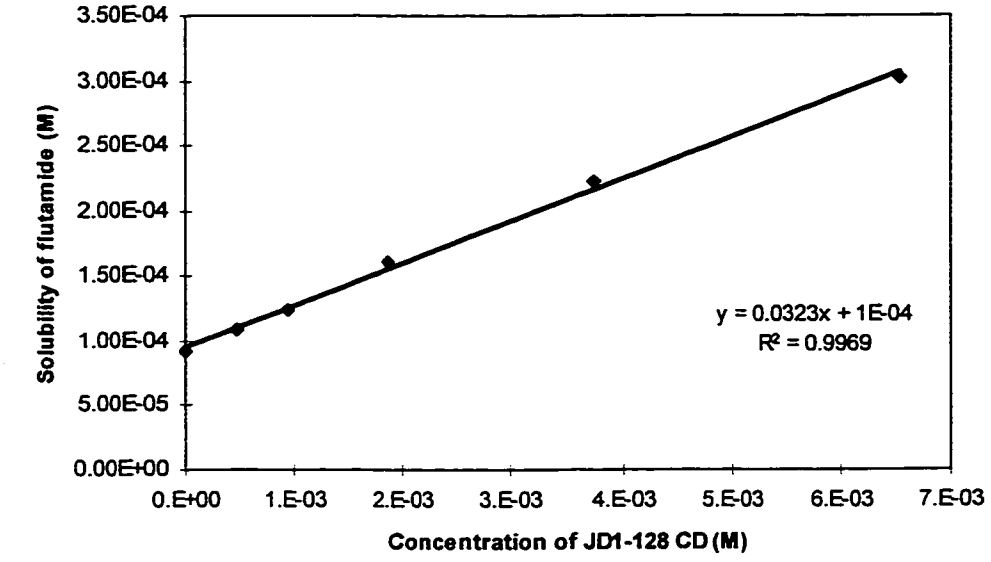


Figure 4.1.4. Phase solubility study of flutamide in JD1-128 CD (n=2).

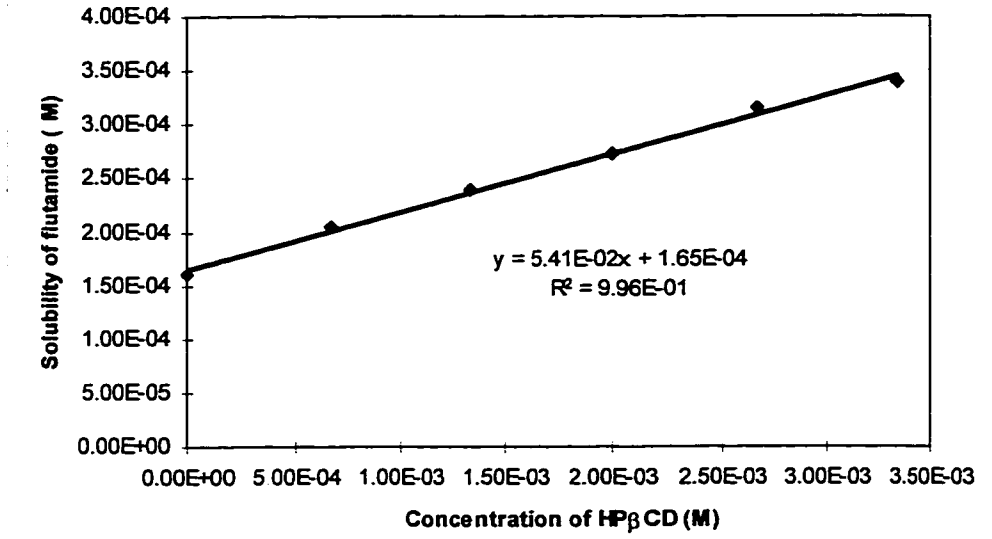


Figure 4.1.5. Phase solubility study of flutamide in HPβ CD (up to 0.0035 M) (n=3)

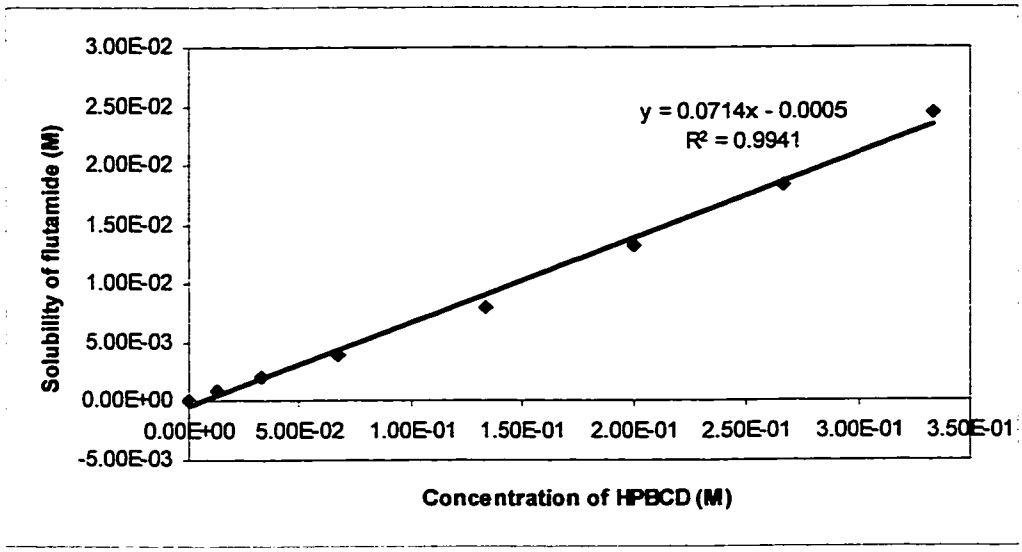


Figure 4.1.6. Phase solubility study of flutamide in HPβ CD (up to 0.35 M) (n=3).

4.2. Temperature effects on the solubility of flutamide in ßCD, JD1-128 CD and HPßCD

The solubility of flutamide in ßCD (0 to 1.8%, w/v), JD1-128 CD (0 to 0.8%, w/v) or HPßCD (0 to 5%, w/v) increased proportionally with increases in temperature. Thermodynamic parameters calculated from these data are shown in Table 4.2.1, 4.2.2 and 4.2.3.

The thermodynamics of flutamide going into HPßCD solution were characterized by a negative $\Delta G°$, indicating that dissolution proceeded spontaneously; $\Delta H°$ for the formation of the flutamide-HPßCD complex was a positive, hence dissolution is endothermic and requires energy. The thermodynamics of flutamide in ßCD and JD1-128 CD were found to have a negative $\Delta G°$ and a negative $\Delta H°$.

The driving forces for inclusion complexation between CD and a guest molecule may include Van der Waals interactions, hydrogen bonding, hydrophobic interaction, release of high-energy water molecules from the cavity of CD and release of strain energy in the ring of CD (Komiyama and Bender, 1978). In any case, there must occur a decrease in the free energy (a negative $\Delta G°$) of the system. Van der Waals forces generally generate from dipole-dipole interactions and are quite weak. Water has a crucial role in the complex formation, since hydration of the cyclodextrin complex is energetically favored as compared with the separate hydration of the components. This phenomenon is called hydrophobic interaction, and as a matter of fact primarily it is not due to the mutual attention of the two components, but rather to

the intrinsic cohesion of the water. There is namely a decrease in the energy of the system caused by the increase of the solvent-solvent interaction, since the contacting surfaces between the solvent and guest molecule, as well as between the solvent and cyclodextrin cavity are reduced.

As demonstrated by the calorimetric data (Szejtli 1982), the compensation effect can be observed in these systems. This means that there is an approximately linear correlation between $\Delta H°$ and $\Delta S°$. In other words, if the enthalpy of formation is small (little heat is liberated during complexation), the entropy value is large, implying that the system has a higher degree of disorder after complex formation, than before. This applies to the cases of complexing with phenol, indole, 1-phenylalanine, tyrosine, 2-aminobezoic acid, 2-nitrophenol and acetic acid. Reversed, if $\Delta H°$ is large, the entropy is negative, i.e. complex formation will result in a higher order of the system.

Unlike the usual complex formation with CD, which results in a relatively large negative $\Delta H°$ and a $\Delta S°$ that can be either positive or negative (Loftsson and Brewster, 1996), flutamide-HPßCD complex formation has a slightly positive $\Delta H°$ and a large positive $\Delta S°$. The large $\Delta S°$ (+20 $cal \cdot K^{-1} \cdot mole^{-1}$) for the complexation of flutamide with CD can be attributed to the transfer of flutamide from aqueous medium to a more apolar medium, such as the cavity of HPßCD. This transfer requires breakdown of structural water molecules around flutamide, resulting in a large positive $\Delta S°$ and a small positive $\Delta H°$. Thus, HPßCD-flutamide complex formation appears to be governed by hydrophobic interaction.

The thermodynamic data available in the literature suggest that cyclodextrins are not very discriminating host molecules. The difference in this experiment may be due to the different natures of HPβCD compared to βCD and JD1-128 CD. Both βCD and JD1-128 CD used in this experiment are pure compounds, but the HPβCD used is an amorphous mixture of thousands of geometric and optical cyclodextrin isomers with a molar of substitution of 0.8. The thermodynamic parameters obtained in the experiment are actually the sum of contributions from different isomers which may have different encapsulation capabilities.

Another possible experimental error involved may be due to the different method in obtaining the thermodynamic parameters. Cramer *et al.* (1967) calculated the value of $\Delta H°$ from the temperature dependence of the rate of constant of the complex formation (from the slope of the log K_s *vs* 1/T correlation); Lewis and Hansen (1973), in turn, measured it directly by calorimetry. The comparison of these two results suggests that only thermodynamic data obtained from direct measurement should be regarded as reliable.

4.3. DSC

The DSC of the free flutamide, ßCD, JD1-128 CD and HPßCD showed sharp endothermal peaks at 110 °C and 300 °C, respectively, and a small exothermic peak at 270 °C. The DSC of the physical mixture also contained the endothermal peaks of the two and the small exothermic peak, whereas the DSC of the complex had no

endothermal peak for free flutamide. It was also found that the endothermal peaks of HPβCD were appreciably broadened, which may due to the change of crystal nature after the complex formation. All of these were taken as an indication of inclusion complex formation with flutamide (Figure 4.3.1, 4.3.2, 4.3.3).

Table 4.2.1. Thermodynamic parameters for complexation of flutamide with βCD.

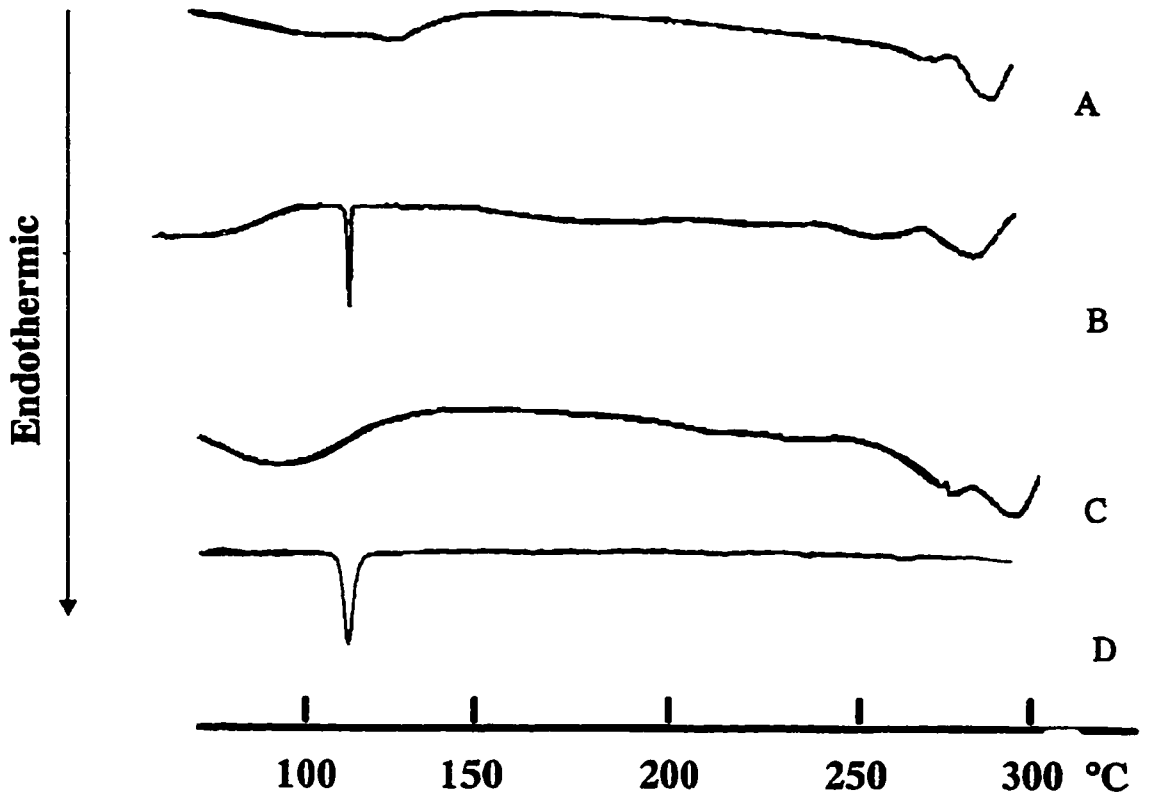
STABILITY CONSTANTS K_S (M^{-1})							ΔG°(298 K)	ΔS°(298K)	ΔH°
293 K	298 K	305 K	308 K	313 K	318 K	323 K	(kcal.mole^{-1})	(cal.K^{-1}.mole^{-1})	(kcal.mole^{-1})
167	164	163	142	145	140	138	-3.07	5.74	-1.36

Table 4.2.2. Thermodynamic parameters for complexation of flutamide with JD1-128CD.

STABILITY CONSTANTS K_S (M^{-1})					ΔG°(298 K)	ΔS°(298 K)	ΔH°
293 K	298 K	308 K	313 K	323 K	(kcal.mole^{-1})	(cal.K^{-1}.mole^{-1})	(kcal.mole^{-1})
505	334	302	269	240	-3.52	0.08	-3.49

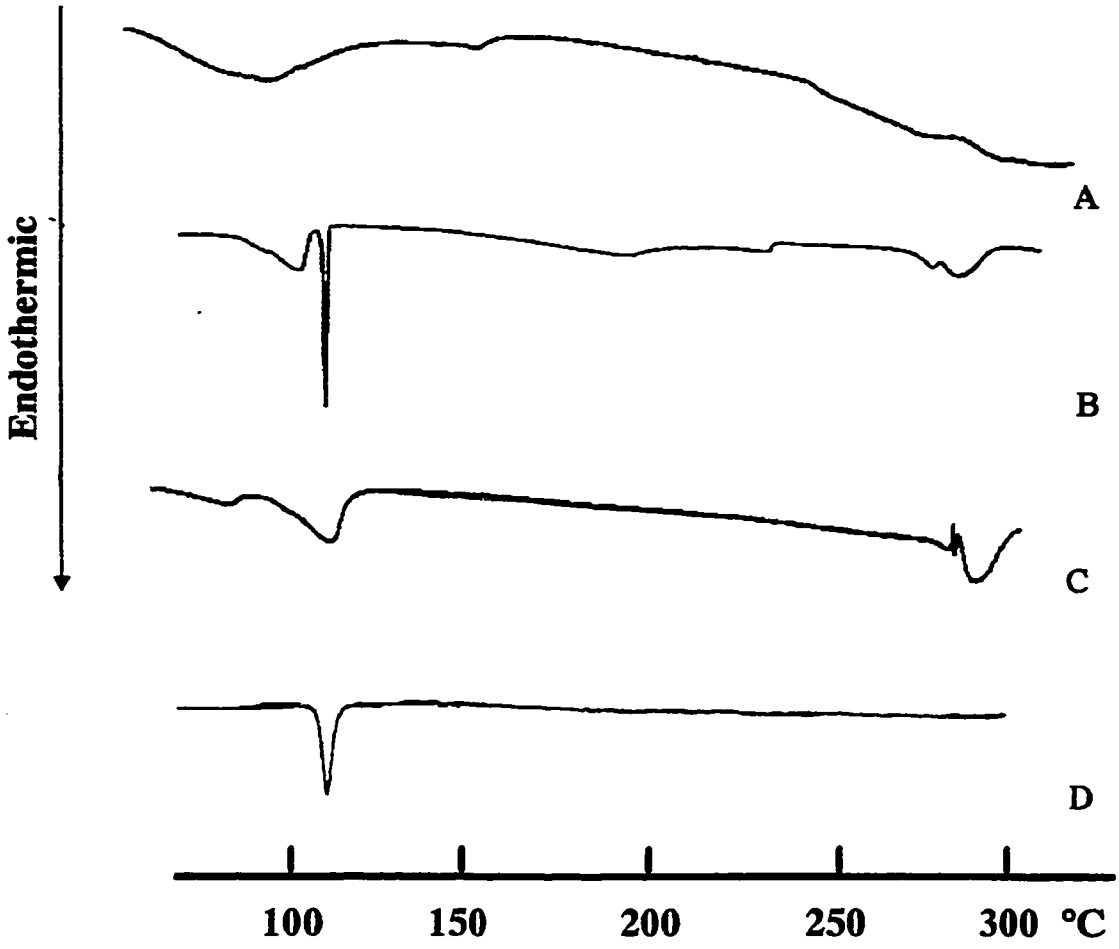
Table 4.2.3. Thermodynamic parameters for complexation of flutamide with HPβCD.

STABILITY CONSTANTS K_S (M^{-1})				ΔG°(298 K)	ΔS°(298 K)	ΔH°
303 K	310 K	318 K	323 K	(kcal·mole^{-1})	(cal·K^{-1}·mole^{-1})	(kcal·mole^{-1})
341	386	444	452	-3.48	21.14	2.85



A: Flutamide βCD complex
B: Flutamide βCD physical mixtrue
C: βCD
D: Flutamide

Figure 4.3.1. DSC spectra for the flutamide-βCD inclusion complex (A), physical mixture of flutamide and βCD (B), βCD (C) and flutamide (D).



A: Flutamide JD128CD complex
B: Flutamide JD128CD physical mixtrue
C: JD128CD
D: Flutamide

Figure 4.3.2. DSC spectra for the flutamide-JD1-128 CD inclusion complex (A), physical mixture of flutamide and JD1-128 (B), JD1-128 CD (C) and flutamide (D).

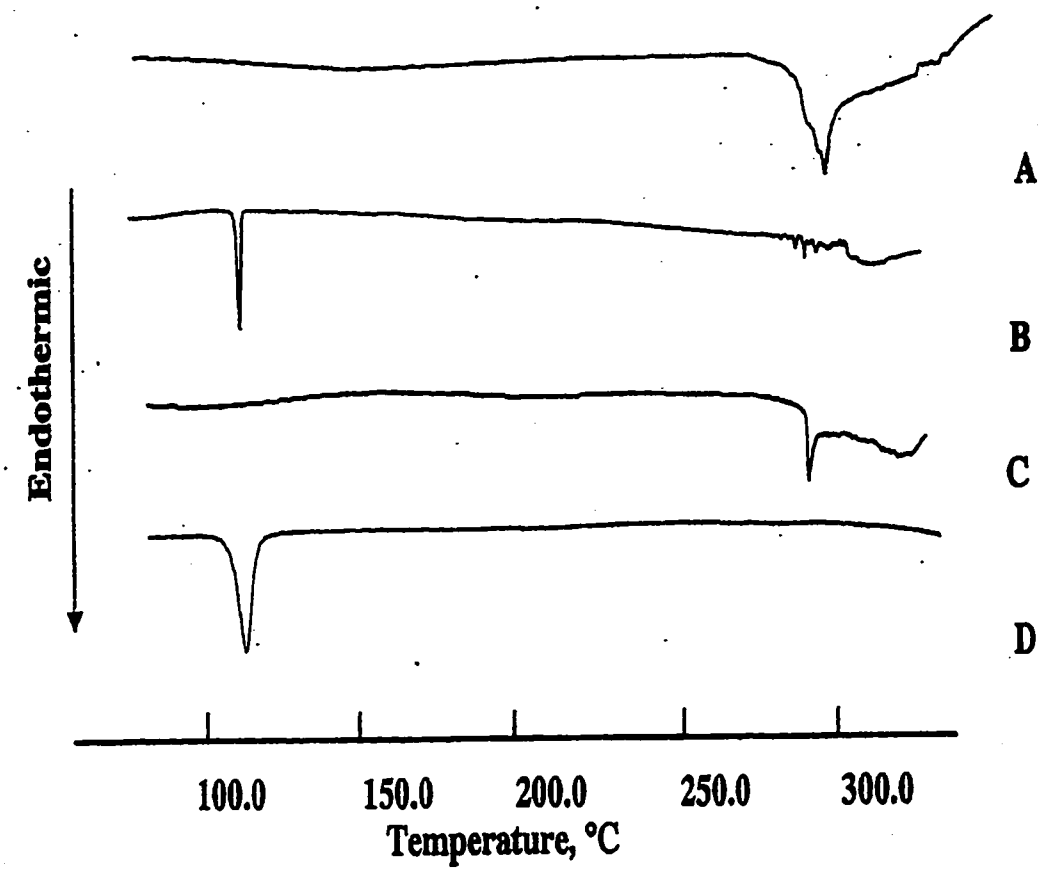


Figure 4.3.3. DSC spectra for the flutamide-HPβCD inclusion complex (A), physical mixture of flutamide and HPβCD (B), HPβCD (C) and flutamide (D).

4.4. NMR

DSC is only one of the analytical methods used to confirm the formation of inclusion complexes. Although the DSC test was positive for flutamide complexation for all the three CDs, NMR was used for further confirmation.

4.4.1. ^{19}F NMR

In this experiment, the ^{19}F NMR chemical shifts of fluorine atoms in flutamide as a function of the concentration of HPßCD and JD1-128, are shown in Table 4.4.1.1. and 4.4.1.2. The observed maximum chemical shift change reached 1.36 ppm, in 33.3 mM HPßCD. This was taken as further proof of the formation of an inclusion complex between HPßCD and flutamide.

The free and complexed forms of flutamide gave rise to only one NMR fluorine signal. This may due to the fast averaging of the exchange between free and bound state at equilibrium:

$$\text{flutamide (free)} + \text{CD (free)} \overset{K_s}{\rightleftharpoons} \text{flutamide-CD inclusion complex}$$

The stability constant K_s can be derived from NMR parameters in the case of 1:1 complexes, in the presence of a large excess of CD, using the Benesi-Hildebrand method (Benesi *et al.* 1949; Florence *et al.* 1990):

$$\Delta\delta_{obs} \cdot ([CD]_{total})^{-1} = K_s \cdot (\Delta\delta_c - \Delta\delta_{obs})$$

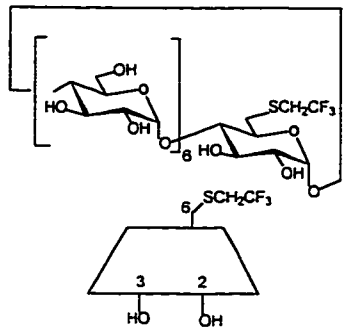
where $\Delta\delta_{obs}$ is the ^{19}F chemical shift difference between free flutamide (in the absence of HPβCD or JD1-128 CD) and the observed value from each sample.

$[CD]_{total}$ refers to the total concentration of HPβCD or JD1-128 CD, including free and complexed CD. $\Delta\delta_c$ represents the ^{19}F chemical shift difference between free flutamide and the pure CD-complex. The negative slope of a plot $\Delta\delta_{obs}\cdot([CD]_{total})^{-1}$ against $\Delta\delta_{obs}$ will generate K_s. The K_s for flutamide-HPβCD inclusion complex, obtained from this procedure, is 357 M^{-1} at 298 K, which is quite close to the value obtained from the phase solubility test (356 M^{-1} at 298 K). Also, the K_s for flutamide-JD1-128 inclusion complex, obtained from this procedure, is 347 M^{-1} at 298 K, which is quite close to the value obtained from its phase solubility test (334 M^{-1} at 298 K).

In principle, the direction of change of the chemical shift should reflect changes in the environment that arise on inclusion of the guest. The induced shifts will depend on the extent of penetration of the residue into the cyclodextrin cavity. Shifts experienced by the protons of the host are essentially due to ring-currant and magnetic anisotropy effects created by the aromatic drug, although down field shifts of the aromatic protons of "guest" are caused by variations of the local polarity (Djedaini *et al.* 1990). The stability constants for the 1:1 complexes have been calculated from the dependence of the ^{19}F NMR chemical shift on the concentration of cyclodextrin by Guo *et al.* (1991) and Brown *et al.* (1991). The presence of the fluorine substituents of a drug facilitates the study of its inclusion by cyclodextrins using ^{19}F NMR spectroscopy. The $\Delta\delta_{obs}$ in both this study and the literature was less than 2 ppm, which may seem too small compared with the whole ^{19}F spectrum (960 ppm). Although the above literature showed the results from the triplicate samples, this study

was based on only one sample points, because of the limited spectrometer time available. This may introduce variation in the K_s calculations.

The CF_3 group of flutamide is attached to the aromatic benzene ring. Since the stability constant can be estimated from the chemical shift changes of flutamide fluorine, we may assume that the benzene ring is the part of molecule which penetrates the inner cavity of HPβCD or JD1-128 CD.



On the other hand, the ^{19}F NMR chemical shifts of fluorine in JD1-128 CD under the same conditions were found to have no significant change. Since fluorine substitution in JD1-128 CD was a "SCH_2CF_3"on C_6 position, the whole chain where the fluorine is situated may not be long enough to fall into the CD inner cavity. Therefore, the inclusion complex formation may not be able to affect the fluorine atom's environment in a significant manner.

In conclusion, the solubility of flutamide can be increased markedly by forming an inclusion complex with HPßCD and JD1-128. DSC and ^{19}F NMR both indicated the formation of an inclusion complex. Stability constants (K_S) were calculated for a 1:1 complex using established methods based on solubility data and using a recently reported NMR method. This work confirms the utility of ^{19}F NMR spectroscopy for characterizing CD complexes with flourine-containing drugs and provides a rationale for further *in vivo* studies on a flutamide-CD formulation.

Table 4.4.1.1. Effect of HPβCD on the ^{19}F NMR chemical shift of flutamide.

$\Delta\delta_{OBS}$ (ppm)	[HPβCD] (M)	$\Delta\delta_{OBS}$/[HPβCD] (ppm.M^{-1})	^{19}F NMR CHEMICAL SHIFT (ppm)
0.00	0.0		59.75
0.52	1.7E-3	305.88	60.27
0.81	3.3E-3	245.45	60.56
1.01	1.0E-2	101.00	60.76
1.18	1.3E-2	88.72	60.93
1.36	3.3E-2	40.84	61.11

Table 4.4.1.2. Effect of JD1-128 CD on the ^{19}F NMR chemical shift of flutamide.

$\Delta\delta_{OBS}$ (ppm)	[JD1-128 CD] (M)	$\Delta\delta_{OBS}$/[JD1-128 CD] (ppm.M^{-1})	^{19}F NMR CHEMICAL SHIFT (ppm)
0.00	0.00		59.75
0.33	1.4E-3	230.32	60.08
0.39	2.4E-3	162.09	60.14
0.52	3.4E-3	153.88	60.27

4.4.2. ^{1}H NMR

Because of the low water solubility of flutamide, a high number of scans (512) was required to provide the sensitivity for ^{1}H NMR measurement. Only HPβCD was chosen for this study, since it had the highest binding constant (357 M^{-1}) and water solubility with flutamide according to the previous calculation (4.4.1.). The HPβCD used in the project is a mixture of substitution isomers with a molar substitution of 0.8, and therefore it is pointless to look at the ^{1}H chemical shift change of HPβCD mixture. The ^{1}H chemical shift change of the benzene ring in flutamide will provide useful information, since the HPβCD has no resonance in this region (Figure 4.4.2.1 and Figure 4.4.2.2). Also, as was discussed earlier, the benzene ring moiety appears to be inserted into the hydrophobic inner cavity of HPβCD.

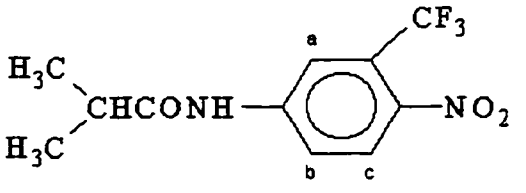


Figure 4.4.2.3 represents the amplified benzene ring region of ^{1}H NMR spectrum (from 3900 Hz to 4300 Hz) for flutamide. In order to confirm the position of flutamide protons in the ^{1}H NMR spectrum, the doublet at 4000 Hz was decoupled and collapsed to a singlet at 4105 Hz (Figure 4.4.2.4). Proton "a" is located at 4267.39 Hz and proton "b" and "c" are located at 4000 Hz and 4105 Hz, respectively.

Figure 4.4.2.5 shows the ^{1}H NMR chemical shift change of the aromatic flutamide protons in the HPβCD-flutamide inclusion complex. Proton "b" has shifted towards "c" and overlapped, while proton "a" has not changed. The formation of inclusion complex between flutamide and HPβCD in which the aromatic moiety of flutamide is inserted into the hydrophobic cavity, is supported by these chemical shifts of the aromatic protons.

Based on the above physico-chemical experiments, HPβCD was found to be the best solubilizer for flutamide among the 3 CDs. Due to the small increase of the flutamide water solubility in the process of JD1-128 CD and limited intrinsic water solubility of JD1-128 CD, no further *in vivo* experiment were undertaken with this cyclodextrin.

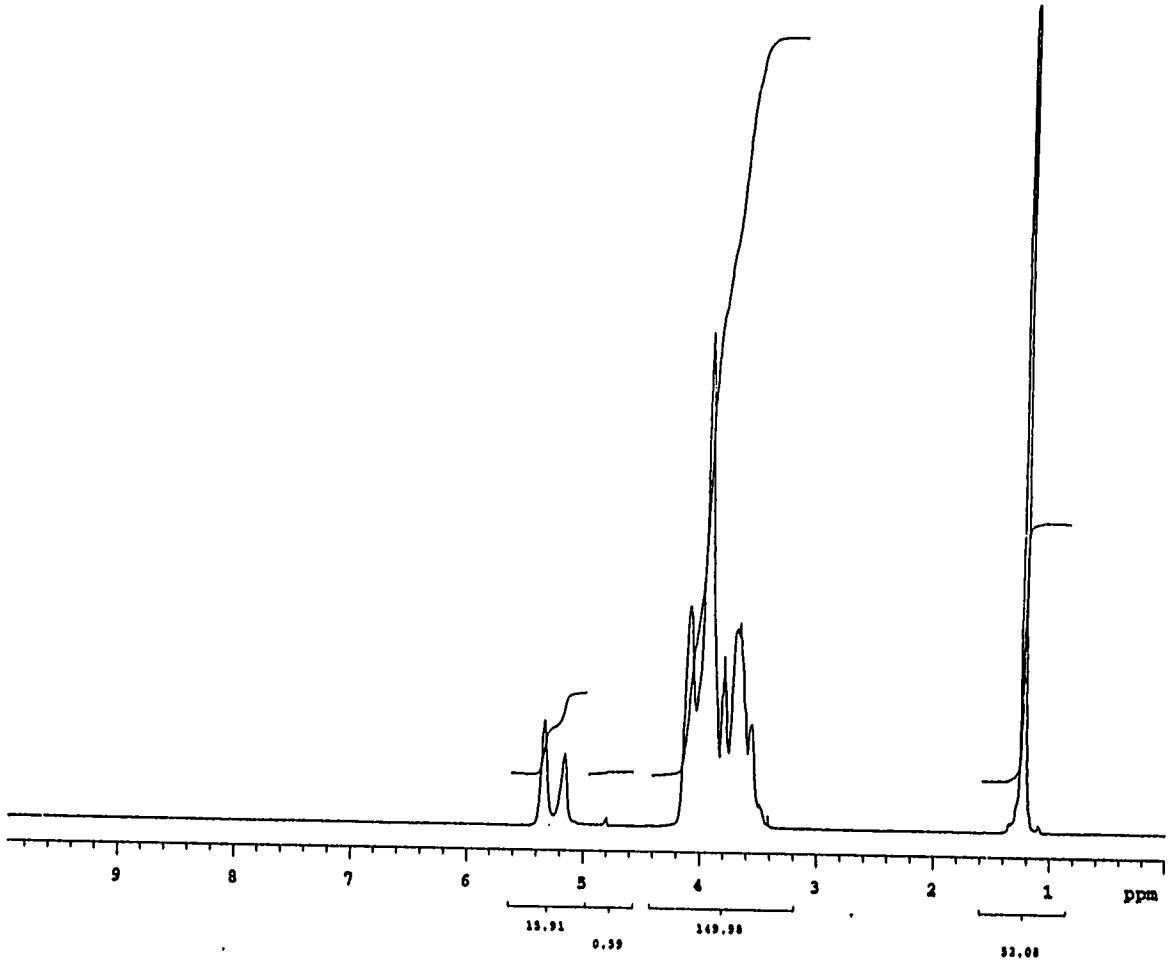


Figure 4.4.2.1. ^{1}H NMR spectrum of HPβCD

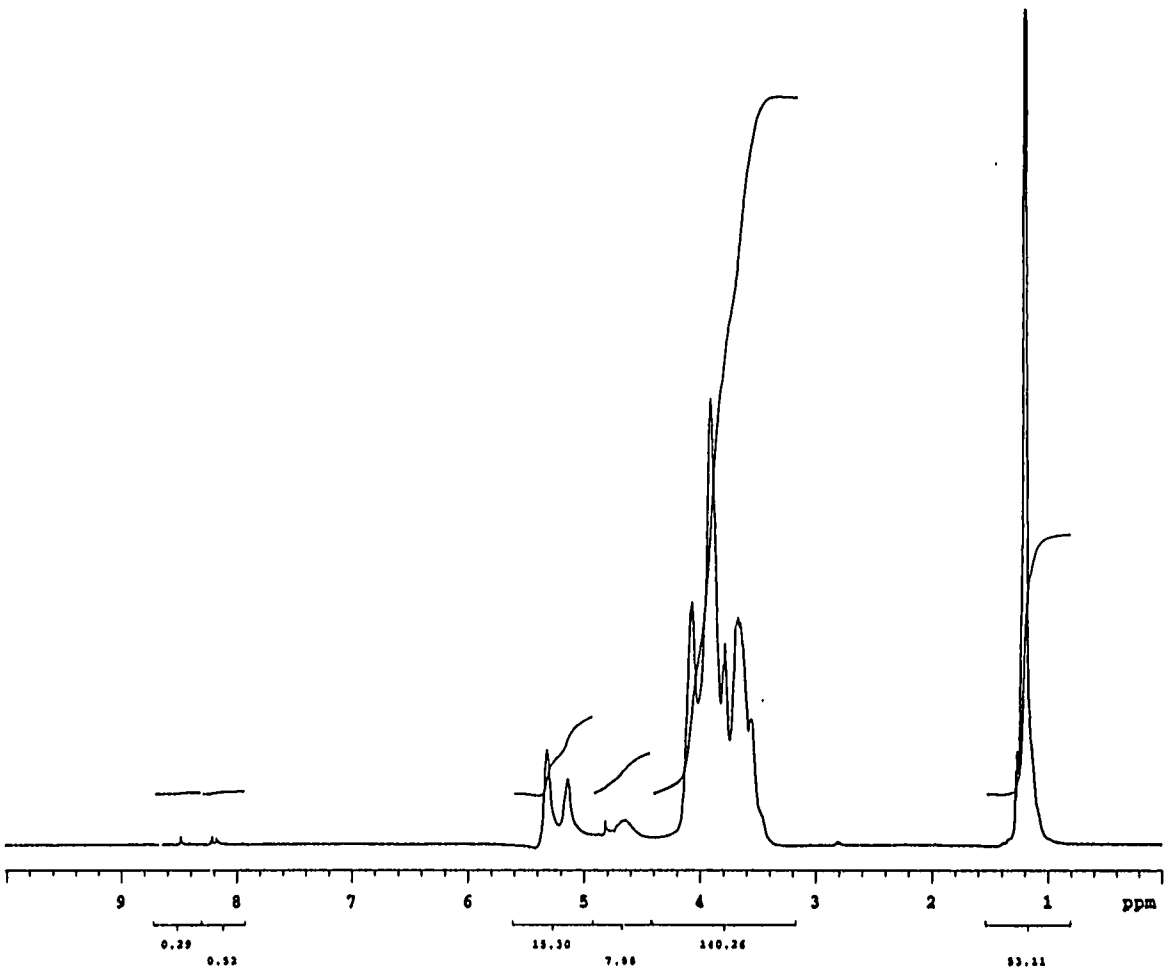


Figure 4.4.2.2. ^{1}H NMR spectrum of HPβCD-flutamide inclusion complex

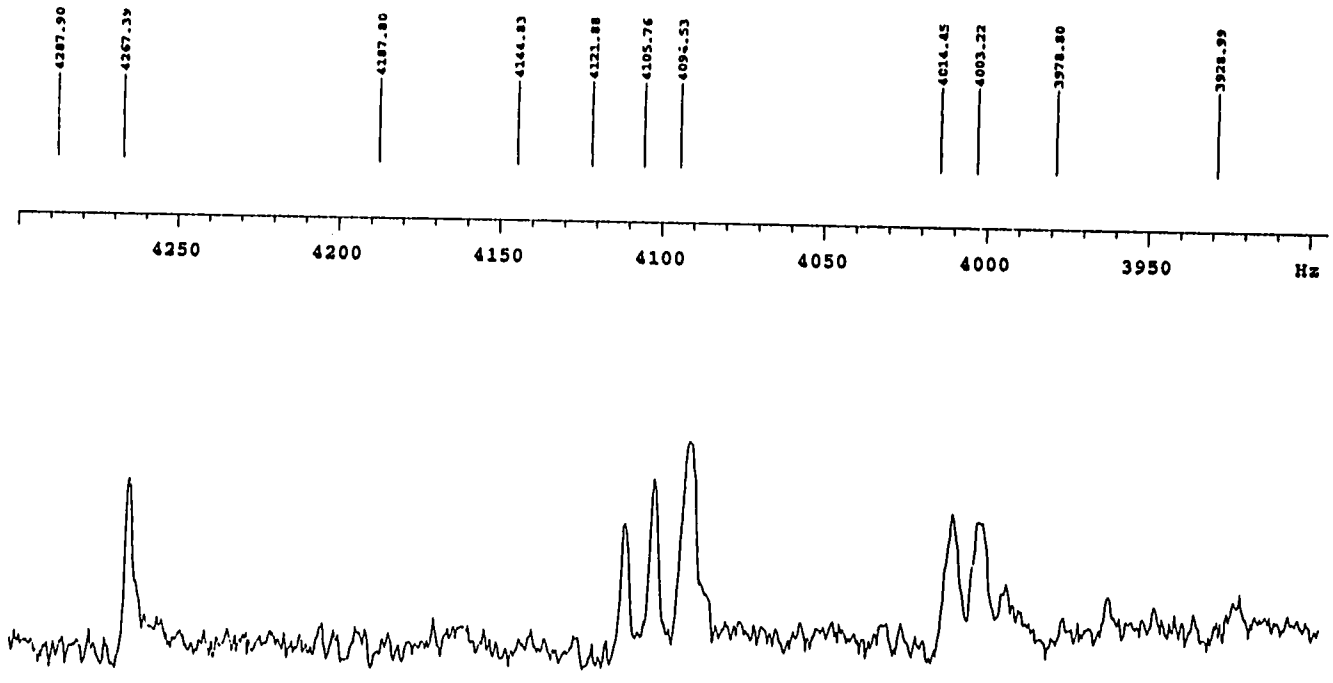


Figure 4.4.2.3 ^{1}H NMR spectrum of flutamide

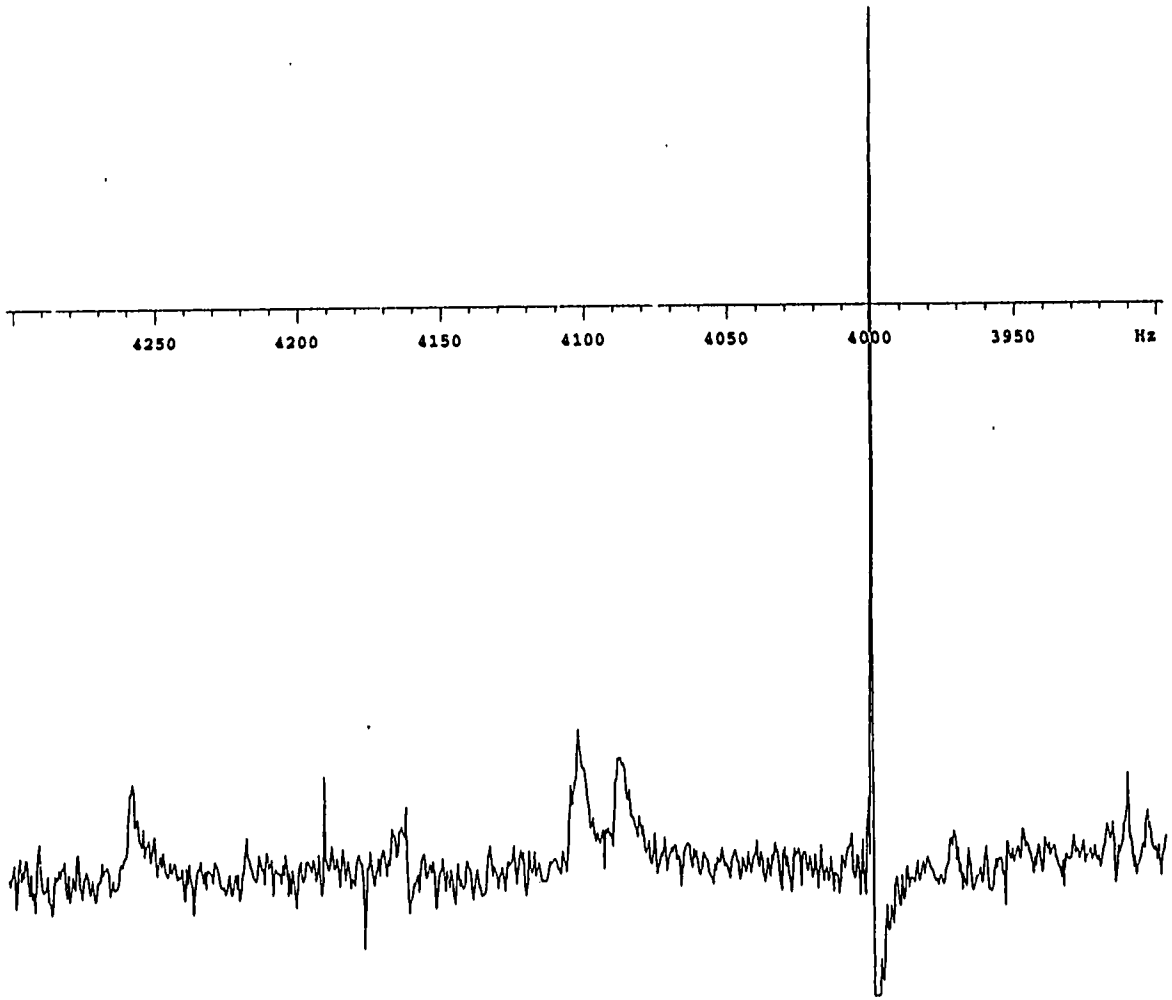


Figure 4.4.2.4 Decoupling of doublet at 4000 Hz for ^{1}H NMR spectrum of flutamide

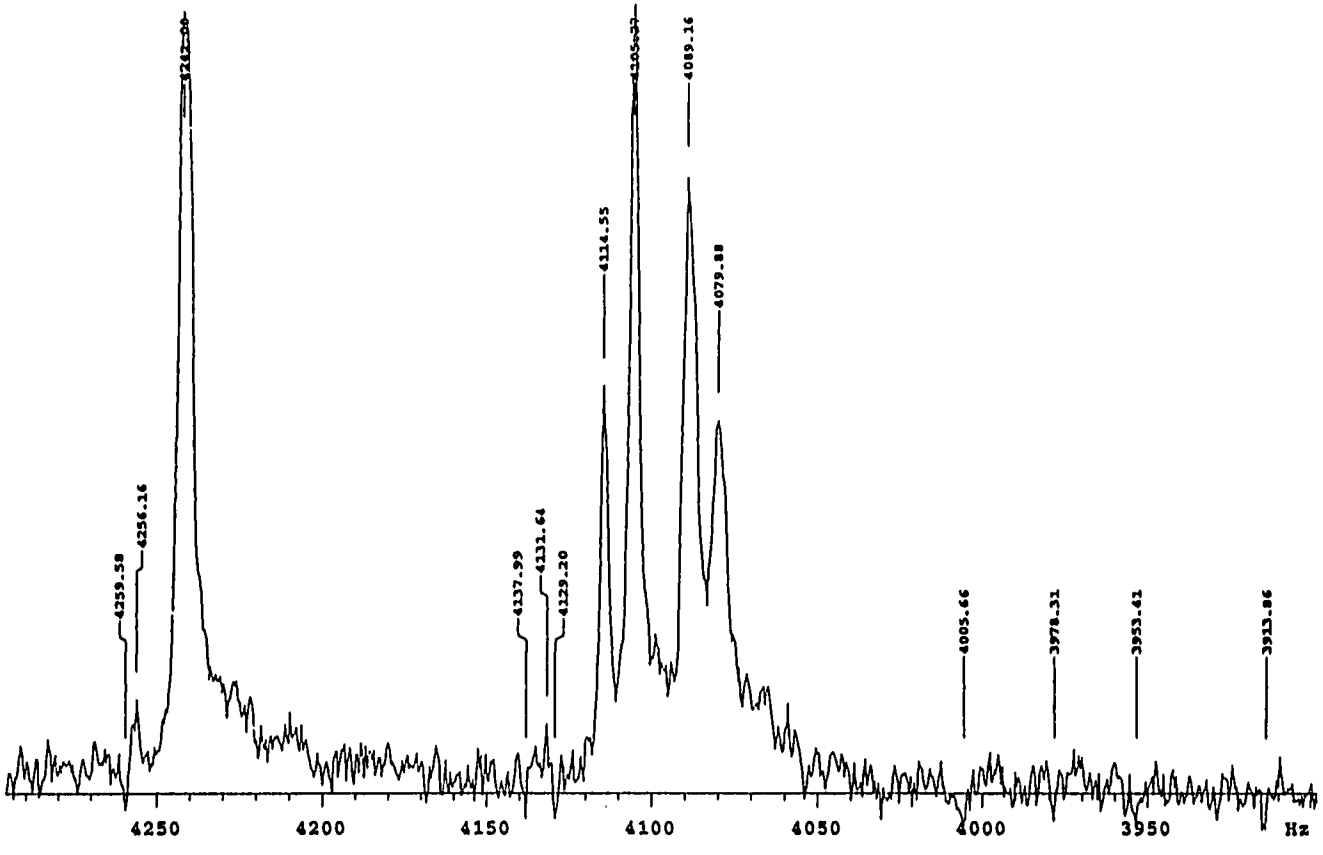


Figure 4.4.2.5.^{1}H NMR spectrum of HPβCD-flutamide inclusion complex.

4.5. *In vitro* drug dissolution experiments

This experiment was designed to find the effect of HPβCD on drug release. It provided a relative comparison of flutamide release from the two dosage forms, free (powdered) flutamide and the flutamide-HPβCD complex. It was found that the flutamide-HPβCD inclusion complex dissolved much more rapidly than flutamide itself (Figure 4.5.). Flutamide was assumed to be stable in the simulated gastric, since no such issue had been raised in any of the literatures. The observed increase in dissolution rate may be due to the increase in solubility, as well as to an increase in wettability, and rapid dissociation of the complex in the dissolution medium.

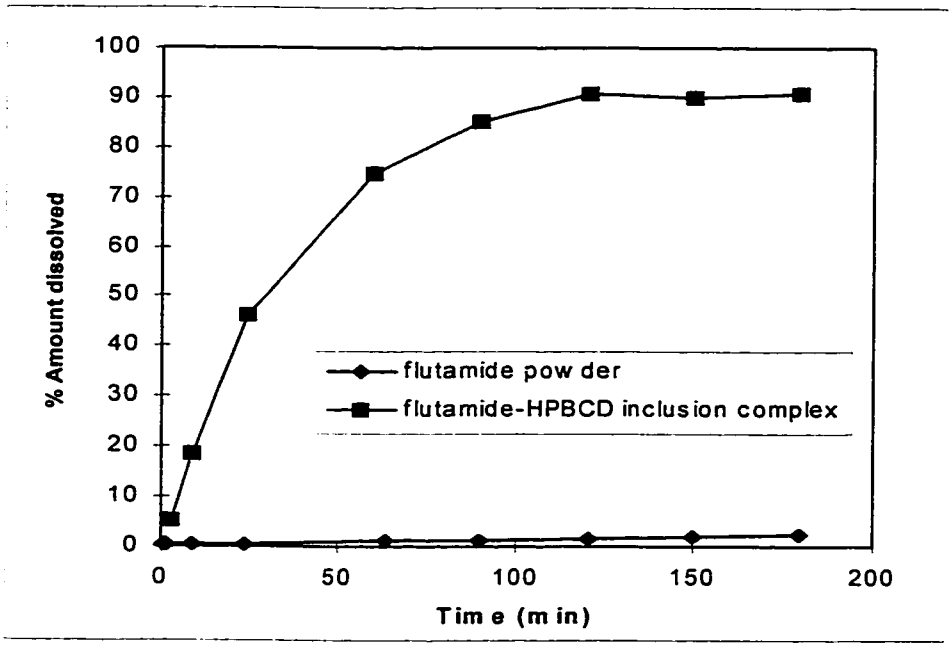


Figure 4.5. Dissolution profiles of flutamide and its HPβCD inclusion complex in simulated gastric fluid at 37°C.

4.6. Oral pharmacokinetics and bioavailability of flutamide from suspension and HPβCD complex dosage forms

The HPLC elution spectrum for flutamide is shown in Figure 4.6.1. The retention times of the internal standard, 2-HY-FLT and FLT were at 5 min, 6 min and 9 min respectively. The individual plasma concentrations of flutamide and 2-HY-FLT attained in each subject after oral administration of suspension and inclusion complex dosage forms are reported in Table 4.6.1 and Table 4.6.2, whereas the profiles of mean plasma concentrations over time are shown in Figure 4.6.2 and Figure 4.6.3. The calculated pharmacokinetic parameters are presented in Table 4.6.3.

Following the administration of the flutamide-HPβCD complex, the mean area under the plasma concentration curve (AUC) (1580±228 ng.h/mL), and the maximum plasma concentration (C_{max}) (1456±79 ng/mL) of flutamide were significantly higher than those obtained after the administration of the suspension form of flutamide (748±206 ng/mL and 230±111ng.h/mL, respectively). Moreover, maximum plasma concentrations appeared at a shorter time after dosing with the HPβCD complex (T_{max} = 0.56±0.10 h) than with the suspension (T_{max} = 2.33±0.29 h) (p = 0.0005).

In conclusion, the administration of the flutamide-HPβCD complex dosage form produces the shorter T_{max} and higher C_{max} than the flutamide suspension formulation. Since flutamide is absorbed by a passive diffusion mechanism, its absorption process could be affected by the increased drug solubility and increased drug dissolution rate showed in the *in vitro* drug dissolution experiment. The

calculated value of the relative bioavailability ($F = (Dose_{suspension}/ Dose_{HP\beta CD}) \times (AUC_{suspension}/AUC_{HP\beta CD}) * 100$) was 221 for flutamide and 225 for 2-HY-FLT. This data clearly shows the increase in the bioavailability produced by the drug inclusion complex.

The use of cyclodextrins to increase the oral bioavailability of a drug through the increase of apparent drug dissolution rate has usually involved the use of the parent cyclodextrins or their more water soluble derivatives (Rajewski and Stella 1996). Such applications have been successful when the rate-limiting step in drug absorption is dissolution of the drug itself and not absorption across the GI mucosa (Szejtli 1994). While many of these studies do not correlate the increased bioavailability with increased dissolution rate, most reports include the administration of uncomplexed drug or commercial formulation to demonstrate the relative bioavailability. Few studies have investigated whether apparent cyclodextrin dependent increases in oral bioavailability are solely a result of increasing the rate at which drug is available for absorption or whether cyclodextrin-induced changes in mucosal permeability play a supporting role. This will be discussed later in the section dealing with the Caco-2 model experiments.

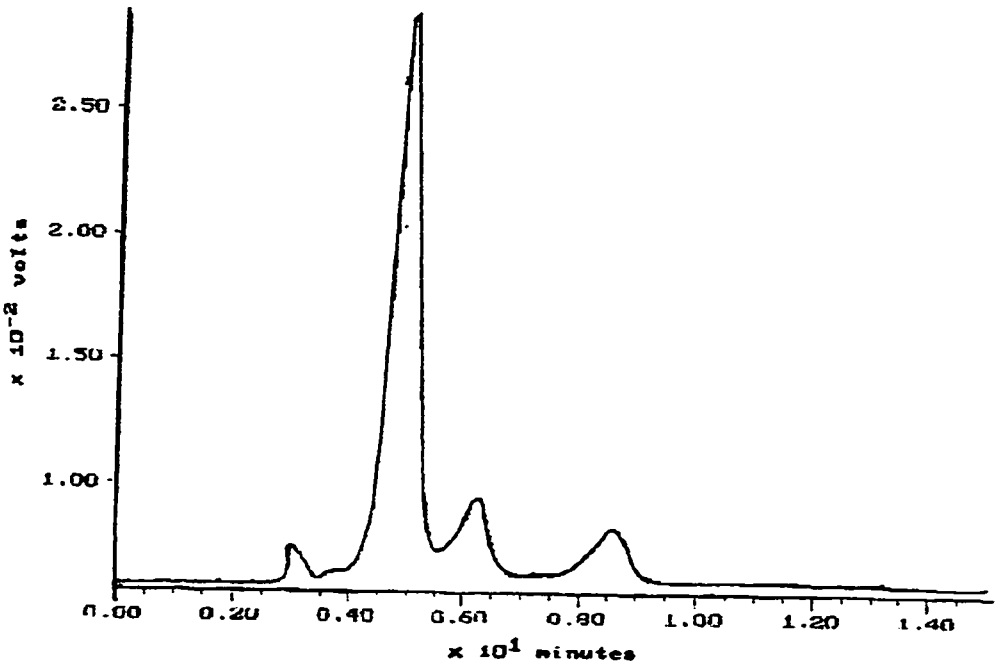


Figure 4.6.1. HPLC chromatograms for 2-hydroxy-5-nitro-benzaldehyde (the internal standard) (retention time = 5 min), 2-HY-FLT (retention time = 6 min) and FLT (retention time = 9 min) using a Radial Pak C18 (10 μm) column with Methanol : Water : Glacial Acetic Acid : Triethylamine (*61:38:1:0.02*, v/v/v/v) as mobile phase and UV detection at 300 nm.

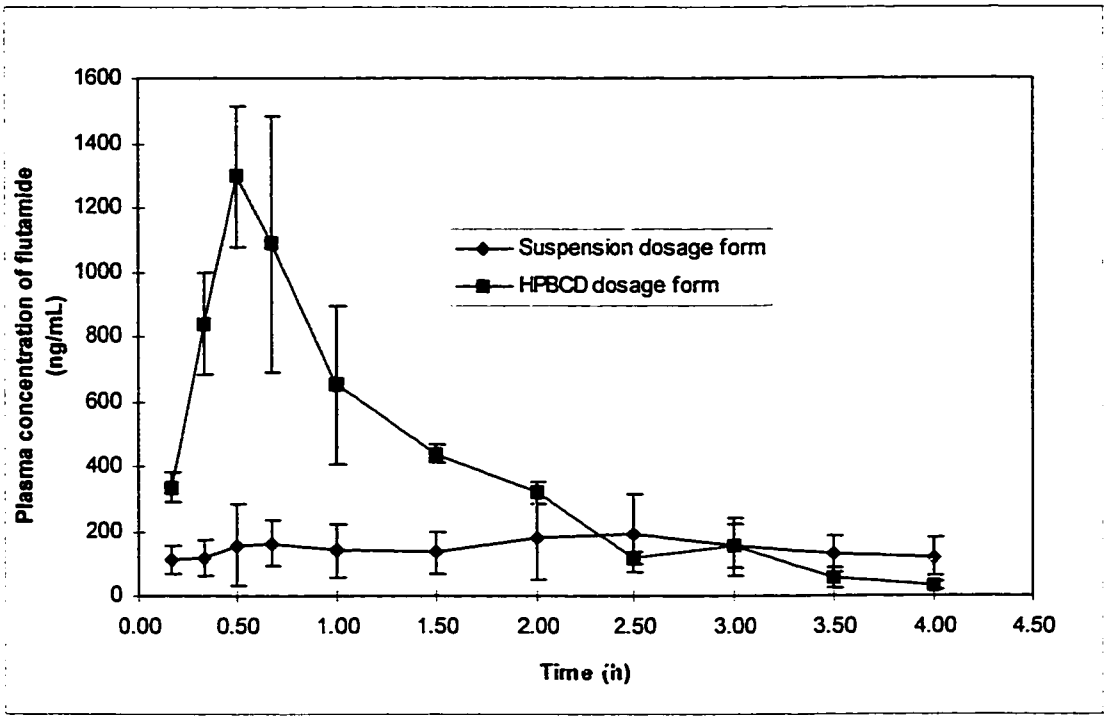


Figure 4.6.2. Plasma concentration profile of flutamide after oral administration of the suspension (n=3) and HPβCD complex (n=3) dosage forms (Dose= 15 mg/kg).

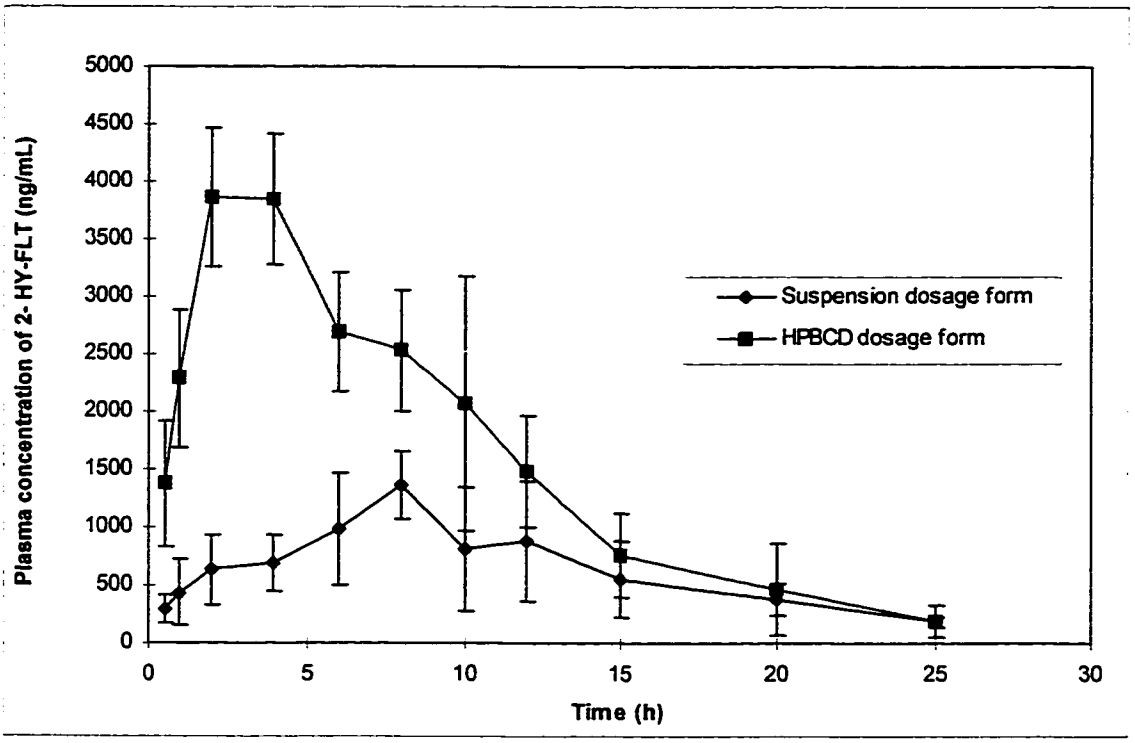


Figure 4.6.3. Plasma concentration of 2-HY-FLT after oral administration of the suspension (n=3) and HPβCD complex (n=3) dosage forms (Dose = 15mg/kg).

Table 4.6.1. Plasma concentration of flutamide after oral administration of the suspension and HPβCD complex dosage forms (Dose = 15 mg/kg)

	Plasma concentration of flutamide (ng/mL)					
	Suspension dosage form			HPβCD dosage form		
Time(hr)	Animal#1	Animal#2	Animal#3	Animal#1	Animal#2	Animal#3
0.17	96	76	156	283	354	367
0.33	119	61	169	703	802	1014
0.5	78	86	302	1371	1470	1051
0.67	139	105	244	982	752	1527
1	125	63	229	757	374	825
1.5	146	63	189	448	460	408
2	152	62	315	353	302	295
2.5	271	56	253	134	234	114
3	189	48	219	169	100	214
3.5	136	72	182	60	79	80
4	103	71	186	42	21	86

Table 4.6.2 Plasma concentrations of 2-HY-FLT after oral administration of the suspension and HPβCD complex dosage forms (Dose = 15mg/kg).

	Plasma Concentrations of 2-HY-FLT (ng/mL)						
	Suspension dosage form			HPβCD dosage form			
Time(hr)	Animal#1	Animal#2	Animal #3	Animal#1	Animal#2	Animal#3	Animal#4
0.5	361	151	359	845	1222	2141	1278
1	700	130	486	1523	2384	2261	2980
2	973	500	416	3752	4727	3349	3607
4	968	618	498	3599	4447	4162	3172
6	734	1536	677	3001	1932	2854	2981
8	1079	1665	1347	2645	2851	1850	2761
10	628	1414	403	3315	1743	1197	2007
12	541	1477	623	983	1379	1430	2147
15	545	880	220	585	785	428	1255
20	421	494	227	206	823	103	730
25	246	152	206	115	123	134	383

Table 4.6.3. Pharmacokinetics comparison after oral administration of the suspension (n=3) and HPβCD complex (n=3) dosage forms (Dose = 15mg/kg).

	Suspension dosage form		HPβCD dosage form	
	Flutamide	2-hydroxyflutamide	Flutamide	2-hydroxyflutamide
AUC (∞): (ng/mL. h)	748±206	18070±4705	1580±228 [a]	40629±5875 [b]
Cmax (ng/mL)	230±111	1364±293	1456±79 [a]	4062±502 [b]
Tmax (h)	2.33±0.29	8.00±0.00	0.56±0.10 [a]	3.50±0.41 [b]

[a, b] Significance of differences from suspension dosage form: $P<0.01$

4.7. Pharmacokinetics of flutamide after *i.v.* administration of co-solvent and HPβCD complex dosage forms

Since in the *i.v* administration experiment, the jugular vein catheter was used as both the injection site and the sampling site, cleaning of the catheter after the injection was essential. The tube washing experiment shows that for method a) (saline wash only), 0.1% of the injected dose (640 μg) was eluted in the final wash with methanol. Although the amount left in the tube was only 640 ng, this could produce a concentration as high as 3200 ng/mL in the first 0.2 mL blood sample. In a preliminary animal experiment, the first blood sampling concentration was around 9000 ng/mL and consequently more than 30% of this may have been due to the unclean catheter. With method b) (blood wash followed by saline wash), nothing above the detection limit was observed in either the final saline wash or in the final methanol wash. It was concluded that method b) was more effective than method a) and that it would be used in the *i.v.* pharmacokinetics experiments.

As shown in Figure 4.7.1, 4.7.2, the initial concentration detected at time 0.08 h was at 3781±494 (ng/mL) after *i.v.* administration of the flutamide HPβCD formulation. Thereafter, flutamide levels decreased rapidly with a $T_{1/2}$ (λz) of 0.40±0.16 h. With the flutamide co-solvent dosage form, the flutamide concentration was 3907±388 (ng/mL) at time 0.08 h after injection. After peak blood levels were attained, flutamide concentration decreased with a $T_{1/2}$ (λz)=0.24±0.06 h, which is not significantly different when compared with the flutamide HPβCD dosage form.

The area under the plasma concentration time profile (AUC) shown in Table 4.7.1, 4.7.2 and the pharmacokinetic parameters calculated in Table 4.7.3. demonstrate that the AUC after *i.v.* injection of the flutamide HPβCD dosage form (1355±162 ng/mL.h) was similar to that calculated after *i.v.* injection of the flutamide co-solvent form (1421±283 ng/mL.h). The volume of distribution (V_z) for the flutamide in HPβCD form (369±191 mL) was no different from that for the co-solvent dosage form (242±24 mL) and similarly, the observed clearance after the flutamide-HPβCD form (735±88 mL/h) was identical to that for the co-solvent dosage form (714±144 mL/h).

Levels of 2-hydroxyflutamide, the major metabolite of flutamide, reached peak values of 760±203 ng/mL at 0.6±0.1 h after injection of the flutamide HPβCD form and 765±154 ng/mL at 0.9±0.2 h after injection of the flutamide co-solvent form. The AUC for 2-hydroxyflutamide after administration of HPβCD form (1058±237 ng/mL.h) is no different ($p = 0.9131$) from what was obtained with the co-solvent form (1076±115 ng/mL.h). Plasma binding may not play an important role here because of the low K_s.

The unchanged pharmacokinetics may be due to the rapid release of flutamide from HPβCD through the dilution effect in blood. The stability constant of flutamide with HPβCD is 356 M^{-1}at room temperature (Zuo *et al.* 1996). This relatively weak binding will lead to the rapid and complete release of the drug on dilution (Stella and Rajewski 1997).

Cosolvents like various alcohols and glycols will increase the solubility of a poorly water soluble drug in a non-linear fashion with respect to co-solvent concentration. Precipitation may occur in such systems at any dilution where the equilibrium drug solubility is lower than the dilution concentration line at a given CD concentration. Rajewski and Stella (1996) mentioned that precipitation of the drug may occur on dilution if there is a nonlinear relationship between the drug solubility and co-solvent concentration. Even though the precipitation didn't show any effect in the experimental data (the C_{max} didn't show significant difference between two dosage forms), it may still occur during storage and the high percentage of alcohol in the cosolvent isn't appropriate for dosage form. On the contrary, as long as the cyclodextrin complex of a drug results from a 1:1 interaction, there is a linear increase in drug solubility with increasing cyclodextrin concentration, dilution of the drug-cyclodextrin complex will not result in drug precipitation. The linear relationship that exists between flutamide solubility and HPβCD concentration shows the advantage of the lack of precipitation of the formulation on dilution. These results demonstrate the capability of an aqueous parenteral formulation of flutamide through use of HPβCD complexation.

Table 4.7.1. Plasma concentration profile for each animal after *i.v.* injection of the flutamide-HPβCD complex dosage form (Dose = 1.6mg/kg)

	Plasma concentrations after *i.v.* injection of HPβCD dosage form ($ng.mL^{-1}$)					
	Flutamide			2-hydroxyflutamide		
Time (h)	Animal #1	Animal #2	Animal#3	Animal #1	Animal #2	Animal #3
0.08	3304	4291	3749	217	90	89
0.17	1804	2098	2309	264	225	270
0.33	1402	1854	1755	328	447	595
0.50	453	432	565	245	445	584
0.67	215	342	342	240	717	773
1.00	153	290	162	550	956	525
1.50	68	55	27	344	341	456
2.00				270	204	265
2.50				98	170	174
3.00				21	93	69

Table 4.7.2. Plasma concentration profile for each animal after *i.v.* injection of flutamide co-solvent form (1.6mg/kg)

	Plasma concentrations after *i.v.* injection of co-solvent dosage form (ng. mL^{-1})					
	Flutamide			2-hydroxyflutamide		
Time (h)	Animal #1	Animal #2	Animal#3	Animal #1	Animal #2	Animal #3
0.08	4023	4224	3474	266	117	188
0.17	1651	2508	1722	650	264	234
0.33	1291	1904	1092	589	598	492
0.5	980	995	545	668	493	692
0.67	577	667	365	795	382	901
1	50	211	74	605	341	367
1.5	32	96	21	421	394	336
2				274	217	269
2.5				93	177	114
3				62	92	71

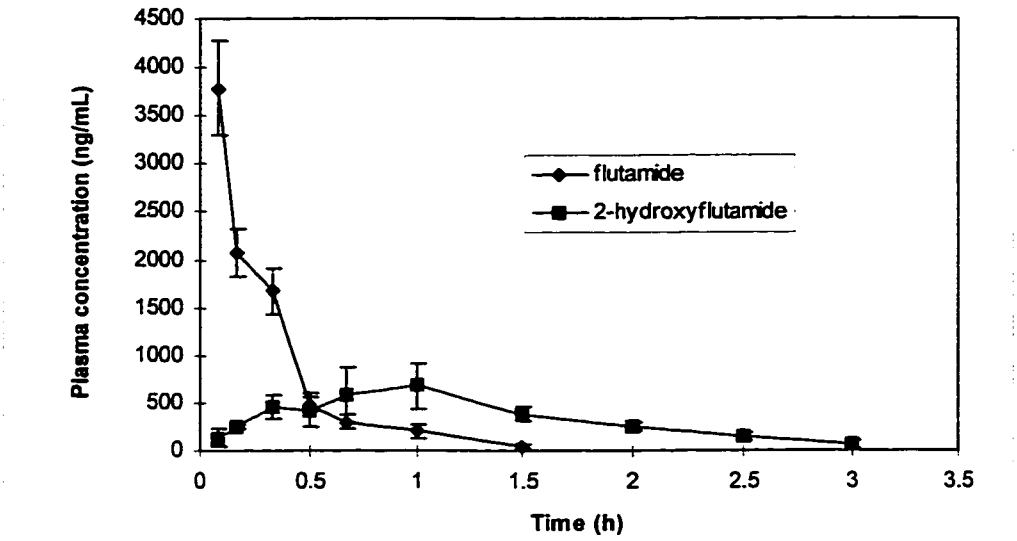


Figure 4.7.1. Plasma concentrations of flutamide and 2-hydroxyflutamide (ng. mL^{-1}) following the *i.v.* administration of flutamide-HPβCD dosage form of flutamide (Dose =1.6 mg/kg, n=3).

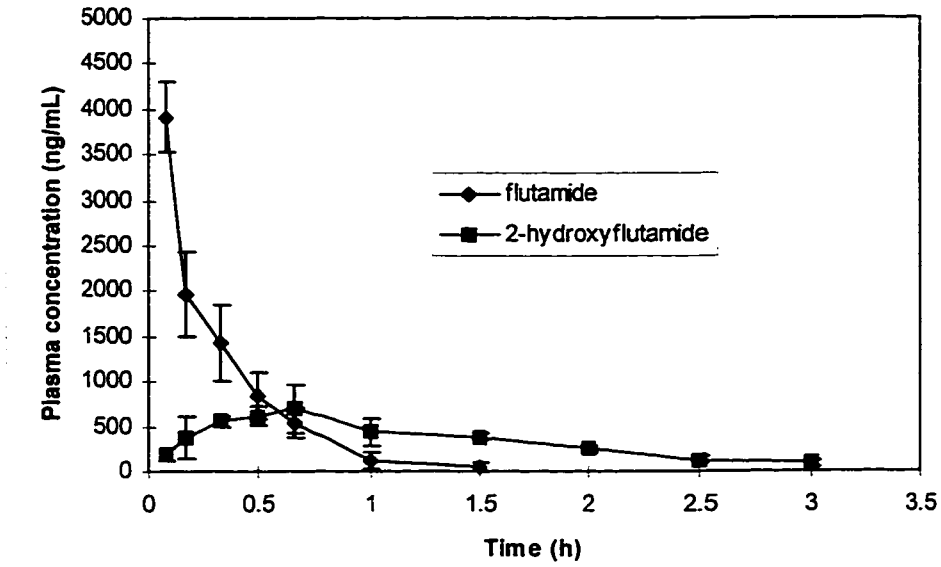


Figure 4.7.2. Plasma concentrations of flutamide and 2-hydroxyflutamide ($ng.mL^{-1}$) following the *i.v.* administration of the co-solvent dosage form of flutamide (Dose=1.6 mg/kg, n=3).

Table 4.7.3. Pharmacokinetic parameters comparison of co-solvent (n=3) and flutamide-HPβCD complex forms (n=3) after *i.v.* injection (Dose=1.6 mg/kg)

	CO-SOLVENT DOSAGE FORM		HPβCD DOSAGE FORM	
	Flutamide	2-hydroxyflutamide	Flutamide	2-hydroxyflutamide
AUC (∞): (ng/mL. h)	1421±283	1076±115	1355±162	1058±237
C_{max} (ng/mL)	7359±1322	765±154	6511±1383	760±203
CL (mL/h)	714±144		735±88	
V_z (mL)	242±24		369±191	
V_{ss} (mL)	193±18		224±52	
$T_{1/2\lambda z}$ (h)	0.24±0.06	0.63±0.13	0.40±0.16	0.57±0.31
T_{max} (h)		0.6±0.1		0.9±0.2

a, b Significance of differences from suspension dosage form: P<0.01

4.8. Caco-2 transport study

4.8.1. Cell growth

As shown in the cell growth curve (Figure 4.8.1.), the cell monolayer TEER reached 200 $\Omega.cm^2$ at day 4, which is defined as the experiment day.

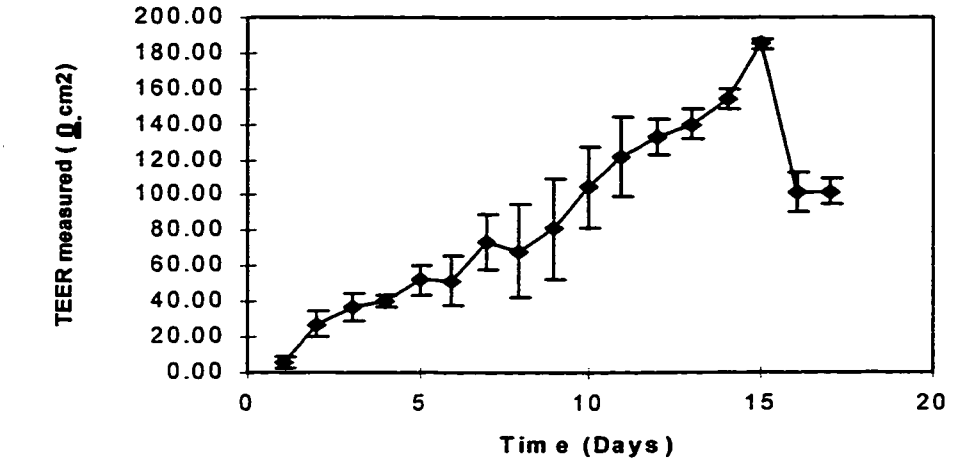


Figure. 4.8.1. Transepithelial resistance of Caco-2 monolayers grown on 6-well transwell insert as a function of incubation times (n=3).

4.8.2. Transport study with different dose of HPβCD

Flutamide prepared in PBS is supposed to contain only the free form of flutamide. When cell culture medium was used as transport medium in the free flutamide cell transport study, dose dependent transport was found (Figure 4.8.2.1). The higher dose has a lower percentage of amount transported. When PBS^+ was used as the transport medium in the same study, the dose dependency phenomena disappeared (Figure 4.8.2.2). Since this is a one sample point experiment, it is hard to get the conclusion of the significant difference in their P_{eff} (Table 4.8.2.1). These results may indicate that the use of culture medium in the transport experiment may affect the free drug transport. This effect may due to the protein binding effect caused by the FBS in the culture medium. Even though the cell monolayer may have better nutrition in its culture medium, PBS^+ is preferred in this study. The integrity of cell monolayer in PBS^+ was also studied and there was no significant change within 4 h of incubation. The reason for using PBS^+ instead of normal PBS is to maintain the cell integrity utilizing the high concentration of Ca^{2+} in PBS^+.

Under different concentrations of HPβCD (0.15 to 2.5%), the P_{eff} values were calculated. TEER didn't change during the experiment. As shown in Figure 4.8.2.3, the percentage amount transported as well as the P_{eff} decreased with the increase of HPβCD. This data indicates that when more free flutamide is available, the P_{eff} increases. The result also indicates that the transepithelial transport is attributed to the passive diffusion of available free drug molecules rather than the collision

complex transfer at the cell surface. Dilution effect may play an important role in the flutamide release from its HPβCD inclusion complex.

Haeberlin *et al.* (1996) recently reported on the cyclodextrin associated absorption enhancement of a modified calcitonin and the octapeptide octreotide *in vitro* using a Caco-2 cell monolayer and *in situ* using isolated rat jejunal sections. They found that β-, γ- cyclodextrin, HPβCD and DMβCD did alter oral absorption of drugs through mucosal membrane permeation enhancement. Hovgaard and Brøndsted (1995) studied the effects of cyclodextrins as absorption enhancers using the Caco-2 monolayer model. βCD (1.8%) and 5% of α, γ and HPβCD were used to compare with 2.5% and 5% of DMβCD. It was concluded that DMβCD was the most powerful in all aspects and caused an increase in the permeability of the cytoplasmic membrane in a concentration dependent manner. No significant beneficial effects on transport were seen for the rest of CDs relative to the control. So, as to the extent of mucosal permeation enhancement of the drug effected by cyclodextrins, the type and dose of cyclodextrin derivative as well as the dilution factor should be taken into consideration.

The free flutamide transport shows the highest P_{eff} of 4.75×10^{-5} $cm.s^{-1}$ in this experiment, which indicates that the free drug itself may not have any permeability problem. The low bioavailability of flutamide may due to the low amount of free drug available, which is caused by the dissolution rate of the formulation.

Since there is no 2-hydroxyflutamide found in the basolateral side, we may

assume that there is no first pass metabolism of flutamide through the GI tract. A more accurate conclusions about the metabolism can only be reached after analysis of the enzyme quality and quantity before utilizing this cultured cell model, because of the quantitative differences in enzyme expression with time in culture together with the qualitative differences in the enzyme properties observed between the parental Caco-2 cell line and its subcultured cells.

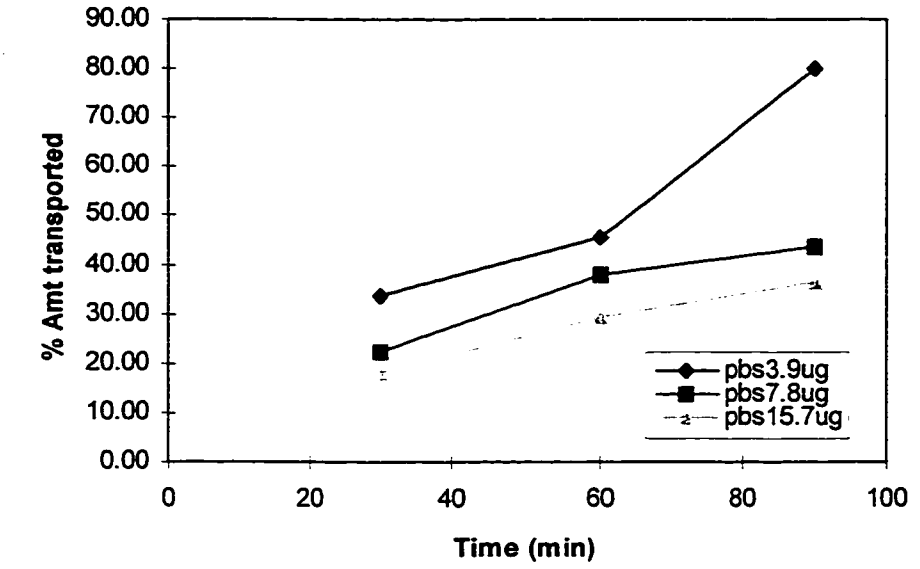


Figure 4.8.2.1. Different doses of free flutamide transported through a Caco-2 monolayer in cell culture medium (n=1).

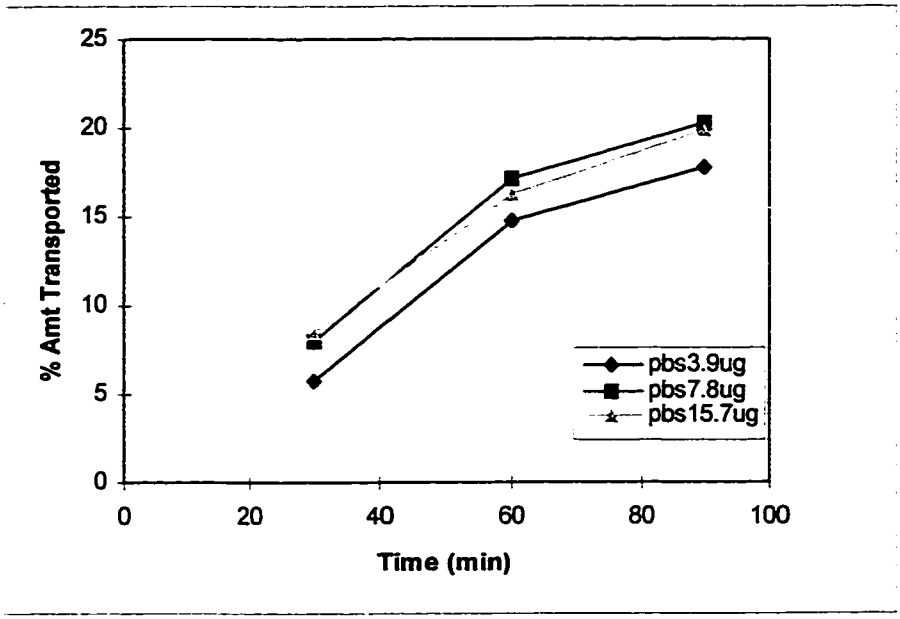


Figure 4.8.2.2. Different doses of free flutamide transport through a Caco-2 monolayer in PBS solution (n=1).

Table 4.8.2.1. P_{eff} of different doses of free flutamide transported through a Caco-2 monolayer in PBS or cell culture medium (n=1).

	P_{eff} (cm/s)	
Dose (μg)	**PBS**	**Medium**
3.9	1.03E-05	3.95E-05
7.8	1.04E-05	1.80E-05
15.7	9.68E-06	1.55E-05

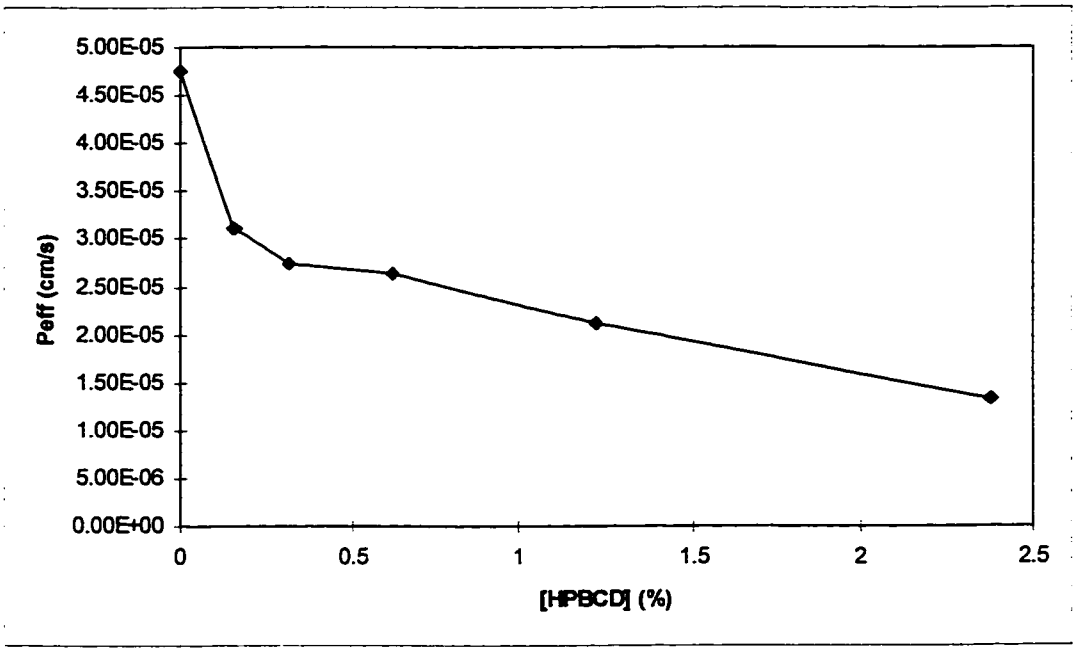


Figure 4.8.2.3. P_{eff} of flutamide in different concentrations of HPβCD (n=1)

4.8.3. Transport of flutamide after the treatment with a calcium chelator:

The concentration of EGTA used in this experiment was 2.5 mM. This corresponds to an extracellular Ca^{2+} concentration of $2\sim4\times10^{-6}$ M. This concentration is at least 10 times higher than the normal intracellular calcium concentration, which is in the 100 nM range (Tsien *et al.* 1984). A decrease in transmembrane resistance was noticed after replacement of the low Ca^{2+} buffer with normal PBS^{+} buffer containing 0.9 mM Ca^{2+}. After 45 min of EGTA treatment, the transmembrane resistance returned to normal values within around 2 h recovery time. It is reported that exposure of the cells to low Ca^{2+} medium for a longer time than 90 min will result in an irreversible decrease in the transmembrane resistance (Artursson and Magnusson 1990). As shown in Figure 4.8.3, the reversible opening of the monolayer tight junction had no significant effect on the transport rate of flutamide. It seems that the transport of flutamide is so fast that the contribution of the widened intercellular spaces to the overall transport is insignificant. Thus, the paracellular pathway is of limited importance for flutamide.

The advantage of using Caco-2 monolayer is that it has a TEER of > $200\Omega.cm^2$. This resistance is high enough to reduce the transport of hydrophilic compounds to a very low level. So, any small change in paracellular permeability is therefore readily detectable.

In general, the opening of the junctional complex had no effect on the transport of the more lipophilic drugs. These drugs partition rapidly into the cell

membranes. The distribution to the intercellular spaces will therefore be limited even after EGTA treatment since the cell membranes cover a much larger surface area than the intercellular spaces. However, the more hydrophilic drugs will have a low solubility in the cell membranes. The transport through the paracellular pathways is therefore more important for these drugs.

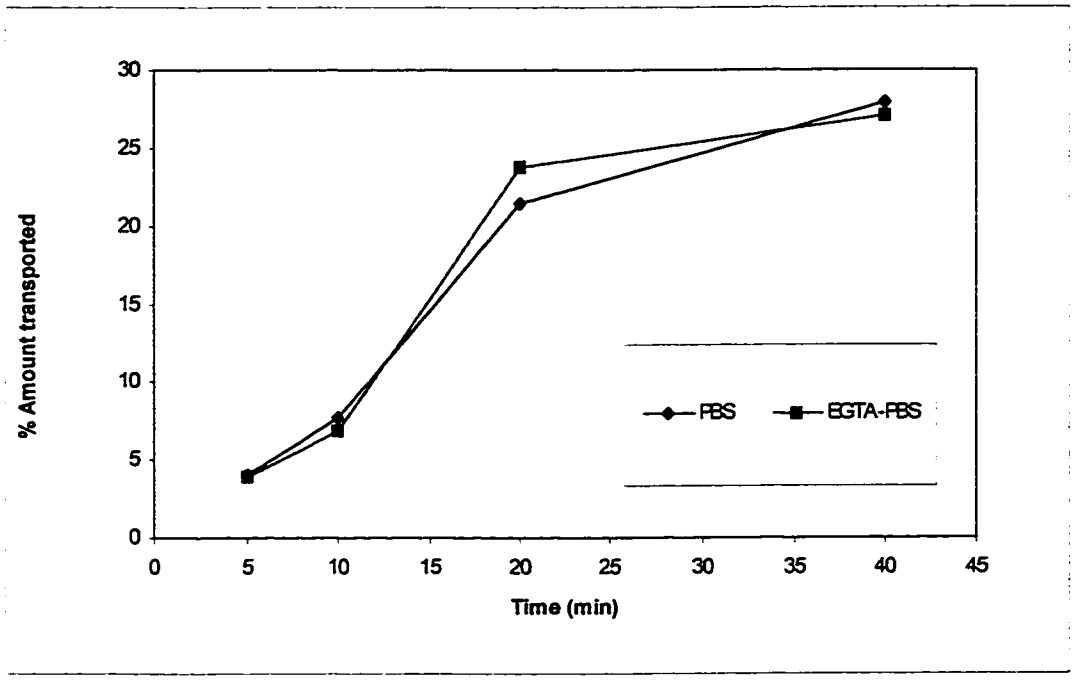


Figure 4.8.3. Flutamide transported under conditions in which tight junctions are open (n=1).

5. Summary and Conclusion

The objective of the project was to solve the problem of relatively low bioavailability, poor water solubility and wettability of the anti-prostate cancer drug flutamide. Three classes of CDs, (ßCD, HPßCD and JD1-128 CD), including one fluorinated-CD, were used to increase the solubility of flutamide. After the extensive studies by phase solubility, thermodynamics, DSC ^{19}F NMR and ^{1}H NMR, HPßCD was chosen for the further *in vivo* investigation. In a rat model, the oral relative bioavailability of the flutamide-HPßCD inclusion complex was compared to that of a flutamide suspension. The *in vitro* dissolution rate of the flutamide-HPßCD inclusion complex was also determined in order to establish its effect on improving the bioavailability of flutamide. Improved solubility of the flutamide-HPßCD inclusion complex also provided the opportunity for *i.v.* dosing. Intravenous pharmacokinetic parameters for the flutamide-HPßCD complex were compared with a standard co-solvent formulation, establish the possibility of developing parenteral delivery form for flutamide. From the investigations described above, the following summary and conclusions can be made:

1). Among the 3 classes of CDs, the solubility of flutamide was increased the most by forming an inclusion complex with HPßCD. The complexes were characterized by an AL isotherm type indicative of a linear increase in flutamide solubility with unchanged stoichiometry. The solubility of flutamide increased 170

times in 50% (w/v) aqueous HPßCD.

2). Thermodynamic parameters of the inclusion complexes were obtained from tests of flutamide solubility in the presence of various levels of CDs at several temperatures. With an increase in temperature, the solubility of flutamide in all 3 CDs increased proportionally but with different patterns. The thermodynamic parameters were calculated and compared from these data. The inclusion complexes with ßCD and JD1-128 CD (the fluorinated ßCD) display the similar behavior. Flutamide-HPßCD demonstrated a very strong hydrophobic interaction in inclusion complex formation.

3). DSC was used to confirm the formation of inclusion complexes. The DSC of free flutamide and 3 CDs showed endothermal peaks at 110°C and around 300°C, respectively, whereas the DSC of the complex gave no indication of free flutamide, but had a wider endothermal peak for all the CDs.

4). ^{19}F NMR chemical shifts of flutamide F atoms in the inclusion complexes, with HPßCD and JD1-128 CD, were also used to estimate the stoichiometry of the complexes. The ^{19}F NMR chemical shifts of flutamide moved downfield as a function of its increased solubility in the presence of HPßCD and JD1-128 CD. The binding constants calculated based on the ^{19}F NMR chemical shift were similar to one calculated from the phase solubility test.

5). ^{1}H NMR further confirmed the inclusion formation and showed the detail of the different proton changes in the benzene ring upon the inclusion complex

formation.

6). The *in vitro* dissolution rate of the flutamide-HPβCD inclusion complex was measured and found much higher than that for flutamide powder.

7). Following the oral administration of the flutamide-HPβCD complex to rat, the AUC (1580 ± 228 ng.h/mL), and C_{max} (1456±79 ng/mL) of flutamide were significantly higher than those obtained after the administration of the suspension form of flutamide (748±206 ng/mL and 230±111 ng.h/mL, respectively). Moreover, maximum plasma concentrations appeared at a shorter time after dosing with the HPβCD complex (T_{max} = 0.56±0.10 h) than with the suspension (T_{max} =2.33±0.29 h). Formulation of flutamide with HPβCD provides a significant increase in flutamide bioavailability compared to the suspension formulation.

8). The pharmacokinetics of flutamide in an aqueous HPβCD solution and in a co-solvent (PEG200/Ethanol/PBS 3:1:2) formulation were studied. The plasma concentration-time profile and pharmacokinetic parameters of flutamide from HPβCD and co-solvent mixture were not significantly different. These results show that flutamide is released rapidly and quantitatively form the HPβCD inclusion complex after *i.v.* injection. HPβCD may be useful in the formulation of a parenteral flutamide dosage form.

9). The mechanism of the enhancing the bioavailability of HPβCD through better absorption of flutamide was investigated *in vitro*. Apical-to-basal transport of flutamide from the HPβCD inclusion complex across Caco-2 cell monolayer was

studied at 37°C. The solutions (cell culture medium and PBS) used in the transport study had also been tested and compared. The results show that PBS is preferred to the cell culture medium in this type of transport study. The tight junction opening experiments proved that the transepithelial pathway was the main transport route for flutamide.

It was also found that with the decreasing HPβCD concentration, the permeability coefficient of flutamide increased. This indicates that the transepithelial transport is attributed to the passive diffusion of available free drug molecules rather than the collision complex transfer at the cell surface. The dilution effect may play an important role in the drug release.

Overall, based on both the *in vitro* and *in vivo* evaluation of flutamide in different classes of CDs, the flutamide-HPβCD inclusion complex represented a valuable oral dosage form and provided a new injectable dosage form for flutamide.

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Appendix 1:

HEALTH SCIENCES LABORATORY ANIMAL SERVICES
TECHNICAL S.O.P. #017
Non-Survival Jugular Catheterization in Rats (Wiebe)

This technique is used by Dr. Wiebe's lab for pharmacokinetic studies to sample blood, inject substances and euthanize the rat at the termination of the experiment. After sampling the cannula should be flushed with heparinized saline to avoid clotting.

MATERIALS:

a) Instrument Pack
   - scissors (2)
   - tissue forceps (2)
   - suture material

b) Masking tape or plexi-glass board & elastics

c) Anesthetic (Methoxyflurane)
   (Sodium pentobarbital - Somnitol)
   - nose cone
   - knock down jar
   - fume hood

d) 0.56 mm i.d./0.60 mm o.d. catheter with 0.5 cm of 0.02 mm silastic tubing attached.

e) #22 scalpel blade

f) 25G needles and 1 ml syringes

g) gauze sponges

TECHNIQUE:

a) Prepare site and materials (as above).

b) Anesthetize the rat.
   1. If using Methoxyflurane the use of a fume hood or gas scavenger is mandatory as methoxyflurane has been shown to be hepato-toxic. The rat is to be placed in a knock down jar and then maintained in surgical plane anesthesia using a nose cone.
   2.. If using sodium pentibarbital, use a 25G needle to inject the rat by the interperitoneal route. Dosage: 30-50 mg/kg BWT.

   As the experiments will require approximately 6 hours of surgical plane anesthesia, if you are using the sodium pentobarbital anesthasia method you will have to supplement the animal to maintain anesthesia after about three hours. This can be done using an inhalation anesthetic (methoxyflurane) to maintain anesthesia, or by topping up the animal with maintenance doses of sodium pentibarbital.

c) The animal is to be shaved as in diagram (A). This is best accomplished by first wetting the area with a mixture of soap and water and using short quick strokes with a #22 scalpel blade to shave the site. DO NOT SOAK THE ANIMAL AS IT MAY INDUCE HYPOTHERMIA.

d) The animal is to be secured on the bench in dorsal recumbency with masking tape or by using a plexi-glass board as per diagrams (B) & (C). It is important that the rat is positioned with its vertebrae as straight as possible and the front limbs at 90 degrees to the spine.

e) The animal should be constantly monitored using the toe pinch (pedal) reflex to ensure that it is maintained in surgical plane anesthesia.

f) An incision of approximately 0.5 - 1.0 cm is made through the skin. If one was to draw a straight line between the tip of the lower jaw and the first nipple (as located on a female rat), the incision should start at the edge of the pectoral muscle and move toward the jaw, as per diagram (D). Using blunt dissection work your way through the fat and fascia until the pectoral muscle is well exposed and the jugular bulges at the cranial border of the muscle. The placement of a needle sheath under the base of the neck will help to visualize the jugular.

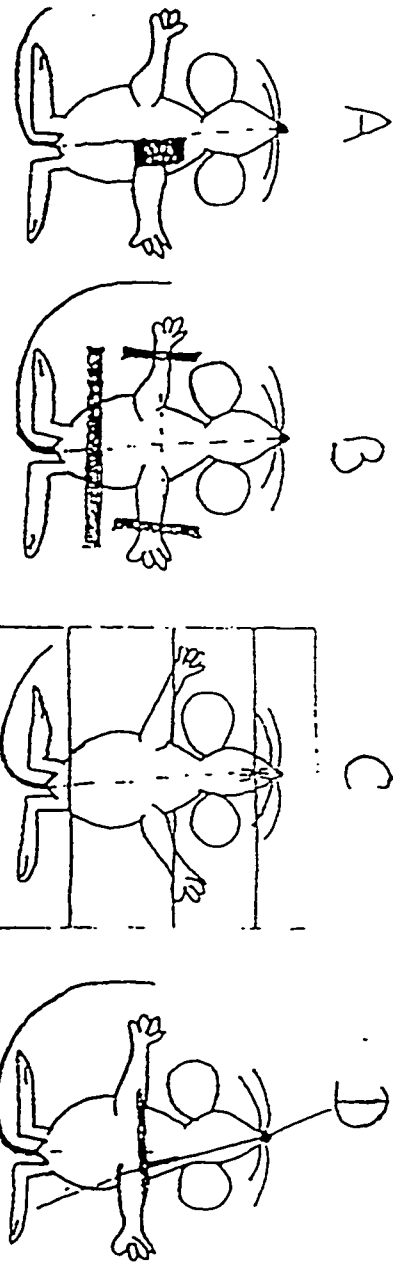
g) Insert the catheter into the vein and secure it using suture. A pair of forceps is used to insert a double threaded suture under the vein. The threads are cut to give two (2) threads. The vein is opened using a pair of fine scissors, and once the catheter is inserted into the vein, the threads are secured on either side of the catheter.

i) At the end of the experiment, ensure that the animal is humanely euthanized by an overdose of sodium pentobarbital given into the jugular catheter. Ensure there are no signs of life and incinerate body.

15:03:94
Director's Signature

Technical Development
Tech-017 (Wiebe) 14/03/94

Tech-017


A
B
C
D

Appendix 2:

Thermodynamic parameters calculations

When a solid (A) dissolves in a solvent, two steps may be considered as occurring: the solid absorbs energy to become a liquid and then the liquid dissolves.

A (solid) $\rightleftharpoons$ A (liquid) $\rightleftharpoons$ A (solution) (Gennaro *et al.* 1990).

For overall dissolution, the equilibrium existing between solute molecules in the solid and solute molecules in solution may be treated as any equilibrium. Thus, for a solute A in equilibrium with its solution we can write,

$$\text{A (solid)} \rightleftharpoons \text{A (solution)}$$

Using the Law of Mass Action we can define an equilibrium constant for this system.

$$K_{eq} = a_{(solution)} / a_{(solid)}$$

where a denotes activity of the solute in each phase. Since the activity of a solid is defined as unity.

$$K_{eq} = a_{(solution)}$$

because the activity of a compound in dilute solution is approximated by its concentration and because this concentration is the saturation solubility, Ks, we can use the van't Hoff equation which defines the relationship between an equilibrium constant (here, solubility) and absolute temperature. This relation is

$$d \log K_s / dT = \Delta H^\circ / 2.3RT^2$$

where $d \log K_s / dT$ is the change of $\log K_s$ with a unit change of absolute temperature,

T; $\Delta H°$ is a constant which in this situation is the heat of solution for the overall process (solid $\rightleftharpoons$ liquid $\rightleftharpoons$ solution); and R is the gas constant, 1.99 cal/mole/deg. The above differential equation may be solved to give:

$$\log K_s = -\Delta H°/2.3RT + J, \text{ where J is a constant.}$$

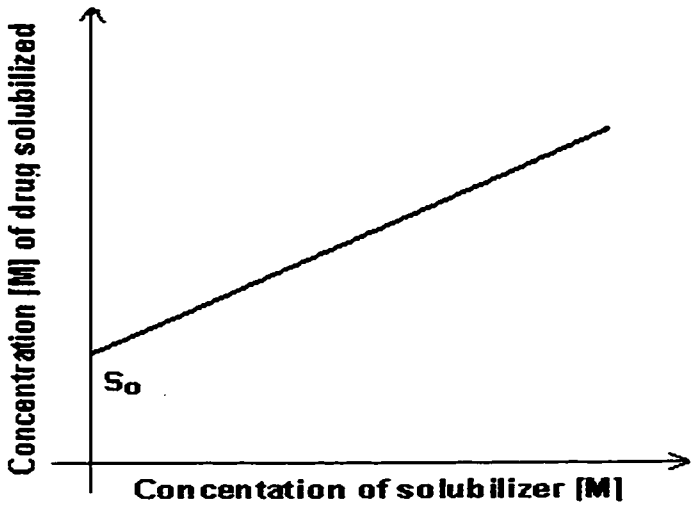
The relation between free energy and equilibrium constant, K_s, for a reaction is give by $\Delta G° = -RT\ln K_s$. Furthermore, we know that the free energy change is dependent on heat-related enthalpy ($\Delta H°$) and order-related entropy ($\Delta S°$) factors as seen in equation

$$\Delta G° = \Delta H° - T\,\Delta S°, \text{ where T is the temperature or } \Delta S° = (\Delta H° - \Delta G°)/T$$

In conclusion, from the calculation of stability constants at different temperature, we may be able to calculate $\Delta H°$ by plotting log K_s versus 1/T. The standard free energy change of complexation $\Delta G°$ can be obtained from its relationship with the overall stability constant K_s. The standard entropy change $\Delta S°$ can thus be obtained from $\Delta G°$ and $\Delta H°$.

Appendix 3:

Calculation of the stability constant from the phase solubility curve



$$CD_{free} + Drug_{free} \overset{K_s}{\rightleftharpoons} [CD.D]_{complex}$$

Based on the definition of stability constant,

$K_s = [CD.D]_{complex} / [Drug]_{free}.[CD]_{free}$ [1]

where $[Drug]_{free} = S_0$, which is the intercept of the phase solubility curve

Rearrange equation [1] :

$[CD.D]_{complex} / [CD]_{free} = K_s \times S_0$ [2]

On the other hand, from the phase solubility curve we may get:

Slope = [D] in complex form / [CD] in both free and complex form.

Which can be simplified as the following,

$Slope = [CD.D]_{complex} / ([CD]_{free} + [CD.D]_{complex})$ [3]

Rearrange [3]:

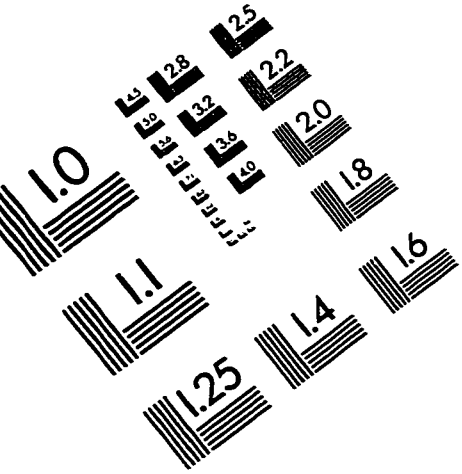
$$\text{Slope} = 1/ ([CD]_{free} / [CD.D]_{complex} + 1) \quad [4]$$

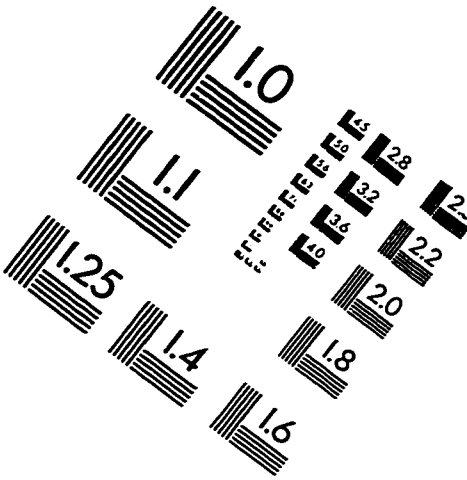
Combine equation [2] and [4],

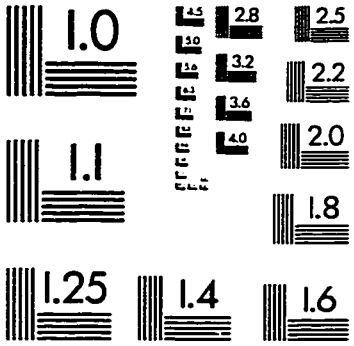
$$\text{Slope} = 1/(1/K_s.S_0 + 1) \quad [5]$$

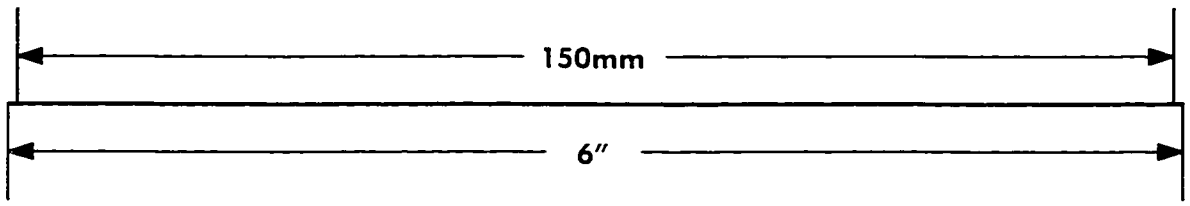
Rearrange equation [4], we get K_s = Slope / S_0 (1-Slope), which may enable us to calculate the stability constant K_s from the intercept and slope of the phase solubility curve.

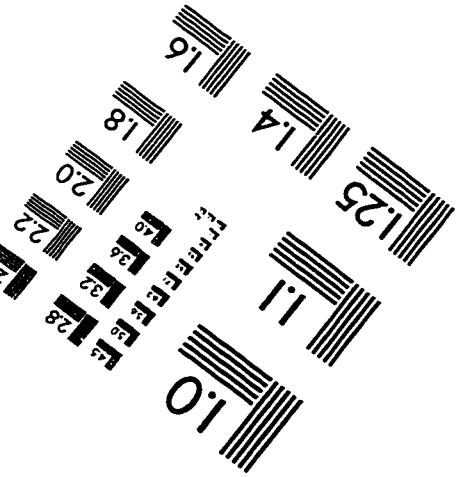
IMAGE EVALUATION
TEST TARGET (QA-3)

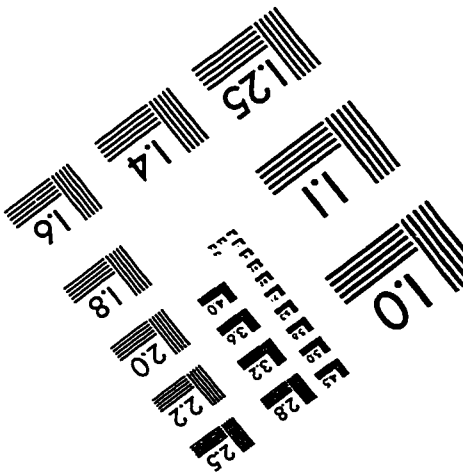












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