Pharmacokinetics/Pharmacodynamics (PK/PD) of Oral Diethylstilbestrol (DES) in Recurrent Prostate Cancer Patients and of Oral Dissolving Film (ODF)-DES in Rats

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Abstract

Prostate cancer is the most diagnosed non-skin cancer and the second leading cause of cancer related death in men in the United States. According to the National Cancer Institute (NCI), an estimated 217,730 men were diagnosed with prostate cancer in 2010 and 32,050 died from the disease. For metastatic disease patients, response to treatment is short-lived (2 to 3 years), and the disease progresses to an androgen-independent form which is irresponsive to hormonal treatment, and usually results in death in less than 18 months (Saitoh et al., 1984).

Estrogens like diethylstilbestrol (DES) have been used for the treatment of advanced prostate cancer (PCa) for more than half a century. The use of DES to treat advanced PCa is associated with excess cardiovascular side effects like deep vein thrombosis, pulmonary embolism, heart attack and stroke. DES was replaced by luteinizing hormone releasing hormone (LHRH) agonists which have a safer side effects profile. There has been renewed interest in DES because it is effective in treating hormone-independent advanced PCa, and LHRH agonists are not. We are interested in optimizing the dosing regimens of oral DES therapy by using patient DES plasma concentrations as a guide. This objective was achieved by developing a robust and efficient LC/MS/MS assay for the quantification of DES in human and rat plasma, and using the assay to quantify DES concentrations in the plasma of 19 patients enrolled in a clinical phase II trial. We are also interested in investigating other routes of administration and/or formulations of DES that will bypass the first-pass liver metabolism and potentially increase bioavailability and decrease DES toxicity. The side effects of DES have been associated with its

metabolism in the liver which is believed to alter the body's coagulation cascade. This aim was achieve by studying the transdermal delivery of a solution of DES in propylene glycol through mouse skin, and establishing the merits of the transbuccal delivery of DES using an oral dissolving film (ODF)-DES in rats.

We quantified DES concentrations in plasma of 19 patients on oral DES capsules for recurrent PCa and correlated DES plasma levels with response by PSA decline. We suggest a DES plasma level of 2 ± 1 ng/ml as a therapeutic target. We also demonstrated the potential merits of ODF-DES in rats; ODF-DES increases significantly the bioavailability (AUC of ODF: 179.07 ng/ml*h, AUC of oral suspension: 65 ng/ml*h) of DES by a factor of 2.7 with minimal effects on the clotting proteins evaluated. The significance of the observation is that a less DES dose from ODF-DES can achieve comparable concentration as a higher dose from oral formulation, which may potential decrease DES side effects.

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ACN	Acetonitrile
ADPCa	Androgen dependent prostate cancer
ADT	Androgen deprivation therapy
AIPCa	Androgen-independent prostate cancer
AT-III	Antithrombin III
AUC	Area under the plasma concentration-time curve
CL	Clearance
C_{max}	Peak plasma concentration
DES	Diethylstilbestrol
DHT	Dihydrtestosterone
DMSO	Dimethylsulfoxide
F	Bioavailability
FBG	Fibrinogen
FI	Factor I
FVII	Factor VII
17HSD	17 β-hydroxysteroid dehydrogenase
IACUC	Institutional Animal Care and Use Committee
IM	Intra muscular
K	Rate constant
LHRH	Luteinizing hormone releasing hormone
LC/MS/MS	Liquid Chromatography Mass Spectroscopy

N	Number
NCI	National Cancer Institute
PBS	Phosphate-buffer saline
PCa	Prostate cancer
PEP	Polyestradiol phosphate
PG	Propylene glycol
PD	Pharmacodynamics
PK	Pharmacokinetics
PSA	Prostate specific antigen
SHBG	Steroid hormone binding globulin
TAB	Total androgen blockade
TDE	Transdermal estradiol
T_{max}	Time to reach peak plasma concentration
$T_{1/2}$	Half-life
V	Volume of distribution

Contents

Chapter 1. Literature Reviews

1.1. Introduction to the Project

Diethylstilbestrol (DES) is a synthetic non-steroidal estrogen that is effective in the treatment of both androgen dependent (ADPCa) and androgen-independent carcinoma (AIPCa) (Smith et al, 1998; Nesbit et al., 1950; Robertson et al., 1996). Oral DES therapy for prostate cancer is associated with excess cardiovascular side effects (heart attack, stroke, deep vein thrombosis, pulmonary embolism, as well as unstable and stable angina), and for this reason, DES was replaced by luteinizing hormone releasing hormone (LHRH) agonist which was considered to be safer (The Leuprolide Study Group, 1984). DES therapy induces castrate level testosterone and there is evidence suggesting that DES may improve survival in patients with advanced prostate cancer compared to other forms of androgen deprivation therapies, namely, orchiectomy and using LHRH agonists (Haapiainen et al., 1986; Bland et al., 2005). The excess side effects of oral DES have led to the near abandonment of DES use for prostate cancer except for clinical trials. The cardiovascular side effects of DES are thought to result from DES metabolism in the liver. The liver metabolism of estrogens has been associated with the alteration in the production of blood coagulation and fibrinolysis proteins synthesized in the liver. Oral estrogens have been shown to increase some blood clotting factors which may increase a patient's risk for clot formation. Other formulations of estrogens have been exploited to treat prostate cancer with reduced cardiovascular side effects. Polyestradiol phosphate, a parenteral estrogen at 240 mg IM was demonstrated to be as efficacious as orchietomy and LHRH agonist, but with no

major difference in cardiovascular mortality (Hedlund et al., 2002). In another study transdermal estradiol (TDE) showed a modest response of 12.5 % in treating AIPCa, but most importantly, TDE was not associated with thromboembolic complications (Bland et al., 2005). The common denominator between the parenteral and transdermal estrogens and the decrease side effects is that both formulations will bypass the first-pass hepatic metabolism. This observation has led us to explore other formulations and routes of delivering DES that will bypass liver metabolism and may potentially reduce thrombogenic toxicities. We are interested in studying the delivery of DES through the buccal route using an oral dissolving film of DES (ODF-DES). Drug delivery through the buccal route (transmucosal) may bypass the liver metabolism increasing DES bioavailability, but potentially decrease the cardiovascular side effects of DES.

1.1.1. Prostate Cancer Prevalence and Risk Factors

Prostate cancer is the most lethal carcinoma (not counting skin cancer) diagnosed in American men and the second most common cause of male cancer death. According to the National Cancer Institute (NCI), an estimated 217,730 men were diagnosed with prostate cancer in 2010 and 32,050 died from the disease. The majority of patients diagnosed with localized prostate cancer are treated for cure with either radiation or surgery. Patients who fail treatment with curative intent and those who present with metastatic disease are candidates for androgen deprivation therapy (ADT). Androgen deprivation therapy is achieved either surgically by orchietomy or medically. Medical ADT involves the use of LHRH agonist, antiandrogens (such as flutamide and megestrol) or estrogens, or a combination of agents from two of the classes, usually

LHRH agonist and antiandrogens. All metastatic prostate cancer initially require testosterone and dihydrotestosterone for growth, hence the use of androgen deprivation therapy as first line treatment for advanced disease since the 1940s (Huggins and Hodges, 1941). For patients with metastatic disease, response to treatment may last 2 to 3 years, after which virtually all patients progress to an androgen-independent state, which is hormone insensitive resulting in death in approximately 16 to 18 months (Saitoh et al., 1984; Grayhack et al., 1987; Harada et al., 1992).

Age, race and family history are the only established risk factors for prostate cancer. Age is the strongest risk factor for prostate cancer. Prostate cancer is very rare at ages younger than 40 years, but the risk increases rapidly after the age of 50 years. Most of the prostate cancer diagnosed (almost 2 out 3) are found in men older than 65 years. African-American men are diagnosed with prostate cancer more than any other races, and are more likely to be diagnosed at an advanced stage. African-Americans are more than twice as likely to die of prostate cancer as white men. Prostate cancer occurs less in Asian American and Hispanic/Latino men than in non-Hispanic white men. The reasons for the ethnic disparity are not clear. There is evidence to suggest that prostate cancer may be inherited or has a genetic component (run in families). Having a father or brother with prostate cancer more than doubles ones risk of developing prostate cancer (risk is higher with an affected brother than father). This risk will increase for men with multiple affected relatives, especially, if the relatives were diagnosed at a young age (Bostwick et al., 2004; American Cancer Society, 2006; US Cancer Statistics for 2010). Other factors have been suggested as risk factors for prostate cancer, but the

association between prostate cancer and these factors is inconclusive. High calorie-fatty diet is one of the factors believed to be a risk factor for prostate cancer. Studies of immigrants from Japan have demonstrated that native Japanese have the lowest risk of prostate cancer, followed by first generation Japanese with intermediate risk, and subsequent generations have risk comparable to the American population. The reason for this change in risk is believed to do with the adaptation to the American diet (high in calories from fat) by the immigrants (Shimizu et al., 1991; Haenszel and Kurihara, 1968). Review of the numerous studies on the association of dietary fat and prostate cancer are almost split in the middle with half finding an association (Zhou and Blackburn, 1997).

Androgens are primary the regulators of normal prostate growth and development, and high levels of androgens have been postulated to be possible risk factors for prostate cancer (Platz and Giovanucci, 2004; Hsing, 2001). Evidence for this hypothesis that prostate cancer has a hormonal component comes from the observation that most prostate tumors respond to androgen blockade. More recently, the Prostate Cancer Prevention Trial (PCPT) demonstrated that the inhibition of the conversion of testosterone to the more potent dihydrotestosterone (DHT) by finasteride, a 5 α-reductase inhibitor, reduced the occurrence of prostate cancer by approximately 25 % (Thompson et al., 2003; Canby-Hagino et al., 2007). Other studies investigating whether differences in endogenous sex hormones are related to risk of prostate cancer have been inconsistent and inconclusive with some studies finding small association and others no association at all (Nomura et al., 1988; Carter et al., 1995; Comstock et al., 1993). Because it was thought that the inconsistencies in the studies may have affected

the outcome of the results, the Endogenous Hormones and Prostate Cancer Collaborative Group was initiated to use uniform statistical methods to provide precise estimate of the association of endogenous sex steroids and prostate cancer on a worldwide basis. The Group concluded that serum concentrations of sex hormones were not associated with the risk of prostate cancer (Roddam et al., 2008).

1.1.2. Sex Hormones and Sex hormone Receptors and Prostate cancer

Testosterone is the main circulating androgen, synthesized in the leydig cells of the testes from androstenedione (A-dione) and the reaction is catalyzed by 17 β-hydroxysteroid dehydrogenase (17HSD) type 3 (Grumbach et al., 2003). The prostate tissue also contains a variety of steroid metabolizing enzymes required for the formation of active androgens and estrogens necessary for the proper functioning of the prostate gland. In the prostate, dihydrotestosterone (DHT) is the predominant androgen and is formed from testosterone by the enzyme 5 α-reductase type 2 (Russel and Wilson, 1994). Both testosterone and DHT bind to the same receptor, the androgen receptor (AR), but generate different physiological functions. Testosterone regulates sexual differentiation, and maintains libido and sexual functions, while DHT is responsible for the development of sex differences during human development (Grumbach et al., 2003) Testosterone is produced in the prostate from androstenedione by 17HSD types 1 and 5, which are predominantly expressed in the prostate (Labrie et al., 2000). Testosterone and DHT are mainly inactivated in the prostate gland by the action of 17HSD type 2 to androstenedione and 5 α-androstanedione, respectively.

Estrogens have also been implicated in the pathogenesis of prostate cancer. Estradiol at physiological concentrations has been shown to stimulate androgen-responsive LNCaP cells, and the stimulation appears to be receptor mediated being abolished by the addition of antiestrogens (Poutanen et al., 1993; Wu et al., 1993). Estrogens bind to estrogen receptors, two forms have been identified: estrogen receptor α (ER α) and the more recently estrogen receptor(ER) β. Both ER α and ER β are expressed in the prostate. Studies with knockout mice have shown that mice with ER β knockout develop prostate hyperplasia, while those with ER α knockout did not. This evidence seems to suggest that the presence of ER β in the prostate may have a protective effect (Krege et al., 1998). It is thought that a balance exists between the production and inactivation of steroid hormones in the prostate. Steroid producing and steroid metabolizing enzymes delicately regulate this balance. Changes in the balance of these steroid hormones or in steroid receptors may be crucial in the development and progression of cancers, including prostate cancer (Soronen et al., 2004).

1.1.3. Prostate Cancer Progression to Androgen-Independent Phase

During the progression to androgen-independent phase, prostate cancer cell growth becomes independent of androgens and is mostly maintained by other mechanisms that are not well understood. Some postulated mechanisms by which this progression may occur are described below:

a) Hypersensitivity pathway, which involves the amplification and increased sensitivity of the androgen receptor to androgens. In this environment, the tumor does not become androgen independent in per se, but develops the ability to use

low levels of androgens because of enhanced ligand binding to the androgen receptor (Chen et al., 2004; Gregory et al., 2001).

b) Promiscuous receptor, which involves mutations in the binding domain of the androgen receptor. This mutations have been shown to expand the specificity of the androgen receptor to not only bind its ligands, but to be activated by non-androgenic steroids and antiandrogens (Chen et al., 2004; Feldman and Feldman, 2001);

c) Outlaw pathway. In this situation the androgen receptor is not altered, but the receptor is activated downstream by ligand independent mechanisms. The growth and proliferation of the prostate tumor will no longer be under the control of androgens. Molecules such as insulin-like growth factor, keratinocyte growth factor, epidermal growth factor and cytokines, especially IL-6 have been shown to have the ability to phosphorylate and activate the androgen receptor (Culig et al., 1994; Culig et al., 2005; Craft et al., 1999).

d) Coactivators and Corepressors: A large number of coactivators and corepressors function as signaling intermediaries between the androgen receptor and the general transcription machinery. These molecules are also involved in the regulation of androgen receptor transcription (Janne et al., 2000). Gregory and colleagues and others, have demonstrated that changes in the levels of the coactivators and corepressors influence the activation of the androgen receptor, but the exact mechanism of this influence still has to be elucidated. (Gregory et al., 2001; Li et al., 2002). Some coactivator proteins have been shown to enhance and broaden the activity of the androgen receptor to respond to

alternative ligands, sensitize the receptor to lower levels of its native ligands and nonnative ligands and also allow the activation of the receptor by nonnative ligands (Scher and Sawyers, 2005).

e) Bypass pathway: In this pathway, the androgen receptor pathway can be bypassed completely and prostate cancer cells develop the ability to survive independent of ligand-mediated androgen receptor activation. One of the best studied bypass mechanism involves the regulation of apoptosis. In androgen dependent prostate cancer cells, activation of androgen receptor by androgens stimulates cell proliferation and abolition of androgens leads to apoptosis of the cells. With the bypass pathway, androgen independent prostate cancer cells have the ability to up regulate anti-apoptotic molecules, and by doing this, may ensure the survival of the tumor cells in the absence of androgens (Colombel et al., 1993).

Because of the many pathways by which prostate cancer can transition from androgen dependent to androgen-independent disease, it is critical to develop agents that are capable of treating both androgen dependent and androgen-independent prostate cancer disease. This need becomes even more urgent if one considers the fact that nearly all patients diagnosed with advanced prostate cancer treatable with androgen deprivation therapy will transition to the androgen-independent disease which is not responsive to most androgen deprivation therapy (Saitoh et al., 1984; Grayhack et al., 1987; Harada et al., 1992). DES is one of the only androgen deprivation therapy that has

demonstrated activity in androgen-independent disease, but the oral formulation has excess cardiovascular toxicity making it risky for patients' uses. Hence, the urgent need to either optimize the dosing regimen of the available DES formulation (oral capsules) or develop other formulations, and/or routes of administration of DES to increase efficacy and reduce toxicity.

1.1.4. Mechanism of Action and Beneficial Effects of DES in Prostate Cancer

Hormonal management of prostate cancer using estrogens or orchiectomy was introduced in the 1940s following the work of Huggins (Huggins and Hodges, 1941) and Herbst (Herbst, 1941). DES remained the mainstay of medical treatment of metastatic prostate cancer for about 25 years. In the 1960s and 1970s, the Veterans Administrative Cooperative Research Group (VACURG) published a series of randomized studies aimed at evaluating estrogenic therapies for prostate cancer. They reported the following findings: (a) oral DES therapy at 5 mg/day was effective in reducing cancer related deaths in advanced disease, but exposed patients to excessive cardiovascular deaths, and (b) oral DES therapy at 1 mg/day was almost as efficacious as the 5 mg/day dose in treating advanced prostate cancer, with reduced but significant cardiovascular deaths (The Veterans Administration Co-operative Urological Research Group, 1967). Following the VACURG studies, other nonsurgical treatments were considered, including other oral estrogens, and none was proven superior in efficacy compared to DES (Spetz et al., 2001). In the 1980s the LHRH agonists were introduced, and demonstrated to be as efficacious as DES, but safer in treating advanced prostate cancer in clinical trials. Because of the safer side effect profile of LHRH agonists, DES fell out of favor and was

replaced by LHRH agonist. The cardiovascular side effects of DES include heart attack, stroke, deep vein thrombosis, stable and unstable angina, and pulmonary embolisms.

The main mechanism of DES action in treating androgen dependent prostate cancer is by mediating the inhibition of gonadotropin release from the pituitary gland, thereby blocking the production of leutenizing and follicle stimulating hormones (Malkowicz, 2001). Luteinizing hormone is responsible for the stimulation of the leydig cells in the testes to produce and release testosterone. The mechanism of DES activity in androgen-independent prostate cancer is not well understood. In this setting, DES is thought to exert its effects by one or both of the following mechanisms: (a) DES may have a direct inhibitory or cytotoxic effect on cancer cells. Preclinical studies have demonstrated that DES has direct cytotoxic effects against various androgen-independent cancer cell lines (Robertson et al., 1996), and (b) DES may induce changes in circulating hormone concentrations which may include reductions in leutennizing hormone, follicle stimulating hormone, free and total testosterone, as well as estradiol and dehydroepiandrosterone sulfate, and increase levels of sex hormone binding globulin (SHBG), prolactin and cortisol (Smith et al., 1998; Kitahara et al., 1999). These DES mediated changes may have the general effect on reducing either the production or availability of testosterone and dihydrotestosterone to stimulate the growth and proliferation of prostate cells.

1.1.5. Diethylstilbestrol in Patients with Androgen Deprivation Therapy Failure

Several clinical trials have demonstrated the efficacy of DES in patients with androgen deprivation therapy failure, i.e. patients whose prostate cancer has progressed to androgen-independent stage. Most of the clinical trials were conducted in the 1990s or early 2000s, after which most clinicians largely avoided oral DES therapy. In 1998, Smith and colleagues conducted a phase II trial of 1 mg/day dose of DES in patients whose cancer had progressed while on hormonal therapy. They reported a 43 % PSA response rate (range 22 % - 64 %). PSA response rate was defined as PSA decline of ≥ 50 %. Toxicity for this study was minimal with 90 % of the patients reporting nipple sensitivity, 14 % had gynacomastia and 5 % (one patient) had deep vein thrombosis. The authors also noted that the response rate to DES therapy is related to the prior androgen deprivation therapies. Sixty two percent of the patients with one prior therapy manipulation responded to DES therapy, while only 13 % of patients with two or more therapy manipulations responded to DES (Smith et al., 1998). In another study, Farrugia and colleagues investigated 1 mg/day dose of DES in 38 patients whose disease progressed on hormonal therapy. Aspirin (75 mg/day) and hydrocortisone (40 mg/day) were given together to reduce the risk of thromboembolic events and the patients were treated for a median of 5 months (0.5 – 21 months). Eighty three percent of patients had improvement in their symptoms, with 72 % having PSA response of ≥ 50 %. Thromboembolic events occurred in 18 % of the patients and one patient had an upper GI bleed (Farrugia et al., 2000). Higher doses of DES have also been studied in patients with androgen-independent prostate cancer. In one study, 18 patients were treated with

3 to 4 mg/day dose of DES plus 100 mg/day aspirin. Eleven patients were receiving DES as a third hormonal maneuver. Overall, the patients demonstrated a PSA response rate of 66 %, with a median duration of response of 7.5 months. The authors noted that 45 % of the patients who were on their third hormonal manipulation responded to DES therapy. None of the study patients had a thromboembolic or cardiovascular event (Rosenbaum et al., 2000). Another study involving 242 patients treated with either 1 mg/day or 3 mg/day dose of DES co-administered with low dose aspirin (75 mg), the overall PSA response (PSA ≥ 50 %) rate was 29.1 % (1 mg: 26.1 %; 3 mg: 32.4 %). Nearly half of the patients reported some improvement in bone pain. Thromboembolic complications were seen in 11 % of all the patients (Shahidi et al., 2001). The median number of previous hormonal manipulations in this study was 2 (range 1 - 6) and the median treatment duration was 3.4 months.

These studies demonstrate that DES at doses of 1 mg/day or 3 mg/day has second line activity in the treatment of androgen-independent prostate cancer. The efficacy of DES in this setting has been demonstrated by PSA declines of ≥ 50 % in 29 – 72 % of the patients with some patients reporting symptomatic improvement in bone pain. Despite these benefits, thromboembolic complications make DES use risky in patients. The benefit of low dose aspirin to reduce thromboembolic events during DES therapy is questionable. In two of the three studies (Farrugia et al., 2000; Shahidi et al., 2001) that combined low dose aspirin as part of the DES regimen, thromboembolic events were reported in 12 % and 11 % of the patients, respectively. Considering the observed efficacy of DES in androgen-independent prostate cancer and the excess cardiovascular

complications experienced by patients on DES therapy, it is important to design new dosing regimens or formulations of DES that will decrease DES toxicities and increase compliance and efficacy. We are interested in optimizing the dosing regimens of oral DES using patient plasma DES and FVII concentrations as guides. We are also interested in studying ODF-DES as a novel formulation to deliver DES topically in the buccal cavity, to potentially avoid liver metabolism to increase bioavailability and decrease thromboembolic toxicities.

1.1.6. LHRH Agonist and Prostate Cancer

LHRH agonists are synthetic analogs of the normal human luteinizing hormone-releasing hormone, which is produced in the human hypothalamus and released in pulses. The binding of the LHRH to receptors in the pituitary leads to the pulsatile release of luteinizing and follicle stimulating hormones. Luteinizing hormone stimulates the leydig cells of the testes to produce testosterone. Engineered LHRH agonists have a modified amino acid structure that makes them more potent than human LHRH. Prolonged administration of LHRH agonists results in down-regulation of the LHRH receptors in the pituitary and decreased secretion of luteinizing and follicle stimulating hormones. The result is a decrease production of testosterone from the leydig cells (Chodak, 1989).

The introduction of LHRH agonists essentially ended the use of DES as first-line hormonal therapy for advanced prostate cancer. In a landmark study published in 1984, LHRH agonists were shown to be as effective as DES in treating prostate cancer, but were shown to have no significant cardiovascular toxicities compared to DES (The Leuprolide Study Group, 1984). In the same study, more patients in the leuprolide group

had progression in their disease compared to the DES group. The currently available LHRH agonists include leuprolide (Lupron, Eligard) and goserelin (Zoladex), and are typically given as depot injections lasting three to four months. A 12-month implant of leuprolide (Viadur) is also available.

1.1.7. Oral DES and Thromboembolism

Oral DES increases the risk of thromboembolic complications (Leuprolide Study Group, 1984). Estrogens administered orally are subject to intestinal and hepatic first pass metabolism (De Linieres et al., 1986). This liver metabolism of estrogens leads to high hormone concentrations in the liver and induces the hepatic production of pro-coagulation proteins such as FVII, FX and fibrinogen (Caine et al., 1992; Henriksson et al., 1999) and induction of resistance to the anticoagulant protein C and a reduction of protein S, which is a cofactor of protein C (Scarabin et al., 2003). These changes may tilt the body's coagulation-to fibrinolysis balance to favor more coagulation.

In clinical trials patients taking parenteral estrogens have been shown to have either fewer side effects or side effects similar to orchietomy and LHRH treatment compared to patients taking oral estrogens. Hedlund and colleagues compared intramuscular (IM) polyestradiol phosphate (PEP) with total androgen blockade (TAB), which comprised of either bilateral orchietomy or triptorelin (LHRH) with flutamide. At the end of the follow-up period of approximately 19 months, there was no difference in overall survival between the PEP and TAB groups. Most importantly, from this study, the investigators did not find any significant difference in cardiovascular mortality (3.5 % with PEP versus 3.1 % with TAB) between the groups (Hedlund et al., 2002). In another study, in which patients with

androgen-independent prostate cancer were treated with transdermal estradiol, the responses to therapy were modest, but none of the patients had a thromboembolic event (Bland et al., 2005). Information about estrogens and thromboembolic complications has also been derived from studies in women using estrogens for hormone replacement therapy. In one study comparing transdermal and oral estrogens for hormone replacement therapy in postmenopausal women, more women in the oral estrogen arm developed thromboembolic complications compared to the transdermal group (Scarabin et al., 2003). These observations that transdermal and parenteral formulations of estrogens yield fewer side effects compared to the oral formulations have led us to explore other ways of delivering DES to minimizing the first-pass metabolism. The buccal cavity offers many advantages over the conventional oral route to deliver drugs to a patient. Some of the advantages include possible bypass of first-pass liver metabolism, less GI and liver side effects, improved bioavailability, water not required for administration, and fast onset (depending on formulation and area of oral cavity applied to). We are interested in studying the comparative pharmacokinetics of ODF-DES and oral DES suspension and establish the PK/PD correlation of DES from ODF-DES and oral DES, respectively, in rats.

1.1.8. Estrogens and Clotting Factors

1.1.8.1. Fibrinogen

Fibrinogen (FI) is a glycoprotein synthesized in the liver, and circulates in plasma at levels of 1.7 – 4 mg/ml. The primary physiological function of fibrinogen is in homeostasis. It is a marker of coagulation and inflammation, and also influences

fibrinolysis. Fibrinogen is the last clotting factor in the coagulation cascade, and it is converted by thrombin to fibrin (Figure 1) which forms a clot. High fibrinogen levels have been associated with cardiovascular disease, (including deep vein thrombosis and pulmonary embolism), and fibrinogen is considered one of the single most important factors determining blood clotting time. High levels of fibrinogen result in shorter clotting time (Schultz and Arnold, 1990). Many studies have evaluated the effect of estrogen therapy on fibrinogen levels. Most of the studies were conducted on healthy postmenopausal women using estrogens for hormone replacement therapy. The results from these studies have been far from conclusive. Some of the studies reported reduction in fibrinogen levels (Lindoff et al., 1996; The Writing Group for the Estradiol Clotting Factors Study, 1996), while others showed no change (Boschetti et al., 1991; Ercan et al., 1996), and still other studies demonstrated significant increases in fibrinogen levels from the baseline (Caine et al., 1992; Shipra and Pralhad, 2007). Ercan and colleagues (1996) conducted a study in rabbits to evaluate fibrinogen levels after 10 days of intra-muscular injection of 0.3 mg/kg dose of estradiol and reported no significant change in fibrinogen levels. It is thought that some of the variations in the results of these studies may be explained partly by the differences in study designs, the inclusion and exclusion criteria, type of estrogen used and the duration of the study, and the doses of study drug administered (Conard et al., 1997; Rosenson et al., 1998; Lip et al., 1997).

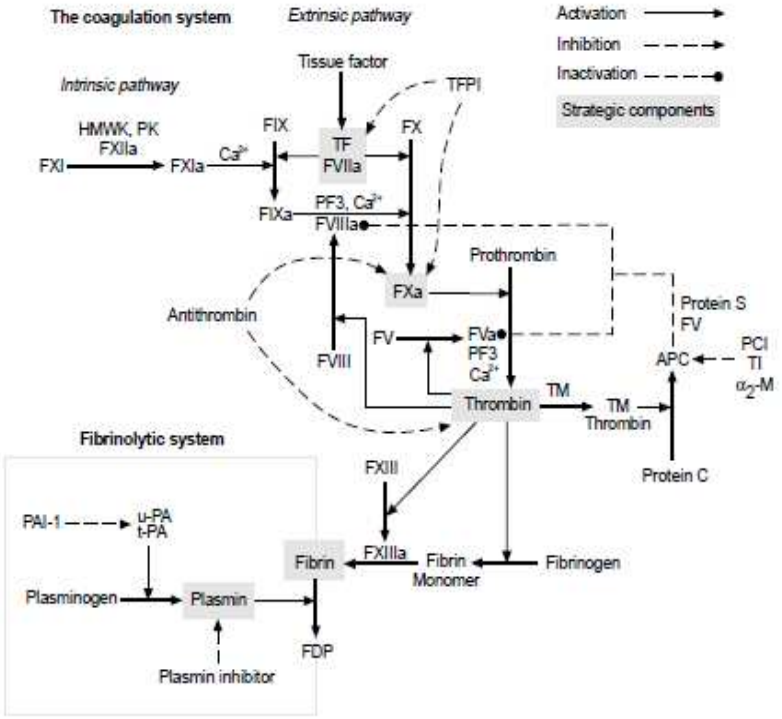


Figure 1. Schematic Representation of the Coagulation Cascade (Shen and Dahlback, 1994).

Blood coagulation is initiated by vascular injury and results in the explosive generation of thrombin, which clots blood.

Coagulation factors are represented by their Roman numerals (a = activated). Abbreviations: HMWK= high molecular weight kininogen,

APC	= activated Protein C,	PF3	= phospholipid,
α_2-M	= α_2-macroglobulin,	PK	= prekallikrein,
FDP	= fibrin degradation products.	TF	= tissue factor,
K	= kallikrein,	TFPI	= tissue factor pathway inhibitor,
PC	= Protein C,	TI	= trypsin inhibitor,
PCI	= Protein C inhibitor,	TM	= thrombomodulin,

1.1.8.2. Factor VII

Factor VII (FVII) is a serine protease and a key enzyme in the extrinsic pathway of the blood coagulation cascade. FVII is activated in the presence of tissue factor. When endothelium wall is damaged, it exposes a surface protein called tissue factor. Tissue factor released at the site of the damage forms a complex with FVII to produce FVIIa. FVIIa then activates factor X (FX), which ultimately leads to the downstream formation of thrombin from prothrombin (Figure 1). Elevated plasma FVII activity has been shown to be a significant predictor of ischemic heart disease and cardiovascular death (Meade et al., 1990; Huntlin et al., 1991). Studies have assessed the effect of oral estrogens on FVII levels. Similarly, as in the case of fibrinogen, most of the studies were conducted in postmenauposal women on estrogen therapy for hormone replacement, and the results have been inconclusive. Most of the studies showed a trend towards increased FVII with oral estrogens compared to transdermal estrogens (Kroon et al., 1994; Lindberg et al., 1989), while other studies reported no significant difference between the two formulations (Conard et al., 1995., Boschetti et al., 1991), and some studies demonstrated a decrease in FVII activity (The Writing Group for the Estradiol Clotting Factor Study, 1996) in subjects on estrogen therapy in general. We intend to quantify FVII levels in plasma of patients on oral DES therapy and compare the levels to FVII levels in normal individuals.

1.1.8.3. Antithrombin

Antithrombin-III (AT-III) is a potent inhibitor of blood coagulation at several steps, including at the point of fibrin formation. It is a glycoprotein produced by the liver and

under physiological conditions; it inhibits activated factor Xa, and thrombin (Figure 1). Thrombin is a key enzyme in blood coagulation and it converts fibrinogen to fibrin for clot formation. The ability of antithrombin III to limit coagulation through multiple interactions makes it one of the primary natural anticoagulant proteins. It is responsible for inhibiting about 80 % of thrombin in plasma (Perry, 1994). Individuals with low antithrombin III levels have been reported to have an increase thrombosis risk. Studies have shown that oral estrogens will significantly decrease levels of AT-III levels, while transdermal estrogens have no effect (De Lignieres et al., 1986; Conard et al., 1997). Higher levels of AT-III will be desirable in an environment of increase potential risk of clotting.

Because ODF-DES is a novel formulation of DES, there are no studies comparing the effect of ODF-DES and other formulations of DES on fibrinogen and AT-III. It is therefore important to quantify the levels of these clotting factors in any study comparing this novel DES formulation to other conventional formulations of DES. We intend to quantify levels of fibrinogen and AT-III in rats after the administrations of single or multiple doses of ODF and oral suspension of DES, respectively.

1.1.9. DES Making a Comeback

In the past several years, there has been a renewed interest in DES therapy for prostate cancer because of the following reasons:

a) The first reason is the evidence that DES is effective in the treatment of both androgen dependent and androgen-independent prostate cancer (Smith et al., 1998). This is important because most advanced prostate cancers will

eventually progress to the androgen independent form, which does not respond to androgen deprivation therapy (Saitoh et al., 1984; Grayhack et al., 1987; Harada et al., 1992). DES has been shown to have efficacy in androgen-independent prostate cancer.

b) The second reason is the high cost of the LHRH. Estrogen therapy represents a very cost effective method of androgen deprivation therapy. A review of studies on androgen deprivation therapy on the relative effectiveness and cost-effectiveness of the various methods revealed that overall survival was equivalent between LHRH agonist, orchietomy and DES, but DES cost less than 1- 2 % of the cost of an equivalent LHRH agonist. The problem with DES was its association with excess cardiovascular toxicities compared to the other androgen deprivation therapies (Aronson et al., 1999; Bayoumi et al., 2000)

c) The third reason is the discovery of the new estrogen receptor (ER) β and its implication in the pathogenesis and treatment of prostate cancer. ER-β is the second isoform of the ER, the first being ER-α. ER-β shares highly homologous sequences to ER-α and has similar binding affinity to estradiol, DES, and anti-estrogens (Kuiper et al., 1996; Shughrue et al., 1998). Although the exact role played by ER-β in the development and progression of prostate cancer is not entirely understood, some studies have reported significant loss of ER-β in cancer cells compared to normal cells (Horvath et al., 2001; Pasquali et al., 2001). Studies in knockout mice have also revealed that rats with ER-β, unlike their counterparts without ER-β, but with

ER-α, do not develop prostate hyperplasia. These findings have led to the speculation that ER-β may have a protective effect in prostatic tissue.

d) The fourth is a better understanding and appreciation of the toxicities associated with LHRH has arisen. These side effects include anemia, hot flashes, depression, osteoporosis, and loss of muscle mass (Oh, 2001; Oh, 2002; Smith et al., 2002; Smith, 1996). Estrogen, including DES are known to reduce hot flashes in castrated men, increase bone mineral density and stimulate erythropoiesis thus can offer patients these quality of life benefits in addition to their activity in treating androgen dependent and androgen-independent prostate cancer (Scherr et al., 2002).

Even with these benefits, most clinicians are still reluctant to prescribe oral DES to their patients because of the thromboembolic complications associated with the DES therapy. In some patients, DES may be the only therapy that affords them relieves from their prostate cancer, but at the same time, the thromboembolic complications of DES may result in their premature death. This makes it critical to develop and optimize new dosing regimens and/or new formulations of DES that may minimize the toxicity and maximize its efficacy.

1.1.10. ODF Dosage Form

Edible ODF preparations have been used by the nutraceutical industry to deliver products such as Listerine film for removing of bad breath for many years (Wu et al., 2002). These preparations dissolve in saliva and the medications are absorbed in the buccal cavity and require no water to take. An ODF-DES formulation will present the

following advantages over conventional oral formulations like tablets, capsules and oral dissolving tablets:

a) ODF-DES delivered to the buccal cavity has the potential to bypass first-pass liver metabolism (Shojaei, 1998). This will lead to less GI and liver associated side effects, such as the induction of clotting factors.
b) Prolong duration of action, as the buccal cavity is less permeable than the GI tract, hence it will take a longer time for the ODF-DES film to be absorbed compared to the oral formulation (Harris and Robinson, 1992).
c) Improved bioavailability as a result of less first passes metabolism.
d) Easy drug administration and withdrawal if the need arises, as the film can be spit out.
e) It is not necessary to take the ODF-DES film with water. Patients can be counseled to place the film in the buccal cavity and the drug will dissolve in saliva and absorbed.

1.1.11. Overview of Oral Transmucosal Drug Delivery

The oral route of drug delivery is the most preferred to the patient and clinicians compared to other routes of drug delivery. However, the oral route has some disadvantages that include hepatic first-pass metabolism and enzymatic degradation that make the administration of certain classes of drugs especially peptides and drugs with high first pass metabolism, problematic. This has led to the consideration of other absorptive mucosae as potential sites for drug absorption. Transmucosal routes of drug delivery, which include, the mucosal linings of the nasal, rectal, and vaginal, ocular and

oral cavities have some distinct advantages over the oral route for systemic drug delivery. These distinct advantages include possible bypass of the first pass intestinal and hepatic metabolism, avoidance of pre-systemic elimination within the GI tract and a better enzymatic flora for drug absorption.

Although the rectal, vaginal and ocular mucosae offer certain advantages for drug delivery, they are all associated with poor patient acceptability, and thus these sites are reserved mostly for local drug applications rather than systemic drug delivery. Compared to other cavities for drug delivery, the oral cavity is highly acceptable by patients, and the mucosa is relatively permeable with rich blood supply, and has a short recovery time after stress or damage (Rathbone and Hadgaft., 1991; Squier, 1991). In addition, lack of the langerhans cells in oral cavity makes the mucosa cells very tolerant to allergens (Wertz and Squier, 1991). The oral cavity mucosa is also easily accessible and could be considered to afford reasonable patient compliance, which makes it attractive for drug delivery.

1.1.11.1. Structure of the Oral Cavity

The oral cavity is divided into two regions, the vestibule and the mouth proper. The vestibule is bounded externally by the lips and cheeks and internally by the gums and teeth. The mouth proper is formed by the hard and soft palates, the floor of the mouth and tonsils (Figure 2). The oral cavity is lined by the oral mucosa, which is made up of stratified squamous epithelium in the outside, followed by a basement membrane, a lamina propria and a submucosa as the innermost layer (Figure 3). The oral epithelium undergoes continuous division to form intermediate layers, which eventually mature and

move to the surface and are removed. The turnover time of the oral mucosa is estimated to be about 5-6 days (Harris and Robinson, 1992). The structure, thickness and permeability of the oral mucosa vary depending on the site and also on the amount of stress endured by the area (Table 1). The mucosae of the gingivae and the hard palate which are subject to much more mechanical stress are keratinized while those of the soft palate and sublingual and buccal regions are not keratinized (Ghandi and Robinson, 1988). The keratinized epithelia contain two main types of lipids (ceramides and acylceramides) which provide barrier function. The non-keratinized epithelium contains ceramides and neutral, and polar lipids such as cholesterol sulfate and glucosyl ceramides, and are more permeable to water than the keratinized epithelia (Fox, 1998).

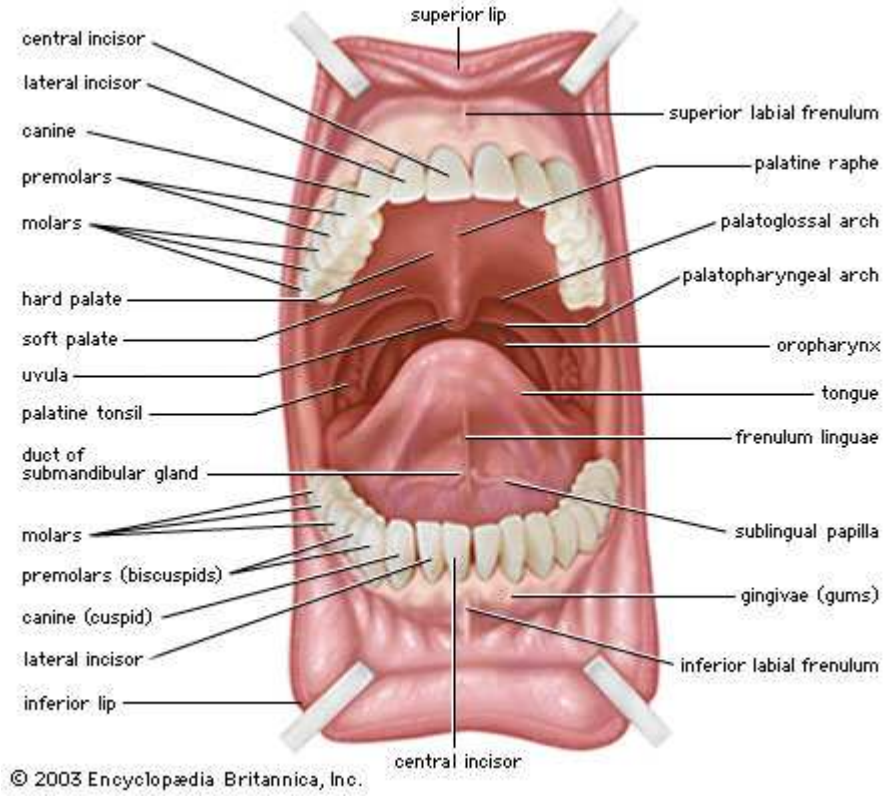


Figure 2. The Oral Cavity (Encyclopedia Britannica, accessed on June 2nd 2011)

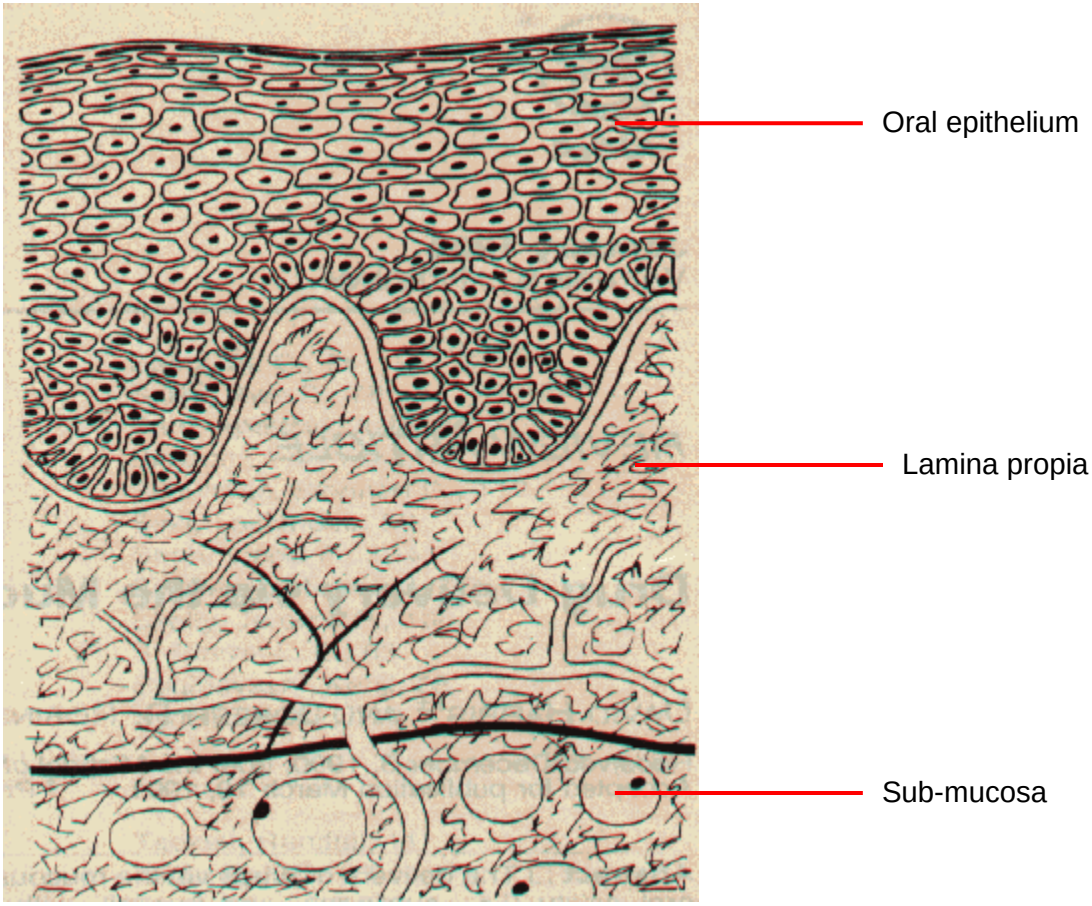


Figure 3. Cross Section of Buccal Mucosa (Shojaei, 1998)

Table 1. Regional Variation in the Composition of Oral Mucosa

Oral Cavity Membrane	Thickness (mm)	Surface area (cm^2)	Structure	Permeability
Buccal Mucosa	500-800	5.2	NK	permeable
Sublingual Mucosa	100-250	26.5	NK	very permeable
Gingival Mucosa	~200	--	K	less permeable
Palatal mucosa	~250	20.1	K	less permeable

K= keratinized

NK= nonkeratinized

References: Harris and Robinson, 1992; Ghandi and Robison, 1988

1.1.11.2. Permeability and Function of the Oral Cavity

The permeability of the oral mucosa is 4-4000 times greater than that of the epidermis, but less than that of the intestinal mucosa (Galey et al., 1976). There are significant differences in permeability between different regions of the oral cavity. The sublingual mucosa is more permeable than the buccal mucosa, which in turn is more permeable than the palatal (Harris and Robinson, 1992). This order is based on the relative thickness and keratinization of these mucosae, with the sublingual being the thinnest and non-keratinized, the buccal is the thickest and non-keratinized and the palatal in the middle in thickness and keratinized (Table 1).

The functions of the oral cavity include:

- Providing entry source for the intake of food material and water
- Undertaking chewing and mixing of food
- Lubricating food with saliva, helping cool down hot food, helping in the formation of food bolus, and providing antiseptic function
- Having taste buds to identify food material
- Initiating the digestion of some food material like carbohydrates
- Controlling speech with the help of the tongue and vocal cords

1.1.11.3. Salivary Glands and Saliva Production

Salivary glands produce saliva, which is an aqueous mixture and plays a vital role in the dissolution and absorption of drugs from oral mucosa drug delivery systems. There are

three main, paired salivary glands and about 600- 1000 minor salivary glands (Amerongen and Veerman, 2002). The major glands secret majority of the saliva and about 30% of mucus, while the minor glands secret a small portion of total saliva along with about 70% of mucus (Tabak et al, 1982). The major salivary glands are:

a) *Parotid*: This is the largest salivary gland measuring about 5.8 cm x 3.4 cm, with a weight of about 14.28 g. It is unilobar and irregular in shape and made up of 3 superficial and 2 deep processes, totaling 5 processes. It is intimately associated with the cranial nerve (CN) VII and its branches, sensory and autonomic nerves, the external carotid artery and branches, posterior facial vein and parotid lymphatics. This gland contains many fat cells and it is more of a serous gland compared to the other two glands,
b) *Submandibular*: It is the second largest salivary gland and thought to weigh half the size of the parotid gland and measures about 5 cm in length. The submadibular gland is innervated by the sympathetic innervations from the superior cervical ganglion and parasympathetic innervations from the superior submandibular ganglion from the lingual nerve, and
c) *Sublingual*: The sublingual gland is the smallest of the three glands. The gland is innervated by the sympathetic nerves from the cervical chain ganglia and by the parasympathetic nerve from the submandibular ganglion.

Minor glands: There are about 600-1000 minor salivary glands and are concentrated mostly in the buccal, labial, palatal, and lingual regions. Most of the minor glands are

innervated from the lingual nerve, except those from the palate that are innervated by the palatine nerves.

1.1.11.4. Role Played by Mucus and Saliva in Oral Transmucosal Drug Delivery

The oral epithelial cells are surrounded by mucus secreted by goblet cells. Mucus is made up of complex protein and carbohydrates. These mucus complexes are usually attached to cell surfaces and function in cell-cell adhesion and as a lubricant to allow cells to move relative to each other. Mucus is thought to play a role in the bioadhesion of transmucosal drug delivery systems to the oral cavity. This bioadhesion helps the delivery system to stay in place for the drug to be absorbed. In the oral mucosa, mucus is secreted from the major and minor salivary glands, with about 70% secreted by the minor glands (Kontis and Johns, 1998). Mucus generally has the following composition: water ~ 95 %, glycoproteins and lipids ~0.5 – 3.0 %, mineral salts ~ 1 % and free proteins ~ 0.5 – 1.0 %. The net charge of mucus at physiological pH is negative attributed partly to the presence of sialic acid and sulfate residues (Gandhi and Robinson, 1988). The negative charge is thought to play a role in the bioadhesion of transmucosal delivery systems like ODF-DES to the buccal mucosa.

Saliva is a complex aqueous fluid made up of organic and inorganic material. Saliva composition is determined by the flow rate which itself is depended on the following factors: type of stimulus, time of the day, length of stimulus on gland, the age and gender of the individual, the overall health of the individual, and the presence or absence of medication use. An adult secretes 1.0 to 1.5 liters of saliva per day. The salivary pH

ranges from 5.5 to 7 depending on the flow rate. At high flow rates, sodium and bicarbonate levels increase in secretions. The basal salivary flow rate from the three major glands is about 0.001 to 0.2 ml/minute/gland, and when stimulated, the rate increases to 0.18 to 1.7 ml/minute/gland. The presence of saliva in the oral cavity is clinically important for two main reasons:

a) Aids in drug penetration. Drug penetration across the moist mucous membrane occurs much more readily compared to the non-mucous membrane.
b) In the clinical setting, drugs are administered to the oral cavity mucosa in the solid form, which must therefore dissolve in the saliva before it can be absorbed across the oral mucosa.

The presence of excess saliva in the buccal cavity is not good for oral transmucosal drug delivery, because the saliva will wash away drug particles and end up being swallowed. This is one of the reasons why the buccal mucosae with few salivary glands affords a better environment for drug delivery compared to the sublingual mucosae.

1.1.11.5. Oral Cavity Routes of Drug Delivery

There are three different categories of drug delivery within the oral cavity:

a) Sublingual delivery: Systemic delivery of drugs through the mucosal membranes lining the floor of the mouth.
b) Buccal delivery: Systemic delivery of drugs through the mucosal membrane lining the cheeks (buccal) mucosa.
c) Local delivery: Drug delivered to treat topical conditions in the oral cavity.

The decision to select one mucosa over the other for systemic drug delivery is dependent on the anatomical and permeability differences that exist between the different oral mucosa sites. Although the sublingual mucosa is more permeable than the buccal, it is not considered suitable for oral transmucosal drug delivery. The sublingual area lacks a sizable distribution of smooth muscle or immobile mucosa, and it is also constantly being washed by large amounts of saliva, making it difficult to place a transmucosal delivery device. Because of its very high permeability and rich blood supply, the sublingual route will produce rapid onset of action, but with low bioavailability, making it appropriate for the delivery of drugs with short delivery periods and/or infrequent dosing regimen such as nitroglycerine sublingual tablet for the rescue of angina pectorial attack. The buccal mucosa on the other hand is the preferred route of systemic transmucosal drug delivery, due to the following reasons (Gandhi and Robinson, 1994):

a) The buccal mucosa is less permeable compared to the sublingual, and will not give a rapid onset of action, making it more suitable for sustain release type of formulations.
b) Unlike the sublingual mucosa, the buccal mucosa has a large distribution of smooth muscle, which provides a large surface area, and relatively immobile muscle making it more suitable for retentive systems, used for transmucosal drug delivery.

1.1.11.6. Advantages of Mucoadhesive Buccal Drug Delivery (Aungst, 1996; Rowe and Robert, 1998)

Drug administration via the oral mucosa offers several advantages over conventional administration:

1. Ease of administration and termination of therapy in emergency.
2. Permitting localization of the delivery system and drug to the oral cavity for a prolonged period of time.
3. Capable of administration to unconscious and trauma patients.
4. Offering an excellent route for the systemic delivery of drug which bypasses first pass metabolism, thereby offering a greater bioavailability.
5. Significant reduction in dose can be achieved because of increase bioavailability, thereby reducing, dose dependent side effects.
6. Drugs that are unstable in the acidic environment of the stomach or are destroyed by the enzymatic or alkaline environment of the intestine can be administered by this route.
7. Activation of drug molecules is not need, drugs are absorbed mostly by passive absorption.
8. Maximized absorption rate due to intimate contact with the absorbing membrane and decreased diffusion barriers compared to the skin.
9. The buccal mucosa is highly supplied with blood vessels and offers a greater permeability than skin.

1.1.11.7. Oral Mucosa Blood Supply

The main arteries supplying the oral cavity are the facial, maxillary and lingual arteries, which are derived from the external carotid artery. Blood from the capillary beds collects into veins carrying blood away from the oral cavity. The most prominent vein is the lingual vein, which empties into the internal jugular vein. Drug absorbed through the oral mucosa will be delivered to the internal jugular vein, which ensures that it bypasses the liver and is delivered to systemic circulation, thus avoiding the first pass hepatic metabolism.

In summary, buccal mucosa is very promising for the delivery of transmucosal drugs for systemic conditions. The avoidance of the first pass liver and GI metabolism of drugs coupled with the other advantages enumerated in Section 1.1.10 makes this route very attractive for drugs like DES. DES as other estrogens undergoes extensive first-pass liver metabolism. This first pass metabolism causes deactivation of DES and changes in the synthesis of clotting proteins that may increase a patient's risk of thrombosis. The use ODF-DES may potentially deliver DES to systemic circulation, bypassing the liver metabolism, and improving bioavailability and the side effect profile of DES.

Chapter 2. Objectives and Specific Aims

2.1. Hypothesis

Hypothesis A

Our first hypothesis is that “the establishment of the pharmacokinetic/pharmacodynamic (PK/PD) correlation of oral DES in recurrent prostate cancer patients will potentially lead to the development of more rational dosing regimens for the treatment of advanced prostate cancer”.

Hypothesis B

Our second hypothesis is that “formulations and /or routes of administration of DES to bypass the first-pass liver metabolism will potentially increase the bioavailability and decrease the cardiovascular toxicity experienced with the oral administration.”

2.2. Objectives

1. To establish the correlation between plasma concentration of DES with biomarkers of efficacy (PSA) and toxicity (FVII) in patients with advanced prostate cancer on oral DES therapy. We shall develop and validate an LC/MS/MS assay to quantify DES in the plasma of patients with recurrent prostate cancer, and use the assay to quantify DES concentration in plasma samples of 20 patients on 1-2 mg/day dose of oral DES therapy for recurrent prostate cancer. The ultimate goal is to be able to optimize oral DES dosing regimen to increase efficacy and decrease toxicity in this patient population.

2. To establish the potential merit of oral dissolving film (ODF)-DES over oral DES therapy. We shall establish the comparative PK parameters of DES from ODF and oral suspension in rats, and determine the relative bioavailability of ODF-DES compared to the oral suspension. The short term (single dose, 1mg/day) and long term (1 mg/day for 7 days) effects of ODF and oral suspension of DES on two key clotting proteins shall also be studied in rats. This study will help to determine if ODF-DES will increase DES exposure without increasing potential risk for clotting.

2.3. Specific Aims

Aim I

To develop and validate an LC-MS/MS assay method for the quantification of DES in the plasma samples of patients with recurrent prostate cancer and rats, and in rat liver tissue. In order to quantify DES in the plasma of patients on DES therapy, we need a robust and sensitive assay method. The goals of this aim are:

a) To establish a specific, sensitive and accurate assay for the quantification of DES in human and rat plasma, and rat tissue samples
b) To use the assay to quantify clinical and preclinical plasma tissue samples.

Aim II

To correlate plasma DES levels with efficacy (prostate specific antigen, PSA) and toxicity (Factor VII, FVII) biomarkers of patients with advanced prostate cancer in clinical Phase II trial at the University of Texas MD Anderson Cancer Center, in Houston TX.

The working hypothesis for this aim is that plasma DES and FVII concentrations can be used as a guide to optimize oral DES regimens for patients with recurrent prostate cancer on oral DES therapy. The goals of this objective are:

a) To establish the safety of the dosing regimen of oral DES used in this patient population.
b) To investigate the need for therapeutic drug monitoring to decrease oral DES toxicity.

Ultimate Goal:

To optimize the dosing regimen of oral DES in patients with recurrent prostate cancer, so as to increase efficacy and decrease thrombogenic toxicities.

Aim III

A. To identify potentially improved formulations of DES that will decrease thrombogenic toxicity and increase bioavailability.
B. To establish the potential merit of ODF-DES over DES oral suspension by:

- Establishing the comparative PK of buccal delivery of ODF-DES using oral suspension as reference in rats.
- Establishing the PK/PD correlation of ODF-DES in rats.

Aim III-A

To assess the feasibility of transdermal delivery of DES by studying the permeation of a solution of DES in propylene glycol (PG) using the Franz® diffusion cell. The *working hypothesis for this aim is that* sufficiently high transdermal flux of DES through excised mouse skin can be achieved upon topical application of a solution of DES in propylene glycol. A solution of DES in propylene glycol will be prepared and applied to excise mouse skin as the barrier using the Franz® diffusion cell. We shall achieve this aim by establishing the feasibility of transdermal delivery of DES solution in Severe Combined Immunodeficiency (SCID) nude mice skin by determining:

- Flux, J
- Permeability coefficient, K_p
- Diffusion coefficient, D
- Partition coefficient, K_m, and
- Lag time, T_{lag} of DES permeation from in propylene glycol through the skin barrier

Aim III-B

To establish the potential merits of ODF-DES over a suspension of DES by establishing the following:

- Enhance bioavailability of ODF-DES

- Reduced thrombogenic side effects of ODF-DES over a suspension of DES.

Aim III-B-1

To establish the relative bioavailability of ODF-DES in rats using oral suspension of DES as a reference. The *working hypothesis of this aim is that* DES from ODF will potentially bypass liver metabolism and increase the bioavailability of DES compared to DES from an oral suspension. ODF-DES will be administered topically to the buccal mucosae and oral suspension will be administered by oral gavage to the rats at a dose of 1 mg/day, respectively. This aim will be achieved by determining the preclinical pharmacokinetic parameters of:

- ODF-DES
- oral DES suspension
- and Evaluating the relative bioavailability of ODF-DES using oral suspension of DES as a reference

Aim III-B-2

To establish the PK/PD correlation of systemic exposure of DES (AUC) from ODF-DES with fibrinogen and antithrombin-III in rats using oral suspension of DES as a reference. *The working hypothesis* of this aim is that ODF-DES will significantly increase the

exposure (AUC) of DES compared to oral suspension without significantly imparting thrombogenic markers (fibrinogen and AT-III). We shall achieve this aim by:

- Determining the relationship of:
  - Plasma levels,
  - C_{max}
  - AUC of DES from ODF and oral suspension with those of fibrinogen and antithrombin-III (toxicity biomarker levels), after single dose (1 mg) of DES.
  - Fibrinogen and AT-III after multiple doses (1 mg/day for 7 days) of ODF-DES and oral suspension of DES.

Chapter 3. Materials and Method

3.1. Materials

3.1.1. Chemicals and Materials

- Acetonitrile HPLC grade (EMD, Gibbstown, NJ, USA) was used in the preparation of the LC/MS/MS mobile phase B.
- Acepromazine used in combination with ketamine and xylazine (all from Sigma Chemical Co., St Louis MO, USA) as the anesthetic combo used in all the pharmacokinetic studies.
- Ammonium Acetate HPLC grade (Sigma Chemical Co., St Louis MO, USA.) used in solution at concentration of 2mM as mobile phase A.
- Daidzein analytical grade (INDOFINE Chemical Company, Hillsborough, NJ, USA) an isoflavone was used as the internal standard.
- Diethylstilbestrol (DES) powder (Sigma Chemical Co., St Louis MO, USA) was the drug used throughout the preclinical studies and for LC/MS/MS assay development and validation.
- DES capsules donated by The University of Texas MD Anderson Cancer Center, Houston, USA and used for clinical trial and preclinical pharmacokinetics studies.
- Dimethylsulfoxide (DMSO) analytical grade (J.T. Baker Co, Phillipsburg NJ, USA) was used as a cosolvent to prepare the stock solution of Daidzein.
- Ethanol 100% (EMD, Gibbstown, NJ, USA) was used as a cosolvent for the measurement of the content of ODF-DES.
- Glacial acetic acid (J.T. Baker Co, Phillipsburg NJ, USA) was used to adjust the pH of the mobile phase to 7.6.
- Heparin sodium salt (Sigma Chemical Co., St Louis MO, USA) was dissolved in normal saline (0.9% sodium chloride) and used at a concentration of 1,000 IU to coat the micro-tubes used for blood collection during the pharmacokinetic studies.
- Human factor VIII ELISA Kit (Assaypro, St. Charles, MO, USA)

- Biotinylated polyclonal human antibody specific for FVII was used to sandwich FVII in standards and samples with a monoclonal antibody coated on the plate
- Chromagen substrate used to impart color to the assay.
- Hydrochloric acid (HCL, 0.5 N) was used as the stop solution for the fibrinogen for the ELISA assay.
- Mix Diluent was used to dilute plasma samples and reagents for the ELISA assay.
- Streptavarin-peroxidase conjugate was used to recognize the polyclonal human antibody specific for FVII bound to FVII in the samples.
- Wash Buffer used to wash antibody that do not coat bound to FVII on the plate.

- Human plasma was kindly donated by the Methodist Hospital, Houston, USA, used to produce the standard curves used to analyze the human samples.
- Methanol HPLC grade (EMD, Gibbstown, NJ, USA) was used to dissolve the drug agent (DES powder) to prepare the stock solution.
- Oral dissolving film DES (ODF-DES) was donated by NAL Pharmaceuticals Ltd., Monmouth Junction, NJ, USA and used during the preclinical pharmacokinetics studies.
- Rat antithrombin III (AT-III) ELISA Kit (GenWay Biotech, Inc., San Diego, CA, USA).
  - Anti-rat antithrombin III antibody conjugate are enzyme labeled antibodies and was used to form complexes with previously bound antithrombin to plate.
  - Chromagen substrate was used to impart color to the assay.
  - Hydrochloric acid (HCL, 0.5 N) was used as the stop solution for the AT-III ELISA kit.
  - Mix Diluent was used to dilute plasma samples and reagents for the AT-III ELISA kit.
  - Wash Buffer also a component of the rat AT-III ELISA Kit.
- Rat Fibrinogen ELISA Kit (GenWay Biotech, Inc., San Diego, CA, USA).
  - Anti-rat fibrinogen antibody conjugate are enzyme labeled antibodies and was used to form complexes with previously bound fibrinogen to plate.
  - Chromagen substrate was used to impart color to the assay.

  - Hydrochloric acid (HCL, 0.5 N) was used as the stop solution for the AT-III ELISA kit.
  - Mix Diluent was used to dilute plasma samples and reagents for the AT-III ELISA kit.
  - Wash Buffer also a component of the rat AT-III ELISA Kit.
- Sodium Chloride, analytical grade (Sigma Chemical Co., St Louis MO, USA), used at a concentration of 0.9 N to perfuse the liver before harvesting.
- Sprague-Dawley rats (males, 260-330 gm) were purchased from Charles River (Houston, USA) and were used for the pharmacokinetic studies.
- Triethylamine ((Sigma Chemical Co., St Louis MO, USA) was used to adjust the pH of the LC-MS/MS mobile phase A to 7.6.

3.1.2. Supplies

- Alcohol wipes (Webcol® Alcohol Preps, Kendall Healthcare Products Co., Mansfield, MA, USA) were used to disinfect animal's skin prior to the administering of anesthesia.

- Cotton swabs (Q-tips, 6 inch) (Sherwood Medical, St. Louis, MO, USA) were used during animal surgery to harvest the livers in preclinical pharmacokinetic studies.

- Gastric gavage blunt needle (20-guage, 2.5 inch, curved, ball-end) (Harvard Apparatus Inc., Holliston, MA, USA), firmly attached to a 1 cc syringe, was used for the oral dosing of rats.

- Gloves (lightly powdered, Latex) were used in handling diethylstilbestrol, other chemicals and animals.

- Inserts (small volume) (Chrom Tech, Inc, Apple Valley, MN, USA) used for holding of samples for LC-MS/MS analysis.

- Insulin syringes (1/2 cc, sterile) (Becton Dickinson & Co., Rutherford, NJ, USA) were used to administer the anesthesia to the thigh muscle of the rats.

- Isothermal pad (model 39 DP, Braintree Scientific, Braintree, MA, USA) was pre-heated to 37 ° C to maintain body temperatures of rat during the preclinical pharmacokinetic studies.

- Membrane filters (47mm, 0.45 µm, hydrophilic polypropylene; Pall Corp., Ann Arbor, MI, USA) were used to filter the mobile phases.

- Paraffin laboratory film (Parafilm M, Pechiney Plastic Packaging, Chicago, IL, USA) was used whenever a temporary seal was required and during the transdermal permeation studies to cover the Franz® diffusion cell.

- Pipette tips (disposable, white: 1-10 µl, yellow: 10-100 µl and blue: 100-1000 µl; Dot Scientific Inc., MI, USA) were used along with Eppendorf® pipettes for measuring and delivering solutions for all experiments.

- Polyethylene microcentrifuge tubes (1.5 ml, Axygen Scientific Inc., Union City, CA, USA) were used for collecting and storing samples from the different experiments including the pharmacokinetic studies (after heparinization).

- Surgical absorbent pads (Medline, Mundelein, IL, USA) were used during all rat surgery to harvest the liver.

3.1.3. Equipment, Apparati and Softwares

- Balance (digital, 0.0001-g sensitivity, Mettler AE100, Mettler Instrument Corp., Hightstown, NJ, USA) was used for all weighting purposes.

- Centrifuge (Marathon 13K/M, B Hermle AG, Germany) was used in the preclinical pharmacokinetic studies to separate plasma from blood cells and during diethylstilbestrol extraction process.

- Columns:

    - Reverse-phase XDB-C_{18} (Agilent Eclipse®: 4.6 mm x 150 mm, particle size of 5 µm; Agilent Technologies, Santa Clara, CA, USA) was used in the LC-MS/MS quantification of DES from both human and rat plasma samples and all pharmacokinetic studies.

- Heating-stirring plate (PC-351, Corning Co., Corning, NY, USA) was used to aid in the mixing and dissolving of DES and other chemicals.

- MS/MS system:

  Unit: Consisted of an ABI 3200 Qtrap triple quadruple mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA, USA). The MS/MS was used to develop the method for the quantification of DES in both human and rat plasma samples, and rat liver samples.

  The Analyst® Software version 1.5 was used to analyze the data.

- LC system:
    - Unit: Consisted of an Agilent Technologies 1200 series liquid chromatography instrument (Agilent Technologies, Inc., Santa Clara, CA, USA).
- Student's t-test was used to compare between two groups, with $p < 0.5$ considered significant.
- NONMEM Software version 7 used for population pharmacokinetics analysis of the plasma DES concentrations from the patients..
- pH-meter (Corning Scholar 425, Corning, NY, USA) was routinely used to measure the pH values of the mobile phases.
- Pipettes (Eppendorf®, three sizes: 1-10 µl, 10-100 µl and 100-1000 µl) were used along with appropriate pipette tips for measuring and delivering solutions for all experiments.
- Pipette-aid (Drummond Scientific, Broomall, PA, USA) was attached to glass pipettes (10 and 20 ml) and was used to transfer liquids whenever needed.
- Spectrophotometer (Model BU-70, Beckman, Fullerton, CA, USA) was used to generate UV absorbance readings for fibrinogen and antithrombin-III.
- Synergy 185 Water Purification System (Molsheim, France) was used for generating deionized water.

- Vortex mixer (Vortex-2 Genie, Scientific Industries, Bohemia, NY, USA) was used whenever sample mixing was required.

- WinNonlin Professional version 3.3 (Pharsight Corp., Mountainview, CA, USA) was used for pharmacokinetic data analysis and generation of pharmacokinetic parameters.

3.2. Methods

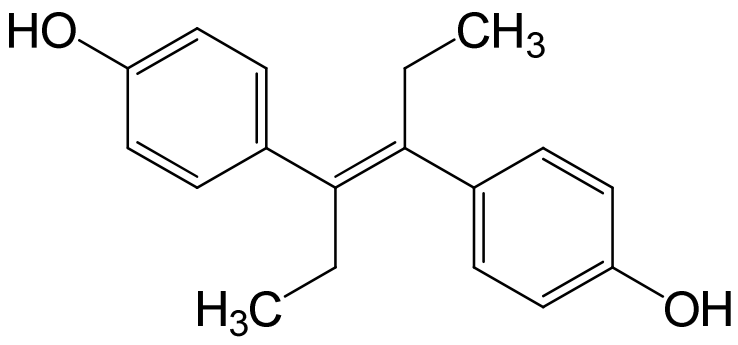
3.2.1. LC-MS/MS Assay

LC/MS/MS assay method for the quantification of DES in the plasma of patients with recurrent prostate cancer and of DES in rats.

3.2.1.1. Rationale:

Following the renewed interest in DES (Figure 4) and the thromboembolic complications associated with its use, we have developed an assay capable of detecting DES with a lower limit of quantification (LLOQ) of 0.78 ng/ml in human plasma. This assay could be employed in quantifying DES in the plasma of patients on oral DES therapy for prostate cancer. Oral DES therapy though very effective in treating advanced prostate cancer, is associated with excess thromboembolic side effects. We believe that by developing a sensitive and precise assay to quantify DES in plasma, a guide to optimize DES dosing regimens in patients to decrease toxicities and maximize benefits can be established, using achieved plasma DES concentrations as quantification measures. To our knowledge, there are no published HPLC or LC/MS/MS assay to quantify DES in human plasma. Two papers published in 1981 and 1982 made mention of quantifying DES in

(a)



(b)

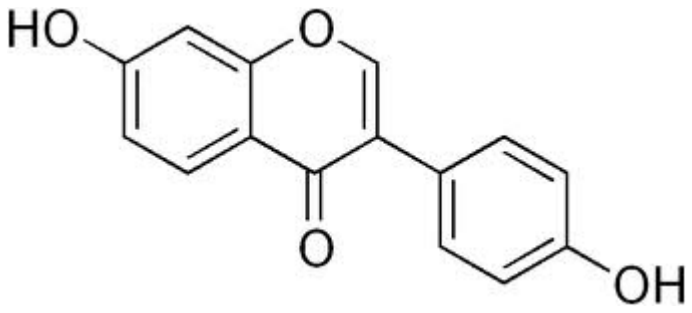


Figure 4. Chemical Structures of (a) Diethylstilbestrol and (b) Daidzein

human plasma, using gas chromatographic/mass spectrometric (GC/MS) (Kemp et al., 1981; Abramson and Miller, 1982), but none of the assays were published. Published assays for measuring DES in plasma are radioimmunoassays (Kemp et al., 1981; Usui et al., 1984; Nakamura, 1986). Radioimmunoassays for DES quantifications in plasma have the major disadvantage of poor specificity, due to cross reactivity with other steroids and metabolites of DES in plasma (Economou et al., 1993). We are employing liquid chromatography tandem mass spectrometer (LC/MS/MS); an instrument widely used in sensitive bioanalytical studies. The developed and validated assay can easily be modified for quantification of DES in rat plasma and liver tissue samples.

3.2.1.2. Experimental Design

The assay was developed using an LC-MS/MS. Ethyl acetate was used as the extraction solvent and Daidzein (Figure 4), an isoflavone, was the internal standard. The linearity of the assay was established in the range of 0.78 – 100 ng/ml. The accuracy and precision of the assay were determined using quality control (QC) samples (samples with known concentrations, n = 6 each). The assay validation was carried out according to the FDA Guidance's for Bioanalytical Method Validation (Guidance for Industry. Bioanalytical Method Validation, accessed in June 2010).

The assay was developed with an AP 3200 Qtrap triple quadruple mass spectrometer. The main MS/MS parameters were set as follows: curtain gas, nitrogen, 30 psi; gas 1, nitrogen, 80 psi; gas 2, nitrogen, 40 psi; ion source temperature, 500 ºC; ion spray source voltage, -4500kv. The transitions for DES and internal standard monitored are m/z 266.9— m/z 237.3 and m/z 253.0—m/z 132.0, respectively. The LC is an Agilent

Technologies 1200 series and the separation was done using an Agilent Eclipse XDB-C18 column (5µm, 4.6 mm x 150 mm), maintained at a temperature of 40 ºC. The gradient mobile phases were: 2.5 mM ammonium acetate, PH 7.6 (A) and 100 % acetonitrile (B), with 35% B (0–1 min), 35 – 95% B (1–5 min), 95% B (5–6 min), and 35% B (7–8 min). The flow rate and injection volume were 1 ml/min and 50 µL, respectively.

3.2.1.3. Stock Solutions and Standards

A stock solution of DES at 1 mg/ml in MeOH was prepared and stored at -20 ℃. The stock solution was diluted to 10 µg/ml using 50 % ACN in water and further diluted with plasma to 100 ng/ml working solution. Serial dilutions were made from the working solution to prepare the calibration standards and quality control (QC) samples. Stock solution of Daidzein at 10 mM prepared in DMSO/MeOH (25/75) was diluted with DMSO/MeOH to 100 µM then with 50% ANC in water to a working solution of 50 µM.

3.2.1.4. Preparation of Plasma Samples

Plasma working stock solution of 100 ng/ml was prepared by spiking drug free human plasma with 10 µL of 10 µg/ml DES. Serial dilutions in the range of 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, and 100 ng/ml were made for the calibration curves. Three sets of QC samples of 2 ng/ml (low), 50 ng/ml (medium) and 80 ng/ml (high) were also prepared using the same methodology.

3.2.1.5. Extraction of DES from Plasma Samples

Ethyl acetate was used as the extraction solvent. A 100 µL aliquot portion from each DES concentration was mixed with 10 µL of 50 µM Daidzein for the human plasma

samples and 5 µM for rat plasma and liver tissue samples, respectively. Five hundred µl of ethyl acetate was added to the mixture and vortexed for 20 seconds. The mixture was centrifuged at 13,000 rpm for 15 minutes at 25 ºC, and the supernatant was collected and air dried at room temperature using an N-EVAD Analytical Evaporator. After reconstitution with 100 µL of 50% ACN in water, the residue was vortexed for 30 seconds and centrifuged at 13,000 rpm for 10 minutes. Fifty µl of the clear supernatant was injected into the LC for analysis.

3.2.1.6. Validation Process

The validation process was guided by the current FDA Guidance's for Bioanalytical Method Validation (Guidance for Industry. Bioanalytical Method Validation, accessed June 2010). The internal standard method was used to quantify the samples. Calibration standards in the range of 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, and 100 ng/ml were prepared by serial dilution from the working standard solution of DES. The range was selected after several trials to cover the concentrations to be observed in our clinical samples. The QC samples, blank and internal standard only samples were also included in the runs.

The slope, intercept and correlation coefficient were calculated and fitted using $1/x^2$ weighted regression analyses. The linearity of the assay was evaluated by comparing the correlation coefficient (r^2) between theoretical and back calculated concentrations of calibration (standard) curves.

Quality control samples at 2 ng/ml (low), 50 ng/ml (medium) and 80 ng/ml (high) were used to determine the intra- and inter-day precision and accuracy. Intra-assay accuracy and precision was determined by calculating QC sample concentration (n=6 for each QC level) using a standard curve prepared within the same run. For inter-assay precision and accuracy, five different runs were performed on three different days and the QC sample concentration (n=18 for each QC level) was calculated using calibration standard curves prepared within the same run. Whenever clinical samples were analyzed, QC samples were included and analyzed using calibration standards within the same run. The result from any patient series was only accepted if the percent deviation (RDS %) between the target and back-calculated concentrations were within ±15% or < 20 % at the LLOQ.

The extraction efficiency of DES and internal standard was determined using calibration standards prepared as follows: (A) Pure analyte, DES in 50 % ACN in water, (B) DES and internal standard spiked in human plasma then extracted, and (C) DES spiked in human plasma, extracted and internal standard added before reconstitution. The extraction efficiency (%) of internal standard was calculated by dividing slope of C by B (C/B), and that of DES was calculated by dividing the slope of C by A (C/A).

The stability of DES was determined using low and high QC samples (n=4 each) under the following conditions: (A) The 12 hours bench top at room temperature and 72 hours (+4 ºC) stability of DES, which were determined by spiking plasma samples and leaving them at the bench top for 12 hours and in the fridge (+4 ºC) for 72 hours, respectively before extraction, (B) The 12 hours stability of the extracted samples inside the

autosampler at room temperature, which was determined by reconstituting extracted samples and leaving them for 12 hours on the autosampler before injecting, (C) The stability of DES pure analyte at room temperature for 12 hours was also determined. The percentage of degradation was calculated by computing the difference in back calculated concentrations between freshly prepared QC samples (considered 100% stable) and QC samples left under the various conditions listed above.

The matrix effect was also determined by comparing the peak areas of analytes of spiked DES and internal standard in the supernatant of extracted blank plasma and extracted pure analyte in 50 % ACN in water. Low and high QC samples were used (n=4 each). Ion suppression was determined using samples spiked with internal standard only, DES only and DES and internal standard, respectively.

3.2.2. Phase II Clinical Trial of Oral DES in Recurrent Prostate Cancer Patients

3.2.2.1. Criteria for Patient Selection

The inclusion criteria included the following:

- Patients must have evidence of rising serum PSA levels (two consecutive increases at least one week apart) after definitive local therapy, which included prostatectomy and/or radiation therapy or on hormonal ablative therapy using LHRH agonist or orchiectomy.

- Patient must have been treated prior with anti-androgens or prostate cancer products.

- Patient must have evidence of progression of disease on anti-androgen withdrawal.

Patient with the following conditions were excluded:

- History of active or unstable angina.
- History of myocardial infarction or cerebral vascular accident within 3 years of registration.
- Prior treatment with bisphosphates within one year.
- On any other investigational drug.
- Psychiatric illness/social situation that may limit compliance.
- Overt psychosis, mental disability or inability to give informed consent or history of noncompliance with medication.

3.2.3. Collection of Patient Clinical Samples

Plasma samples were collected at the University of Texas MD Anderson Cancer Center from 19 of the 20 patients enrolled in this clinical phase II study. One patient dropped out 2 days into the study due to deterioration in his health condition. All the patients received the study medication which comprised of 2 mg/day (1 mg twice a day) dose of oral DES capsules (except patients # 3, 5 and 8, who received 1 mg/day), and were not hospitalized. The patients were required to return to the clinic for follow-ups. Blood samples approximately 3 ml were collected 2-3 hours post dose into pre-heparinized

centrifuge tubes and immediately centrifuged to separate plasma from blood cells. The plasma samples were stored at -80^{o} C pending LC/MS/MS analysis by the validated LC/MS/MS assay method.

3.2.3.1. Preparation of Human Clinical Plasma Samples for Analysis

The study clinical plasma samples were prepared just like the samples used to develop the assay method, with the only difference being that the human samples were concentrated 10 times to increase the concentration of DES in the samples. To achieve this, we started with 1 ml of study sample and added 5 ml of ethyl acetate (extraction solvent). After the extraction and the drying step, we reconstituted the residue with 100 uL of 50 % ACN in water, concentrating the sample by a factor of 10. After analysis, the concentration obtained after interpolation of the unknown concentration from the calibration standard curves was divided by the factor of 10, to obtain the DES concentrations in the patients. The reason for concentrating the samples is that preliminary studies with the clinical samples revealed that DES concentration in the clinical plasma samples were very low.

3.2.4. In Vitro Permeation Experiment

3.2.4.1. Preparation of Permeant Solution

A stock solution of DES was prepared in methanol at a concentration of 2 mg/ml. The DES stock solution was mixed with propylene glycol in the ratio of 1:1 and the mixture was air dried to eliminate the entire methanol. The DES solution in propylene glycol was stored at -20 ℃ until the start of the permeation experiment.

3.2.4.2. Harvesting and Preparation of Mouse Skin Samples

Skin samples from severe combined immunodeficiency (SCID) athymic nude mice (n= 3) were used for this experiment. The mice were euthanized using overdose of carbon dioxide inhalation and after making sure the animals did not show any sign of movement, the skin from the dorsal section were surgically harvested. The subcutaneous fat was carefully removed with a blade, making sure that the integrity of the skin barrier was not compromised. The skin pieces were carefully cut and mounted on top of the 9-unit Franz® Diffusion Cells (Figure 5), and maintained at a temperature of 32 ± 1 ℃ by circulating water through a jacket sur rounding each cell.

3.2.4.3. Diffusate Samples

The procedure for the permeation experiment using the Franz® diffusion cell was described (Franz, 1978; Chow et al., 1984). The receiver medium (diffusate) was composed of 0.2 % Tween 80 in phosphate buffer saline (PBS; 140 mM NaCl, 0.4 mM KH_2PO_4 and K_2HPO_4; pH 7.4). One hundred µl of the permeant solution was applied to the stratun corneum surface of the mouse skin. The donor compartments of the cells were covered with paraffin to prevent the evaporation of the permeat solution. At predetermined time points of 1, 2, 4, 6, 8, 10, 14, 16 and 24 h, aliquots of 0.2 ml of the diffusate was withdrawn from the side arm of the receptor chamber. Equal volume of fresh receiver medium was used to replace the withdrawn volume. All samples were stored at -20 ℃, until the end of the experiment. The samples were analyzed using the validated LC-MS/MS assay described in section 3.2.1.

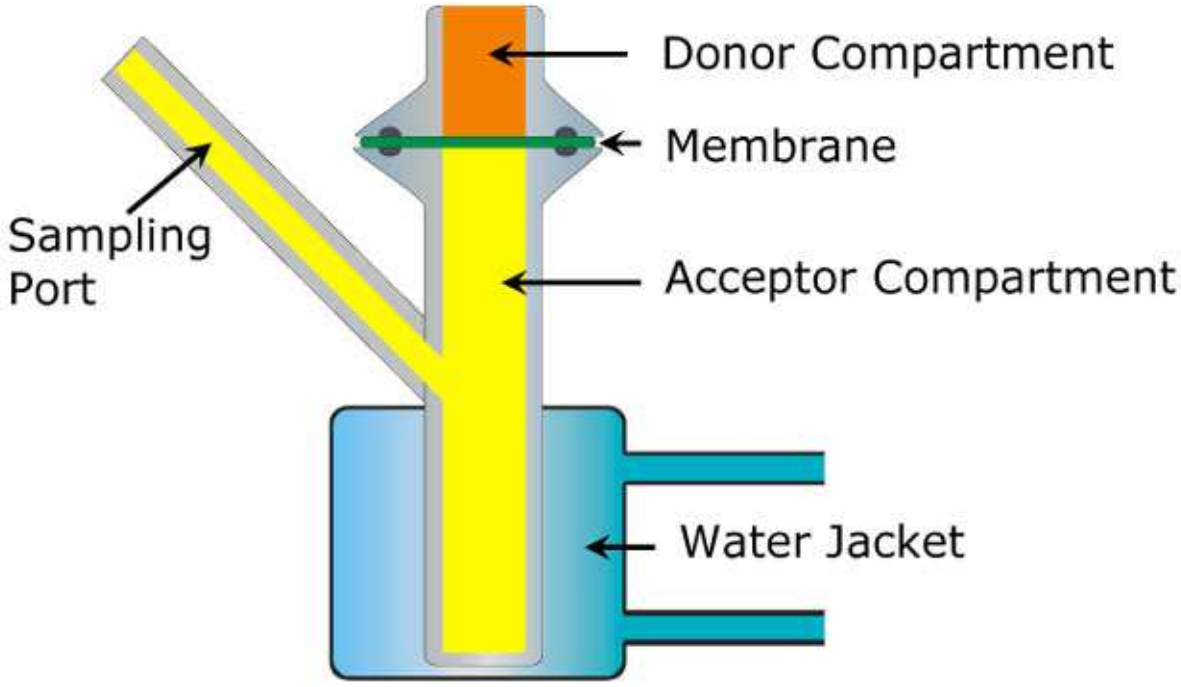


Figure 5. Franz® Diffusion Cell for Evaluation of Transdermal Permeation In Vitro (Franz, 1978).

3.2.4.4. Data Analysis (Barry, 1983; Bronaugh and Maibach, 1985)

A transdermal permeation profile of DES from the solution in propylene glycol was constructed by plotting the cumulative amount of DES permeated (ng) versus time (h). The slope of the linear portion of the plot was determined by regression analysis and represented the steady-state transdermal flux (J) (Figure 6). The x-intercept of the extrapolated linear portion of the graph represented the lag time (T_{lag}). When J is divided by the initial concentration (C_s) of the formulation, the permeability coefficient (K_p) is obtained, (Eq. 1). The diffusion coefficient normalized by skin thickness (D/h^2) and partition coefficient between the skin and vehicle normalized by skin thickness (Km.h) were calculated for this formulation of DES according to Eq. 2 and 3, respectively.

$$K_p = J/C_s \qquad \textbf{Eq. 1}$$

$$D/h^2 = 1/6T_{lag} \qquad \textbf{Eq. 2}$$

$$K_m \cdot h = K_p / (D/h^2) \qquad \textbf{Eq. 3}$$

The data were reported as mean ± SD.

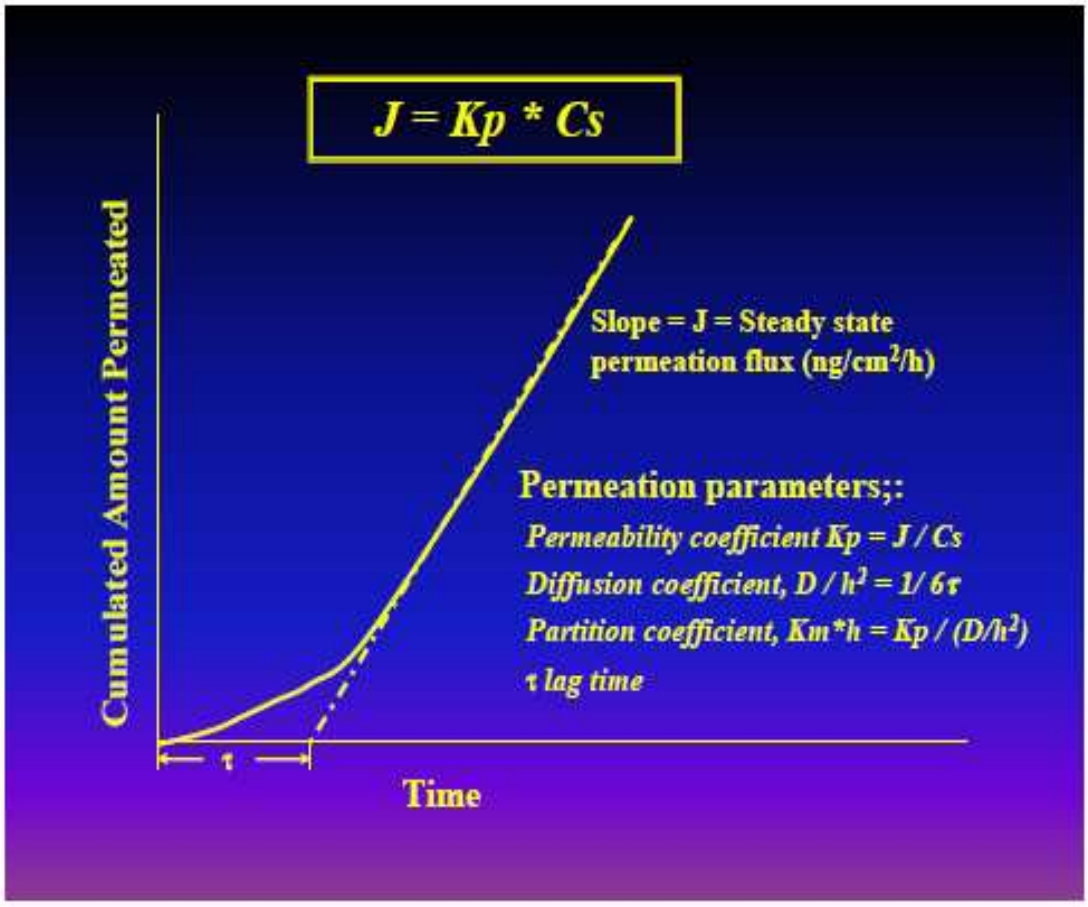


Figure 6. Typical Permeation Profile for Finite Dose Application to the Skin (Bronaugh and Maibach, 1985)

3.2.5. Determination of DES Content in ODF-DES Film.

3.2.5.1. Preparation of ODF-DES Film Samples for Content Measurement

Before using the ODF-DES films, the DES content of the ODF film was measured. Each film (n= 4) was cut into 4 equal pieces with a scissor and carefully weighed. Each piece was placed in a 15 ml vial holding 10 ml of 50 % ethanol in water. The vial was vortexed for 1 minute and sonicated for 1 h to reduce the film to very fine particles. Aliquot portions were collected from each vial and diluted 1000 times with 50 % ACN in water. Fifty µl of the diluted aliquot portions were injected into the LC-MS/MS for DES quantification using the validated LC-MS/MS assay method. The DES concentration in each film portion was quantified using a calibration standard curve prepared alongside the film samples

3.2.5.2. Calculation of DES Content in ODF-DES Films

The DES content from each ODF film was calculated by multiplying each concentration by the dilution factor of 10,000 (film was dissolved in 10 ml and later diluted by 1000 with 50 % ethanol in water). The content of the entire film was obtained by adding the content of the 4 divided pieces of each film. The uniformity of each film was determined by comparing the film portions on 0a mg/mg basis.

3.2.6. Pharmacokinetic Studies in Rats

The animal studies were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Houston.

3.2.6.1. ODF-DES Film

The ODF-DES film was provided by NAL Pharmaceutical (Monmouth Junction, NJ, USA). The film is developed with a patented technology (Figure 7) and is off-white in color and measures approximately 2 cm by 2 cm. The target weight and DES content of each film were 50 mg and 1 mg, respectively.

3.2.6.2. Dosing of ODF-DES Film

The rats were anesthetized with a mixture of ketamine, xylaxine and acepromazine (50: 3.3: 3.3 mg/ml) at a dose of 1 mg/kg. A single film (1 mg of DES) was divided into two approximately equal portions, and each portion was applied topically on each side of the inner cheek of the rat. Serial blood samples were collected by tail vein at predetermined time points of 0.25, 0.5, 1, 2, 4, 8, 12, 24 and 30 h post-dose into heparinized polyethylene microcentrifuge tubes. The blood samples were immediately centrifuged at 13,000 rpm for 15 minutes to separate plasma from blood cells. The plasma was transferred to another polyethylene microcentrifuge tube and stored at -80 ℃ until sample analysis. The control animals were dosed with placebo ODF film and all the other procedures were the same as for the study animals.

Figure 7. Example of Bio-FX ODF: 2 cm x 2 cm (NAL Pharmaceutical, New Jersey, USA)

3.2.6.3. Preparation of Oral Suspension

Oral DES capsules were kindly donated by the Pharmacy Department of the University of Texas MD Anderson Cancer Center in Houston, Texas. The oral suspension was prepared from oral capsule in patented Plus™ Oral Suspending Vehicle (Ingredients: Purified water, lambda carrageenan, simethicone, xanthan gum, microcrystalline cellulose/ sodium CMC, sodium phosphate dibasic, citric acid, potassium sorbate, methylparaben , and propylparaben) from Professional Compounding Centers of America (PCCA). The capsules were gently opened and carefully emptied into a 15 ml polyethylene vial and an appropriate amount (15 ml) of the PCCA Plus™ Oral Suspending Vehicle was added to make a concentration of 1 mg/ml. The mixture was vortexed for one minute and sonicated for 30 minutes at room temperature, until no powder was visible by visual inspection. At this point, the suspension was ready for dosing the animals.

3.2.6.4. Dosing of Oral Suspension of DES

The rats were anesthetized as in Section 3.2.6.2, and each was administered a volume of 1 ml (1 mg DES) of the oral suspension by oral gavage. The rest of the procedure were as described in section 3.2.6.2; except that during the serial blood sampling, the 0.25 h time point was omitted for the oral suspension and an additional time point at 0.75 h was added. The control rats were administered the oral suspending vehicle and treated as the study rats for the remaining procedures. The oral suspension formulation

served as the reference formulation for bioavailability evaluations and during the pharmacodynamics investigation of toxicity biomarkers.

3.2.6.5. Long term dosing of ODF and oral suspension

We wanted to determine the effects of the long term dosing of ODF-DES and oral DES suspension on the clotting factors (fibrinogen and AT-III). One group of animals (n=4) was dosed ODF-DES for 7 days, and on the 5th day, plasma samples were collected daily up to the 8th day. Another group of rats was given oral suspension of DES as described in section 3.2.6.4 daily for 7 days and plasma samples collected daily from the 5th to the 8th day. The plasma samples were analyzed for clotting factors using ELISA kits. At the end of the study, the animals were sacrificed by overdose of carbon dioxide inhalation and liver tissue was harvested from both animal groups to quantify DES in the liver tissue samples. Before harvesting the liver tissue, the liver was perfused with normal saline through the aorta to remove residual blood from the tissues. The samples were stored at -80 ℃ until analysis.

3.2.6.6. Extraction of DES from liver tissue samples

The liver samples were allowed to thaw and carefully weighed then added to appropriate volumes of normal saline with a weight/volume ratio of 0.5. The tissue samples were vortex-mixed and homogenized with a tissue tearor homogenizer until thoroughly homogenized. One hundred µl of the liver homogenate sample was mixed with 10 µl of internal standard (5 µM of Daidzein). After adding 500 µL of ethyl acetate (the extraction solvent), the mixture was vortexed for 30 seconds and centrifuged at 13,000 g for 20 minutes. The clear supernatant was collected and aired dried. The residue was

reconstituted with 100 µL of 50 % ACN in water and ready to be injected in the autosampler for quantifications of DES in liver tissue samples.

3.2.6.7. Extraction of DES from Rat Plasma Samples

The extraction of DES from rat plasma samples was carried out as described in Section 3.2.1.5. The samples were analyzed using the validated LC-MS/MS assay.

3.2.7. PK/PD Studies

3.2.7.1. Determination of Factor VII (FVII), Fibrinogen (FBG) and Antithrombim III (AT-III) Levels

The levels of clotting factors (fibrinogen and AT-III) were measured using ELISA Kits from GenWay Biotech, Inc. (San Diego, CA, USA), While FVIII was quantified using ELISA Kits from Assaypro (St. Charles, MO, USA). Briefly, plasma samples were diluted 10,000X and all reagents were brought to room temperature. One hundred µl of sample was added to the pre-designated wells and incubated for 60 minutes. After washing the wells, 100 µl of anti-AT-III horseradish peroxidase enzyme conjugate was added and incubated for 30 minutes in the dark. A second round of washing was followed by the addition of chromagen substrate solution and incubated in the dark for another 10 minutes and then the absorbance read at 450 nm. The average background was subtracted from the test values for each sample and a standard curve was constructed using a four-parameter logistic fit. The test sample values were interpolated from the standard curve and after correction for serial dilution, the AT-III concentration in the original samples was obtained. The same procedure was applied to quantify the FVII with little variation as suggested by manufacturer (in human plasma samples) and

fibrinogen levels, except that anti-FVII and anti-fibrinogen horseradish peroxidase enzyme conjugates were used for the FVII and fibrinogen assays, respectively.

3.2.8. Pharmacokinetic Data Analysis

3.2.8.1. Clinical Data Analysis

Each patient had sparse samples and the data from the clinical trial was analyzed using the NONMEM version 7. The population pharmacokinetics parameters of elimination rate constant (k), absorption rate constant (k_a) and volume of distribution (V) were generated by fitting the one-compartment model to the patient data. The population clearance (CL) and elimination half-life were calculated using the equations CL = KV, and $t_{1/2}$ = 0.693/K, respectively.

3.2.8.2. Preclinical Data Analysis

Data obtained from the Pharmacokinetic studies were analyzed using the WinNoLin software professional version 3.3. The parameters of time to reach peak concentration (T_{max}), peak concentration (C_{max}), area under the curve (AUC, both truncated and infinite), absorption rate constant (K_a), terminal elimination rate constant ($T_{1/2\ \beta}$), clearance (CL) and volume of distribution (V) were generated by fitting the plasma concentration-time profiles into different PK models. The appropriate model selected was based on (a) the correlation coefficient (R^2) between the observed and predicted data points based on the selected model and (b) the visual inspection for best fit of the observed data points to the model generated concentration time profile (predicted data points).

3.2.9. Statistical Analysis

The student's t-test was used to determine statistical significance between 2 groups with $p < 0.05$ reported as statistically significant.

Chapter 4. Results

4.1. LC-MS/MS Assay

A sensitive and precise LC-MS/MS assay was successfully developed and validated for the quantification of DES in plasma and rat liver tissue. The internal standard was daidzein at a concentration of 50 µM. The mass spectra for DES and internal standard showed maximum intensities at ions 266.9/237.0 and 253.0/132.0, respectively, (Figures 8 and 9). No interference with the peaks of both DES and the internal standard, as evident by the chromatogram of blank plasma (Figure 10). The running time of the assay was 8 minutes, with the retention time of DES and internal standard of 5.3 (Figure 11) and 2.9 minutes (Figure 12), respectively. The assay was linear within the range of 0.78 – 100 ng/ml (Figure 13). The assay was validated using high (80 ng/ml), medium (50 ng/ml) and low (2 ng/ml) quality control (QC) samples. The intra-day accuracy and precision were below 13 % for the high and medium QC samples and below 17 % for the low QC samples (n=6) (Table 2). The inter-day accuracy and precision were below 10% for the high and medium QC samples and below 16% for the low QC samples (Table 2), all of which are below the FDA recommended guidelines (Guidance for Industry. Bioanalytical Method Validation, accessed June, 2010).

Pure DES analyte solution was stable at room temperature for 12 hours, while DES in human plasma at room temperature for 12 h showed a degradation of 1.3% and 12.5 % at the high and low QC sample concentrations, respectively. When DES human plasma samples were stored at 4 ℃ for 72 h, the degradati on was between 21.3 % and 20.5%

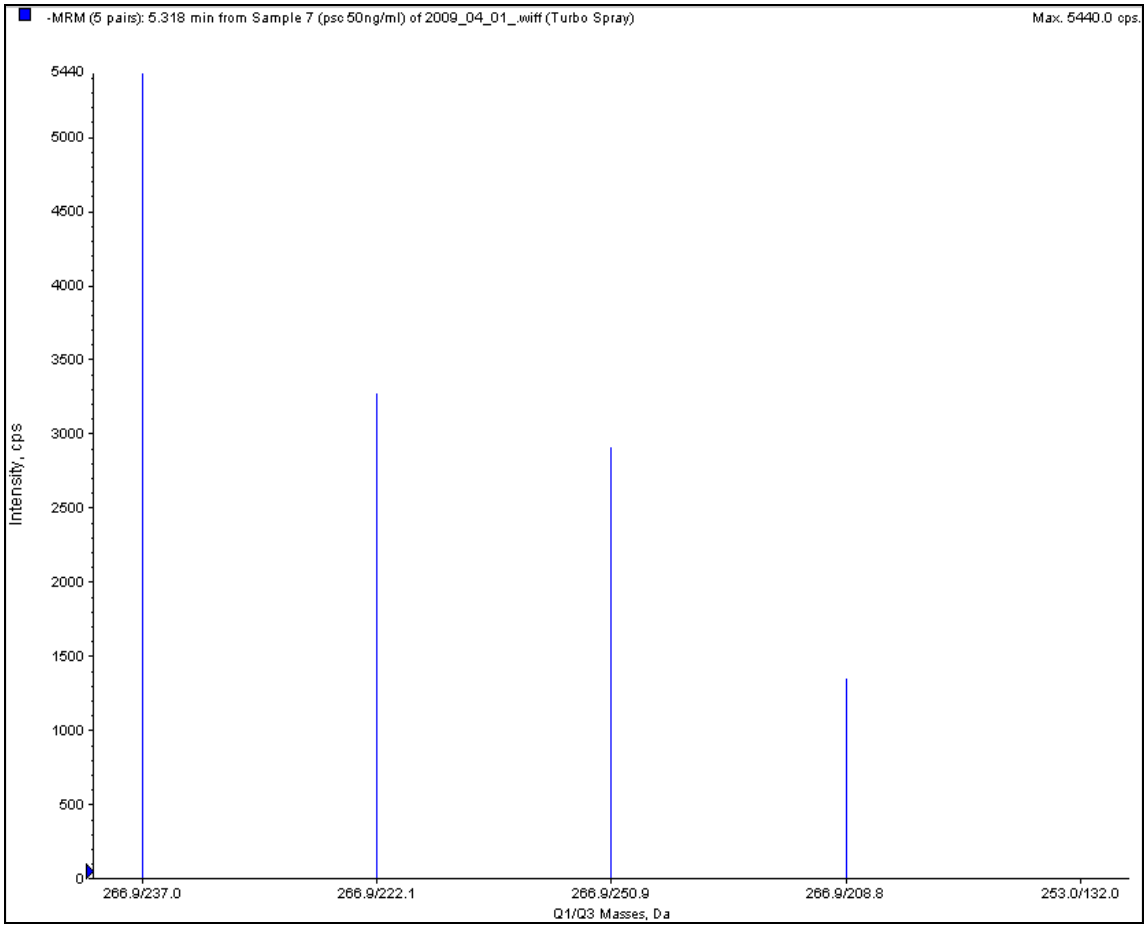


Figure 8. Authentic Mass Spectra of DES (Ions monitored at 266.9/237.0)

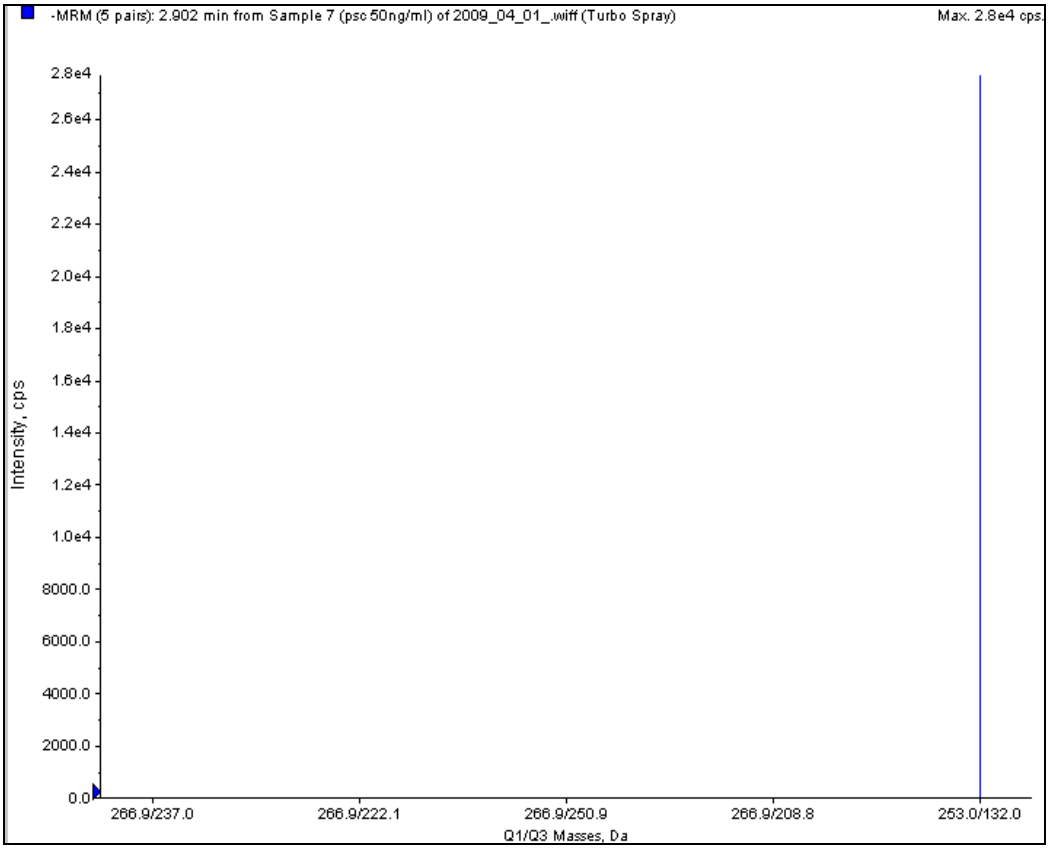


Figure 9. Authentic Mass Spectra of Daidzein (Ions monitored at 253.0/132.0)

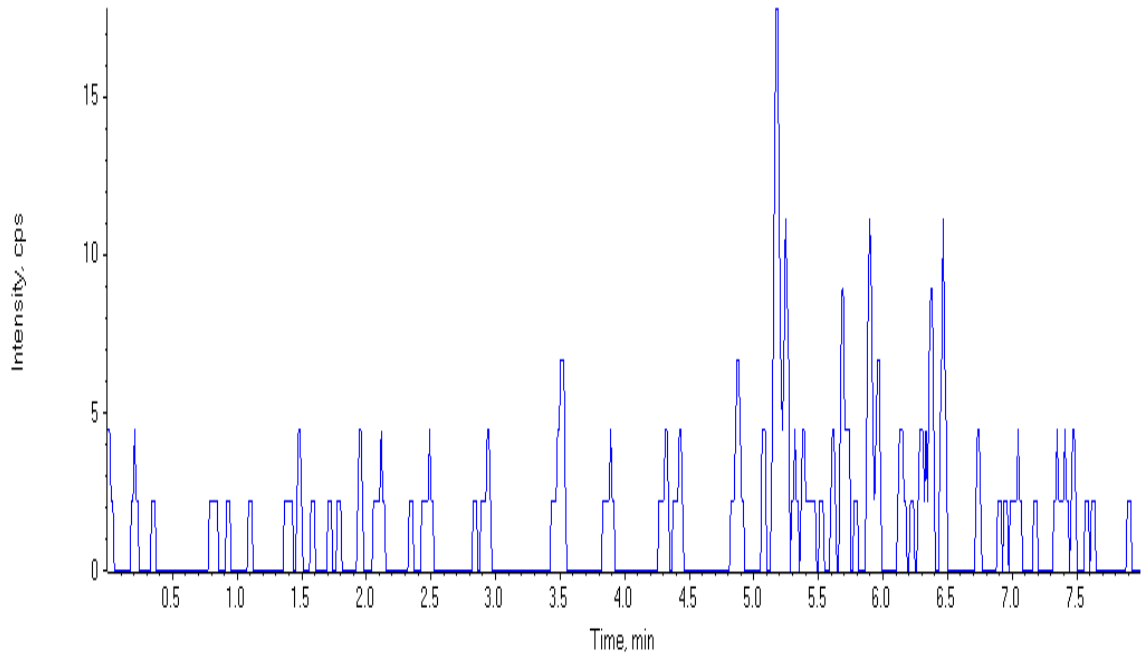


Figure 10. Chromatogram of Blank Plasma

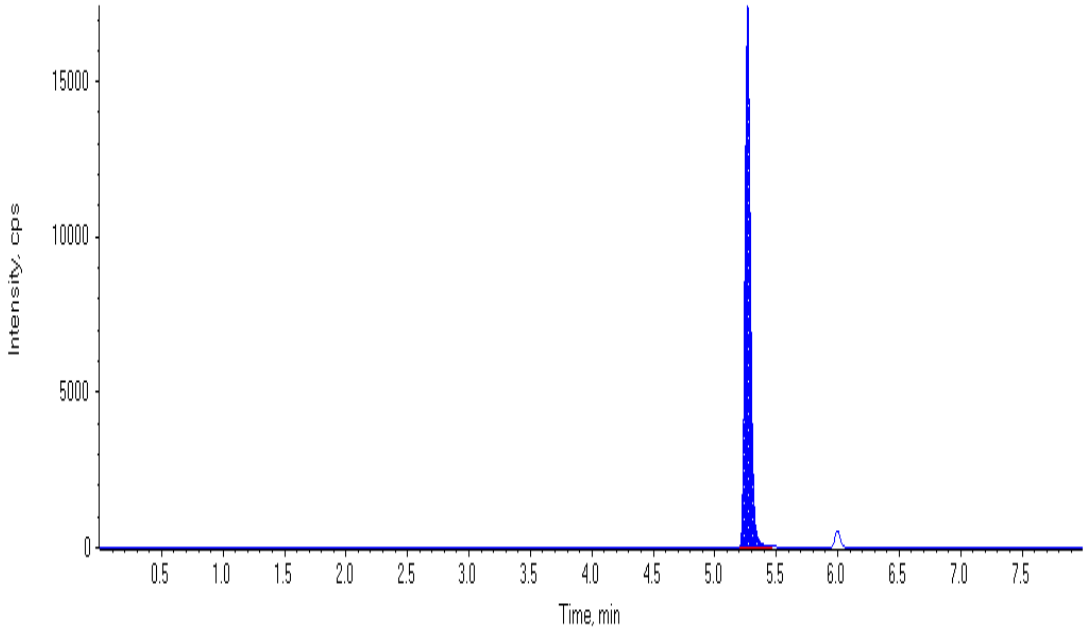


Figure 11. Chromatogram of DES at Lower Limit of Quantification (LLOQ= 0.78 ng/ml)

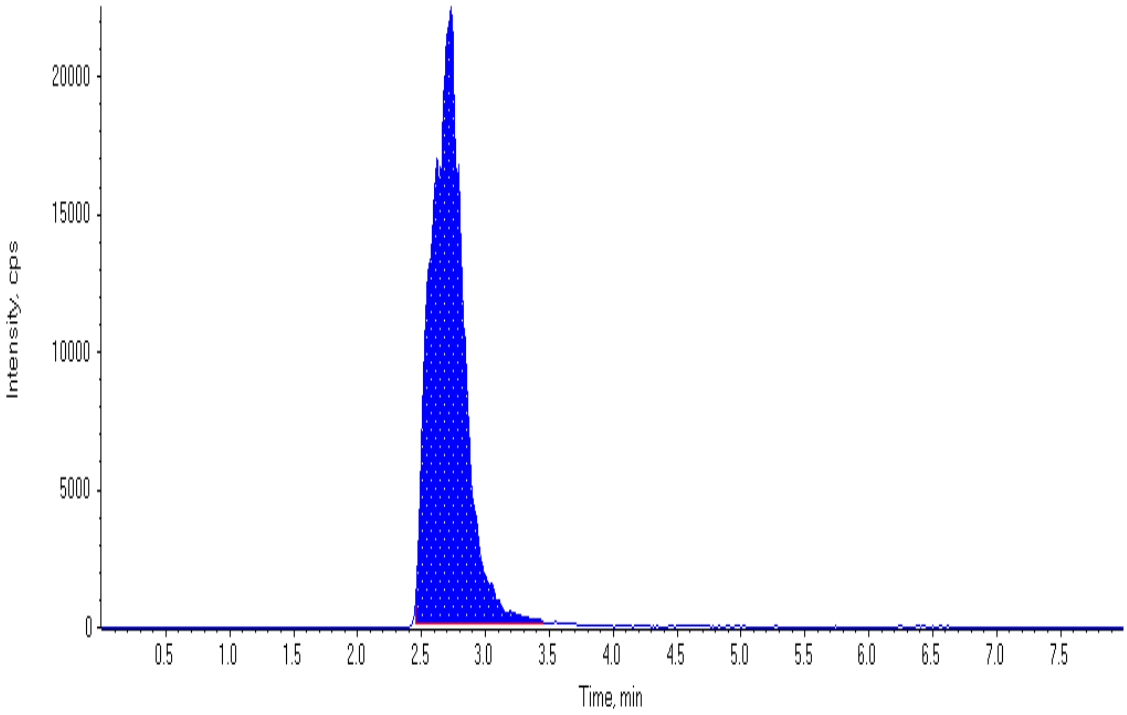


Figure 12. Chromatogram of Daidzein Internal Standard at 50 µM.

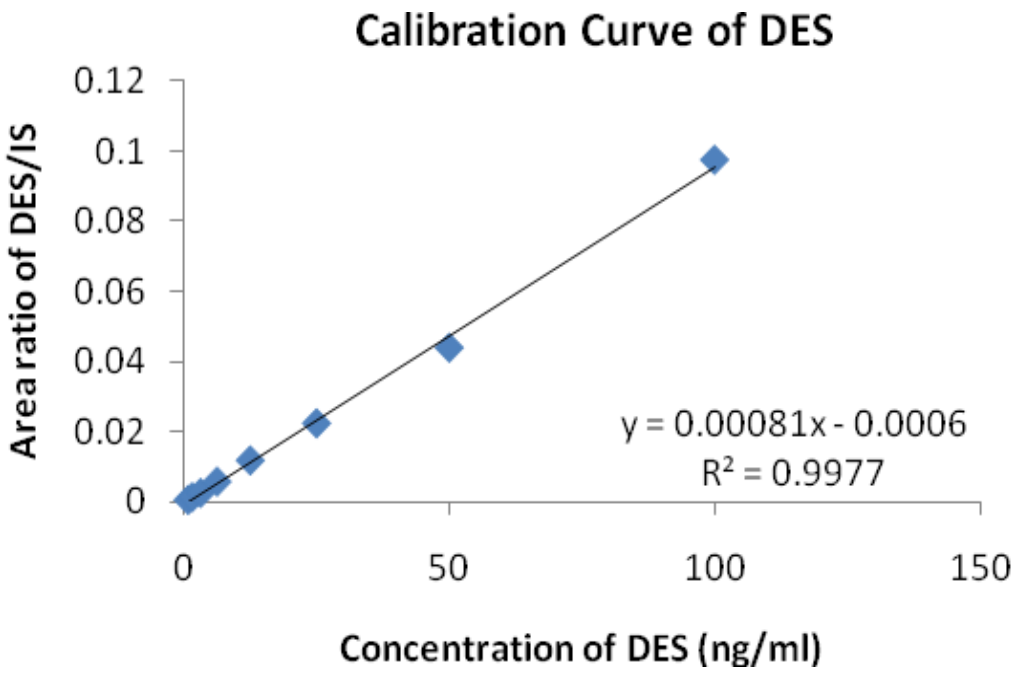


Figure 13. Representative LC/MS/MS Calibration Curve for DES in Plasma.

Linear range was 0.78 – 100 ng/ml of DES in plasma

LLOQ=0.78 ng/ml

at the high and low QC sample concentrations, respectively. The extracted DES human plasma samples ready for injection, but left on the autosampler for 12 h before injecting showed a degradation of 1.4 % at the high QC and 5.5 % at the low QC sample concentrations (Table 3). The recovery of DES and internal standard were 71 and 94 %, 76.72 and 92.66 %, and 68.78 and 86.35% for human plasma, rat plasma and rat liver tissue, respectively. Data showed ion suppression of <19 % at the high QC and <26 % at the low QC sample concentrations as the matrix effect for human plasma (Table 3).

This is the first LC/MS/MS assay method to our knowledge to quantify DES in plasma and a manuscript has been written and is in the process of being published.

Table 2. DES Assay Validation, Accuracy and Precision in Plasma.

Time	Plasma						Tissue			
	QC Sample (ng/ml)	Human Plasma			Rat Plasma			QC Sample (ng/g)	Rat Liver		
Intra-day					n=6						
		Mean (ng/ml)	RE (%)	RSD (%)	Mean (ng/ml)	RE (%)	RSD (%)		Mean (ng/g)	RE (%)	RSD (%)
Day1	**80**	72.5 ± 1.01	-9.44	1.39	79.4 ± 0.79	-0.73	1.00	**160**	153 ± 11.8	-4.41	7.71
2		82.9 ± 2.01	3.63	2.43	77.5 ± 4.16	-3.10	5.36		161 ± 10.4	0.37	6.44
3		90.0 ± 3.74	12.52	4.15	81.2 ± 3.97	1.56	4.88		157 ± 12.5	-2.19	7.96
Day1	**50**	47.9 ± 2.48	-4.13	5.18	51.1 ± 3.52	2.20	6.90	**100**	102 ± 2.37	1.95	2.32
2		52.5 ± 2.67	5	5.09	51.5 ± 4.02	2.90	7.82		91.2 ± 7.68	-8.80	8.42
3		51.8 ± 2.77	3.53	5.36	49.4 ± 1.43	-1.20	2.89		102 ± 10.4	1.60	10.2
Day1	**2**	1.69 ± 0.24	-15.7	14.2	1.95 ± 0.17	-2.67	8.77	**4**	3.95 ± 0.37	-1.25	9.40
2		1.94 ± 0.32	-3.17	16.45	1.99 ± 0.19	-0.42	9.55		4.29 ± 0.30	7.25	7.09
3		2.16 ± 0.15	7.83	6.94	1.75 ± 0.16	-12.8	9.07		3.56 ± 0.47	-11.00	13.26
Inter-day					n=18						
	80	81.8 ± 7.79	2.24	9.53	79.4 ± 1.43	-0.76	3.97	**160**	157 ± 11.0	-2.07	7.00
	50	50.7 ± 3.97	1.47	7.83	50.6 ± 3.14	1.30	6.20	**100**	98.3 ± 8.61	-1.75	8.76
	2	1.93 ± 0.30	-3.67	15.8	1.89 ± 0.20	-5.28	10.39	**4**	3.93 ± 0.47	-1.67	11.94

Assay was Validated Using Quality Control (QC) Samples at High (80 ng/ml for plasma, or 160 ng/g for tissue), Medium (50 ng/ml for plasma, or 100 ng/g for tissue) and Low (2 ng/ml for plasma, or 4 ng/g for tissue) (n=6 each).

The Accuracy and Precision were within the FDA Guidelines.

Data reported as mean ± SD

Table 3. Stability and Recovery of DES and Matrix Effect.

Stability*				
Experimental Condition	2 ng/ml		80 ng/ml	
	Observed Concentration	Degradation (%)	Observed Concentration	Degradation (%)
Pure analyte (12 h at RT)	2.02 ± 0.36	1.0	80.70 ± 7.03	0.8
Plasma samples (12 h at RT)	1.75 ± 0.14	12.5	78.90 ± 4.62	1.3
Plasma sample (72 at +4^{o} C)	1.59 ± 0.15	20.5	63.04 ± 3.13	21.3
Autosapler sample (12h at RT)	1.89 ± 0.28	5.5	78.90 ± 4.62	1.4

*Stability of DES studied in human plasma.

Table.3 Stability and Recovery of DES and Matrix Effect (Continued).

Recovery (n=4)						
	Human Plasma		Rat Plasma		Rat Liver	
	Mean slope (n=4)	Recovery (%)	Mean slope (n=4)	Recovery (%)	Mean slope (n=4)	Recovery (%)
A	0.00112 ± 0.0002	71[a]	0.0109 ± 0.00199	76.72[a]	0.0109 ± 0.00199	68.38[a]
B	0.00085 ± 0.0001	94[b]	0.0101 ± 0.00095	92.66[b]	0.00947 ± 0.00193	86.85[b]
C	0.00080 ± 0.00007		0.00836 ± 0.00423		0.00745 ± 0.00135	

Matrix effect[c] (n=4)		
	Mean area ratio	
	2 ng/ml	80 ng/ml
Pure analyte	2.45×10^{-2}	1.01
Analyte in extracted	1.83×10^{-2}	0.82
Matrix effect (%)	<26	<19

(a) Recovery of DES, calculated by: slope [C/A]

(b) Recovery of IS, calculated by: slope [C/B]

(c) Matrix effect in human plasma, calculated by: area ratio [Analyte in extracted blank / pure Analyte]

4.2. Phase II Clinical Trial of Oral DES in Recurrent Prostate Cancer Patients

The validated LC-MS/MS assay was used to quantify DES levels in the plasma of 19 patients on oral DES capsule for the treatment of recurrent prostate cancer. The patients in this clinical trial had failed first and in some cases second line therapies for prostate cancer. A total of 20 patients with mean age of 71.2 ± 9 (54 -86) years were enrolled in the study and received study medication (Table 4). One patient (# 17) withdrew from the study after just two days because of deterioration in his health condition. Each patient received 1 mg twice daily dose of DES capsule, except three patients (Patients # 3, 5 and 8) who received 1 mg daily dose. Two other patients had their dosing regimen altered during the course of the study; Patient # 2 started on 1 mg daily dose and his dose was increased later to 1 mg twice daily and patient #13 had his dosed decreased from 1 mg twice daily to 1 mg daily due to gynacomastia. Race breakdown of the patients is as follows: Caucasians, 15; Hispanics, 4 and Blacks, 1. Six of the study patients also received concomitant chemotherapy. Patients # 1 and 9 received doxetaxel; patients # 5, 8 and 14 received paclitaxel with # 14 receiving additional carboplatin and patient # 17 received cyclophosphamide and vincristine (patient not included in data analysis). Two patients were on herbal supplements. Patient #10 was on prostasol and # 11 was on prostasol and PC-SPES. The duration of treatment is 63 – 826 days (Table 4). Plasma samples were drawn from the patients 2 – 3 hours post dose for the quantification of DES and the thrombogenic marker, FVII levels. FVII levels were analyzed using human FVII ELISA Kits. Levels of DES obtained after analysis were correlated with the efficacy biomarker, prostate specific antigen (PSA) and FVII.

4.2.1. Patient DES Concentrations

DES levels were detected in all the patients in this study. DES levels ranged between 0.11 and 16.27 ng/ml with a mean value of 1.19 ng/ml. All DES levels in this patient population were below 4.30 ng/ ml, except one value for Patient # 11 that was 16.27 ng/ml, and 87 % of all the DES levels were below 2 ng/ml (Table 4 and Figure 14). Patient # 11 was a unique case, in that he had been on oral DES capsule for more than 9 years, and in addition he was on 2 herbal supplements with estrogenic activity (prostasol and PC SPES) for an unspecified number of years. Another Patient, #10 was taking prostasol after the collection of his plasma samples for DES quantification, and hence the reported DES levels (0.56 ng/ml and 0.86 ng/ml) did not reflect any effect from the prostasol. Two patients had their DES doses altered during the study, Patient #2 had his dose increased from 1 mg/ day to 1 mg twice a day and Patient # 13 had his dose reduced from 1 mg twice a day to 1 mg/day because of gynacomastia. We observed that their DES concentrations reflected these dose changes. The plasma DES levels increased from 0.24 to 0.48 and 0. 43 ng/ml for Patient #2, and decreased from 2.85 and 2.78 to 1.42 ng/ml for Patient #13 (Table 4 and Figure 14). Besides, patient # 16 at least once during the study ran out of study medication for one full week before his follow-up hospital visit. This discontinuation in his DES therapy was reflected when his DES levels were analyzed but below the limit of detection (Table 4 and Figure 14).

In this patient population, DES showed considerable variability within and between patients (Figure 14). If we consider Patients # 1 and 19, DES levels drawn 1 day apart for Patient # 1 were 1.38 ng/ml and 0.40 ng/ml, respectively, (a factor of about 3.5) and

Table 4. Patient Demographics, DES, FVII and Percent Change in PSA Levels Achieved in 19 PCa Patients

patient #	age	Race	days on DES Therapy	DES Regimen (1 mg)	DES (ng/ml)	FVII (ng/ml)	%Reduction in PSA (Days)	Concomitant Meds	Herbal supplement
1	67	W	63	BID	1.38±0.04	1617.81±34.44	49.0 (75)	docetaxel	
					0.40	1562.05±57.83			
2	77	W	210	QD	0.24±0.14	144.88±2.00	35.3 (49)		
				BID	0.48, 0.49	148.03±2.69			
					0.43	144.71±3.47			
3	83	W	186	QD	0.59±0.09	197.90±19.67	99.0 (186)		
					0.40±0.08	214.83±6.99			
4	64	W	299	BID	0.61	273.04±31.23	99.0 (104)		
					0.77	230.03±3.58			
					0.42	244.86±6.65			
5	69	W	146	QD	0.52	191.61±0.50	18.9 (32)	paclitaxel	
					0.97	207.16±6.83			
6	54	W	124	BID	0.43	307.08±1.79	16.9 (63)		
					0.91	274.79±17.10			
7	84	W	699	BID	1.63, 2.05	271.79±68.61	52.6 (31)	celebrex	
					1.39, 0.86	223.37±6.70			

Table 4. Patient Demographics, DES, FVII and Percent Change in PSA Levels Achieved in 19 PCa Patients (Continued)

patient #	age	Race	days on DES Therapy	DES Regimen (1 mg)	DES (ng/ml)	FVII (ng/ml)	%Reduction in PSA (Days)	Concomitant Meds	Herbal supplement
						212.29±12.57			
8	72	B	106	QD	1.17±0.26	155.31±6.76	58.8 (92)	paclitaxel	
9	68	W	126	BID	1.50	227.46±17.09	75.2 (125)	docetaxel	
					1.25	226.23±21.10			
10	70	W	186	BID	0.56	261.15±18.34	96.3 (186)		prostasol
					0.86	255.44±10.27			
						289.37±43.67			
11	79	W	520	BID	16.27±0.20	190.07±0.53			prostasol, PC-SPES
12	64	H	826	BID	0.65, 0.76	268.42±1.08	60.0 (181)		
					0.620, 0.63	262.64±14.69			
					0.517	247.91±25.14			
13	86	W	411	BID	2.78, 2.85	285.41±39.85	41.9 (236)		
				QD	1.42	344.66±4.94			
14	58	H	63	BID	1.58±0.08*	170.97±4.60		Paclitataxel, carboplatin	
15	69	H	365	BID	4.29±0.731*	320.39±30.55	98.4 (243)		
16	71	H	342	BID	BLT	234.83±27.32	35.7 (34)		

Table 4. Patient Demographics, DES, FVII and Percent Change in PSA Levels Achieved in 19 PCa Patients (Continued)

patient #	age	Race	days on DES Therapy	DES Regimen (1 mg)	DES (ng/ml)	FVII (ng/ml)	%Reduction in PSA (Days)	Concomitant Meds	Herbal supplement
					3.19±0.20*	183.74±2.02			
					BLT	244.84±21.47			
18	74	W	166	BID	0.35, 0.507	122.33±15.30			
					0.11	128.48±19.04			
19	65	W	112	BID	2.00±0.21*	168.54±5.78	50.0 (41)		
					3.28±0.10*	164.57±4.94			
20	78	W	334	BID	1.53±0.41*	159.72±16.44	63.3 (104)		

All patients were on 2 mg/day Regimen, except Patient #3, 5 and 8 who were on 1mg/day dose.

Patient # 2 had his DES dose increased from 1 mg/day to 1 mg twice daily on 10/25/07.

Patient # 13 had his dose reduced from 1 mg twice daily to 1 mg daily on 03/03/08.

PSA reduction (PSA response) is defined as reduction from baseline to PSA Nadir and Reduction ≥ 50 % is considered significant.

Normal FVII levels in human ≤ 500 ng/ml.

One Patient (#8) had unstable angina attributable to DES therapy.

BLT= below limit of detection

n=3 for all runs, * n=4

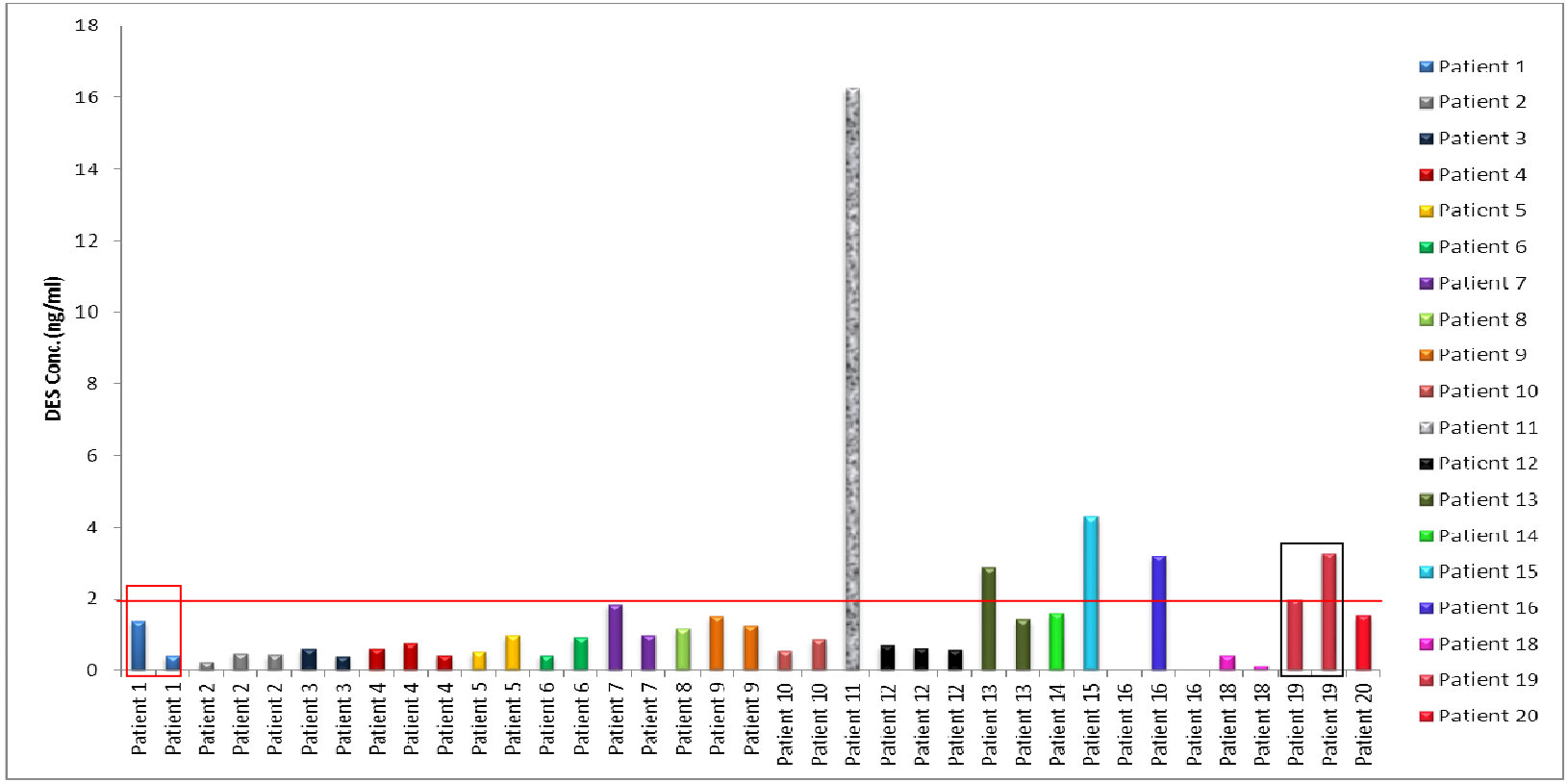


Figure 14. DES Concentrations Achieved in 19 Pca Patients on Oral DES Therapy.

All patients were on 1 mg twice daily regimen, except Patients #3, 5 and 8 who were on 1 mg/day dose.

Patient # 2 had his DES dose increased from 1 mg/day to 1 mg twice daily during the course of the study.

Patient # 13 had his dose reduced from 1 mg twice daily to 1 mg/day.

for Patient #19, levels drawn 42 days apart were 2.0 ng/ml and 3.28 ng/ml, respectively (factor of 1.6). Comparing DES levels between the two patients (Patients # 1 and 19) also shows considerable variability between the patients (0.4 ng/ml vs 3.28 ng/ml). Most of the patients had DES levels below 2 ng/ml (Figure 14).

4.2.2. Response to DES Therapy by PSA Levels

All patients in this study for which baseline PSA levels were available (16 patients) had a response to oral DES therapy (response defined as difference in PSA level from baseline to PSA nadir). The response ranged from 17 % to 99 % and the average DES concentration for this response range is 1.15 ng/ml. Eighty eight percent of the patients had PSA reductions better than 35 %, 63 % of patients had a reduction ≥ 50 %, considered significant reduction (The Prostate Specific Antigen Working Group, 1999) and 31 % had reductions ≥ 75 % (Table 4 and Figure 15). The average DES concentrations were 1.24 ng/ml, 1.23 ng/ml and 1.13 ng/ml for PSA response of ≥ 35 %, ≥ 50 % and ≥75 %, respectively. At DES concentration ≤ 2 ng/ml, 56 % of patients had a significant PSA response ≥ 50 % (Table 4). The median time to PSA response in this population was 98 days for all the patients and 114.5 days for those with significant PSA reduction, with a range of 31 – 243 days.

There is a correlation between PSA response and days on DES Therapy with R^2 of 0.23 (Figure 16a). The correlation improved dramatically when Patient # 13 was omitted, with R^2 of 0.63 (Figure 16b). This Observed correlation supports the need for safe DES dosing regimens and/or formulations that will increase therapy adherence by reducing DES toxicities and thus improve efficacy.

The PSA profiles for four patients (Patients # 11, 7, 2 and 13) on DES therapy were plotted. Patient # 11 is complicated because of the additional herbal supplements with estrogenic activity on his regimen. He started on DES therapy in March of 2002, but his DES therapy at 1 mg twice daily was monitored for inclusion into the study starting in November of 2006. His PSA levels were held below 1.2 ng/ml until April of 2008 when he discontinued DES therapy (Figure 17). The percent decline in PSA for this patient could not be evaluated because his baseline PSA level was not available. His DES levels were monitored once during the study and the levels were 16.27 ng/ml (Table 4). Patients #11 also reported times that he voluntarily stopped his DES therapy because of gynacomastia and only resumed when his PSA level was increasing. This interruption of therapy and the phytosterol that he was taking make the analysis of his response to therapy complicated. As discussed earlier he was on prostatsol and PC-SPES, both of which have estrogenic activity and this combination of herbs and DES may explain his DES plasma concentration that was substantially higher than the average value. He discontinued DES therapy in April of 2008.

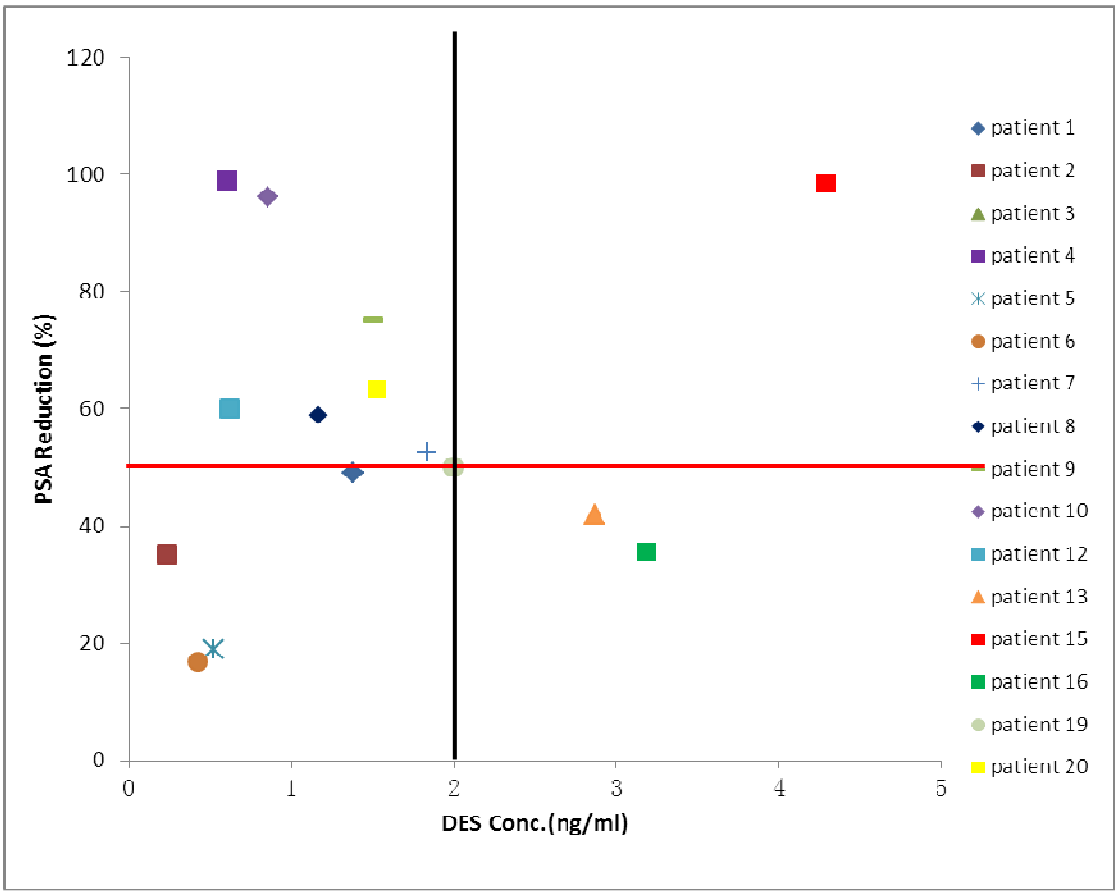


Figure 15. Correlation of Percent Reductions in PSA with DES Plasma Concentrations in 16 Patients on DES Therapy.

All patients were on 1 mg twice daily regimen, except Patients #3, 5 and 8 who were on 1mg daily dose.

Patient # 2 had his DES dose increased from 1 mg/day to 1 mg twice daily during the course of the clinical trial.

Patient # 13 had his dose reduced from 1 mg twice daily to 1 mg/day.

Red line and above, 10 patients who had significant PSA Response (10 patients or 63 %).

Intersection of red and black line, upper left quadrant, number of patients with DES levels ≤ 2 ng/ml who had significant response (9 patients or 56 %).

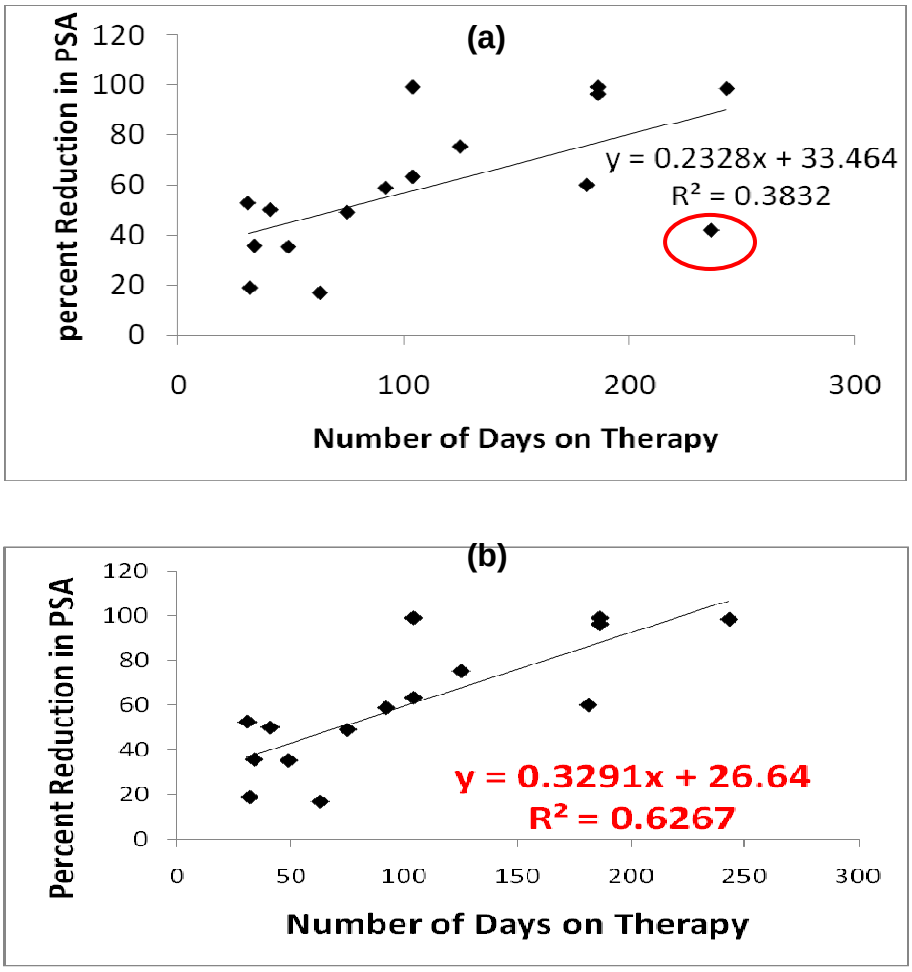


Figure 16. Correlation of Percent Reductions in PSA (PSA Response) and Days on DES Therapy in Patients on Oral DES Therapy for Recurrent Prostate Cancer. (a) Includes outlier Patient # 13, and (b) Without outlier Patient # 13

There is a correlation between PSA Response and Days on DES Therapy. This Observation supports the need for safe DES dosing regimens and/or formulations that will increase DES therapy adherence by reducing DES toxicities and improve efficacy.

Patient # 7 started DES therapy in July of 2006 at 1 mg twice daily regimen and enrolled in the clinical trial in November of 2007. His PSA level at study entry was 15.2 ng/ml and within 31 days of oral DES ttherapy, his PSA dropped to 7.2 ng/ml, a 53 % reduction. His DES levels were quantified twice, 1.84 and 0. 99 ng/ml and these levels suppressed his PSA at or below 7.2 ng/ml until June of 2008 when his PSA started rising. (Table 4 and Figure 18).

Patient #2 started DES therapy at 1 mg/day dose in August of 2007 and his PSA level at clinical trial entry was 33.7 ng/ml. His DES level 61 days after commencing therapy was 0.24 ng/ml, and he had a maximum PSA reduction of 35.3 %, 49 days after starting the therapy (Figure 19). Because patient tolerated therapy with the 1 mg/day dose, his dose was increased to 1 mg twice daily and his DES plasma level almost doubled to 0.48 and 0.43 ng/ml with the doubling of the dose (Table 4). The PSA level of this patient was suppressed below 33.7 ng/ml while on DES therapy, until March of 2008 when he discontinued DES therapy (Figure 19).

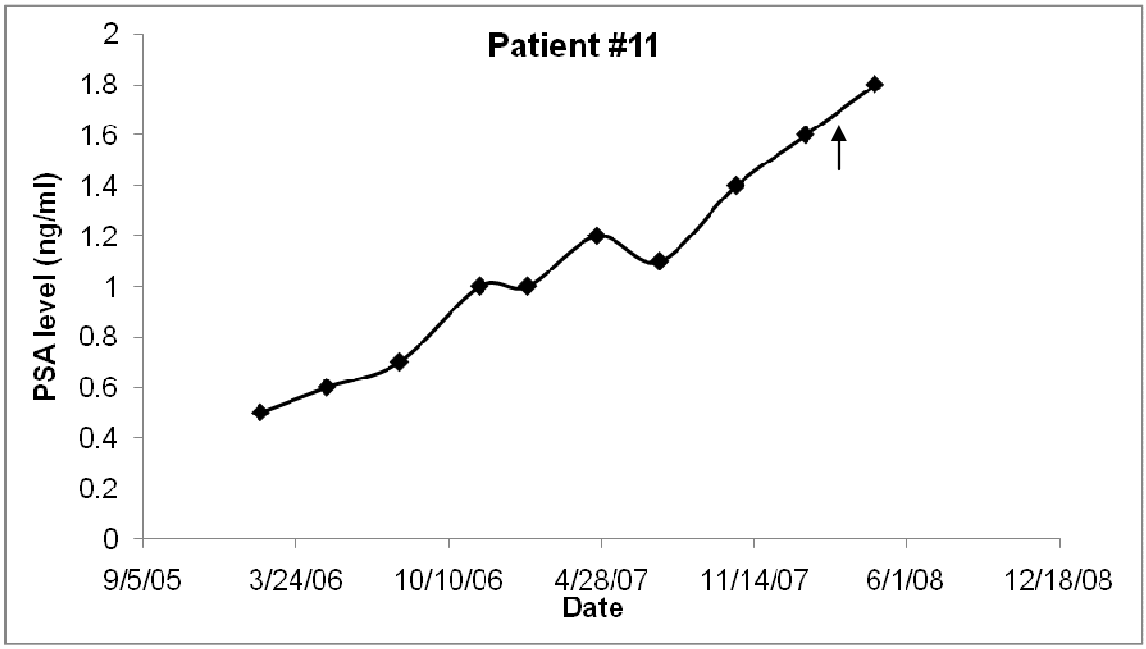


Figure 17. PSA Profile of Patient # 11.

Patient #11 had been on- and- off Oral DES therapy since March of 2002 and discontinued DES therapy on 04/23/08 (↑). Monitoring of DES therapy started 11/20/06.

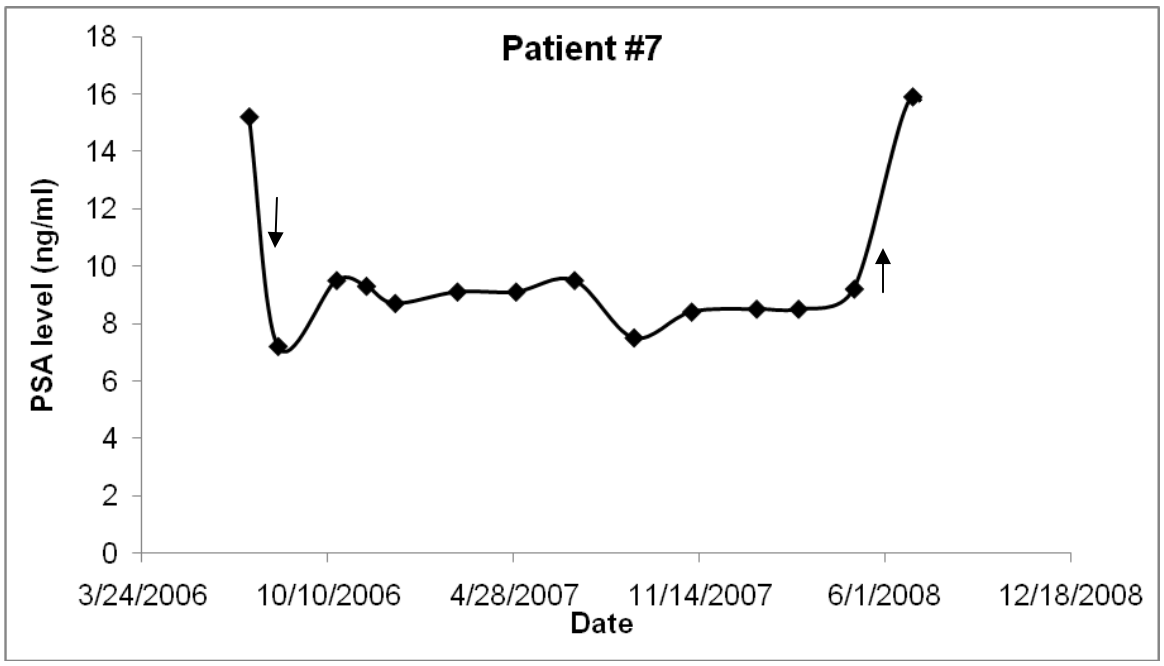


Figure 18. PSA Profile of Patient # 7.

Patient started DES therapy on 07/17/06 (↓) and discontinued DES therapy on 06/15/08 (↑)

Patient #13 is another patient who had his DES therapy modified during the course of this study. He started DES therapy at a regimen of 1 mg twice daily in July of 2007 and enrolled in the study in March of 2008. His PSA at study entry was 169.9 ng/ml. The DES level drawn after 236 days of DES therapy was 2.78 ng/ml, and this DES level suppressed his PSA level to below 150 ng/ml. Patient's DES therapy was decreased to 1 mg daily, after March 2008, yielding DES concentration of 1.42 ng/ml. Around March 2008 when his DES regimen was decreased, an upward trend in the levels of PSA was observed (20). Patient #13 achieved a maximum PSA reduction of 42 % during the course of his DES therapy.

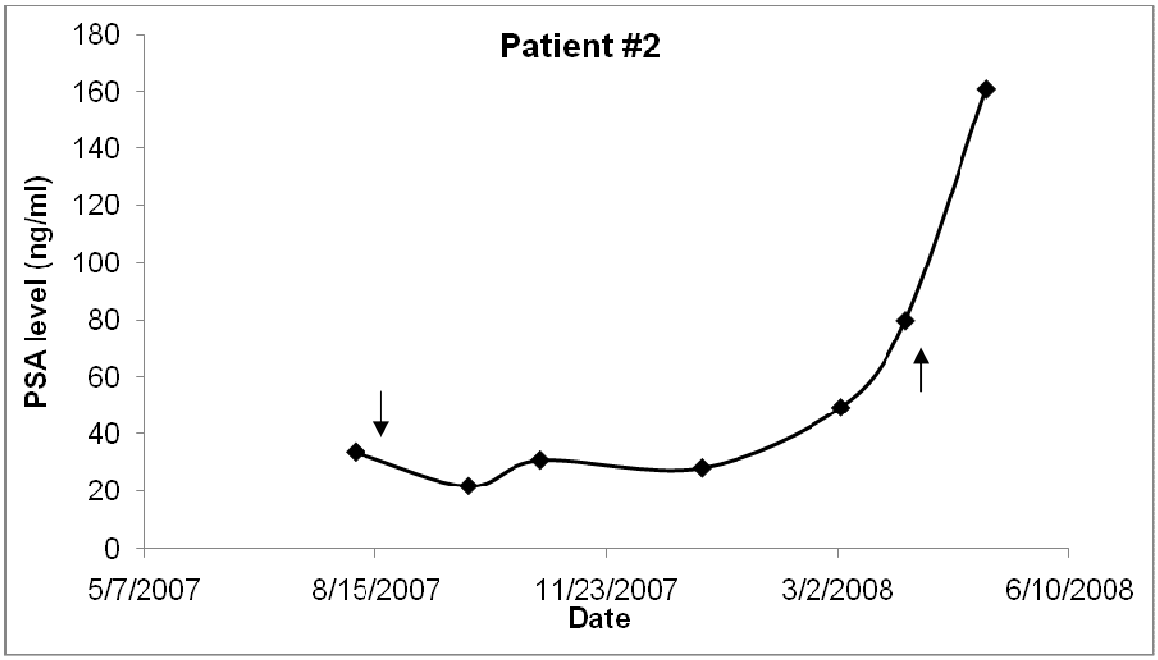


Figure 19. PSA Profile of Patient # 2.

Patient started DES therapy on 08/06/07 (↓) and discontinued DES therapy on 03/03/08 (↑).

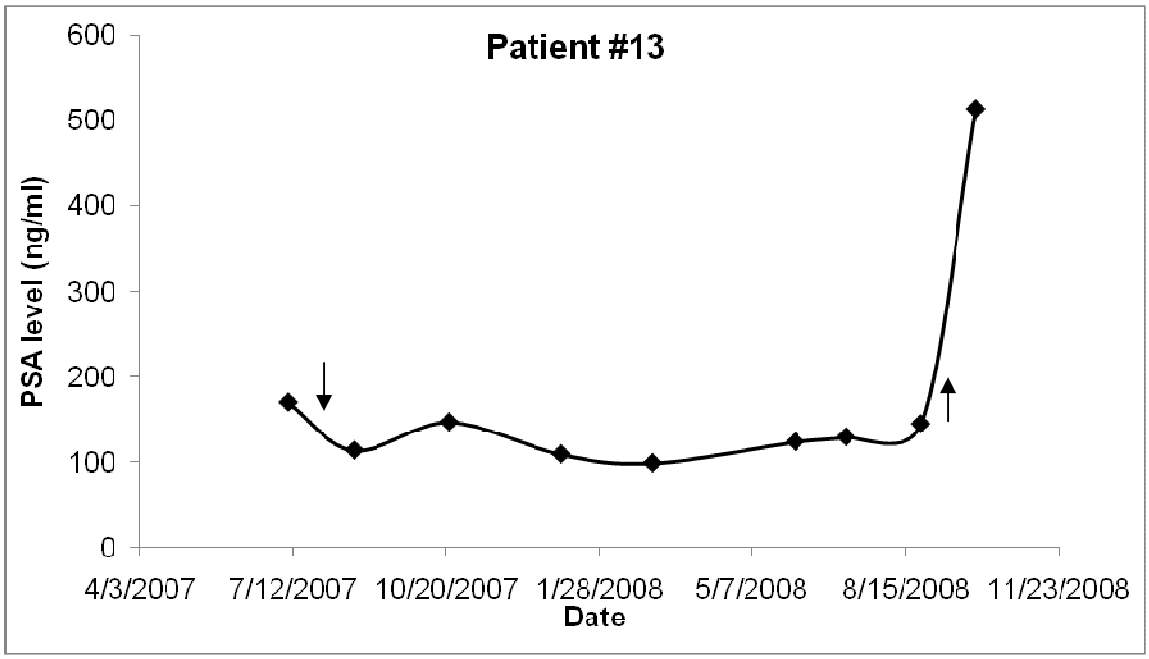


Figure 20. PSA Profile of Patient # 13.

Patient started DES therapy on 07/11/07 (↓) and discontinued DES therapy on 08/25/08 (↑).

4.2.3. Correlation of DES Concentrations to FVII Levels and Toxicity

Factor VII is a critical clotting protein and the first clotting factor in the extrinsic pathway of the coagulation cascade. When it combines with tissue factor, it becomes activated and plays a role in activating factors X and IX (Figure 1). Factor VII levels were determined for 19 patients in this clinical trial. The FVII levels in this study population ranged between 133 – 348 ng/ml for all the patients, except for Patient #1 whose levels were 1562 – 1617 ng/ml (Table 4). The values less than 500 ng/ml are comparable to those from normal subjects. None of the patients was diagnosed with a heart attack, stroke or pulmonary embolism, except Patient # 8 who was diagnosed with unstable angina, which was attributable to his DES therapy.

4.2.4. Population Pharmacokinetic Analysis of Clinical Data

Because each patient had sparse samples collected several days and in some cases months apart, we needed special software to analyze the population pharmacokinetics parameters of oral DES. We employed the NONMEM software which has the ability to integrate sparse clinical data to derive population PK parameters. The population PK parameters of elimination rate constant (k), volume of distribution (V), and absorption rate constant (k_a) were derived by fitting the one-compartment model into the clinical data. The population k, V, and k_a of DES from this clinical study population as determined using NONMEM statistical software are 1.49 h^{-1}, 795 L, and 9.97 h^{-1}, respectively. The population clearance (CL) and $t_{1/2}$ were calculated using the PK

equations; CL = KV and t ½ = 0.693/k, giving 1,184.55 L/h and 0.47 h, respectively. The diagnostic plots for data are shown on Figure 21.

4.3. In Vitro Permeation of DES from Propylene Glycol Solution

The transdermal permeation of DES from a solution (2 mg/ml) in propylene glycol through excised SCID nude mouse skin was evaluated (n=3). The permeant solution of DES in propylene glycol was applied to the donor compartment of the Franz® diffusion cell on top of the stratum corneum of the mouse skin. Samples were collected from the sampling port of the Franz® diffusion cell for analysis with fresh medium replaced immediately. The samples were analyzed using the validated LC/MS/MS assay described in Section 4.1. Before we started the study, we evaluated whether DES is a candidate for transdermal delivery by comparing the physicochemical properties of DES

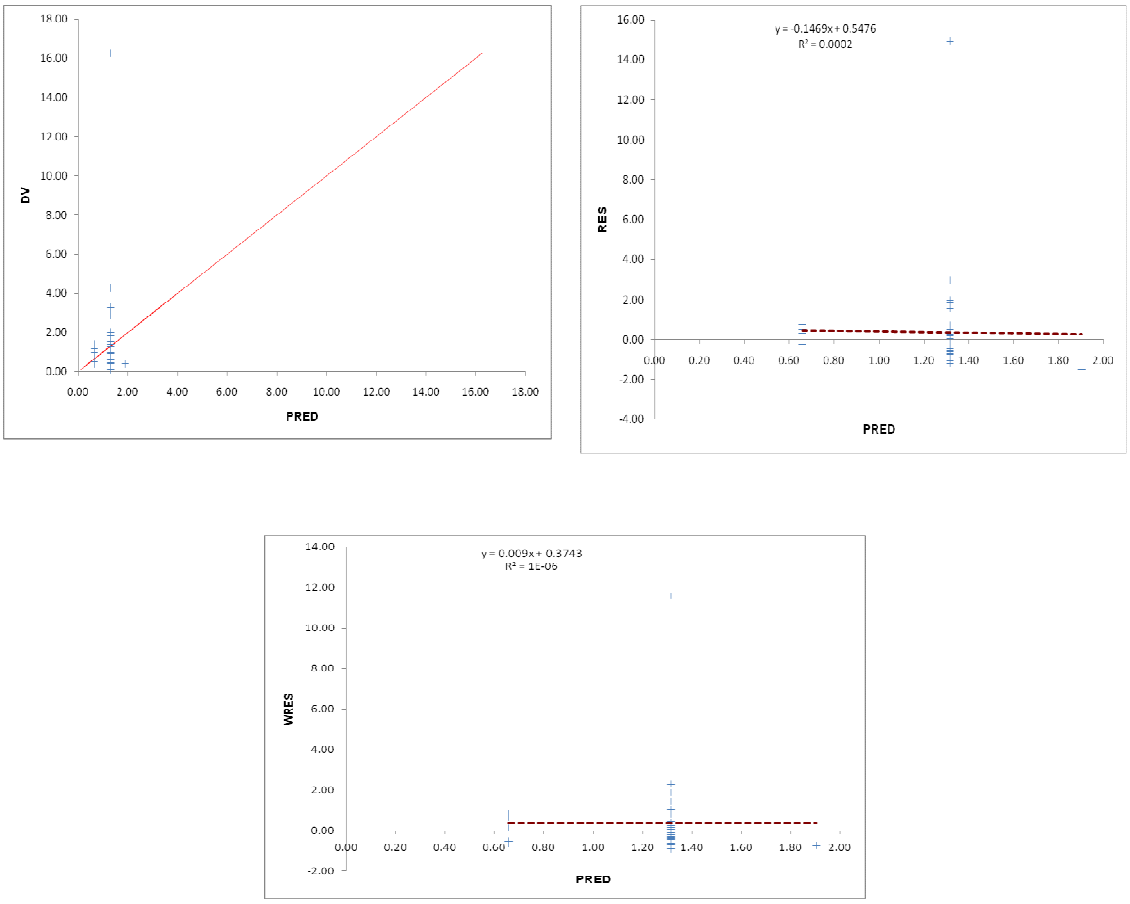


Figure 21. NONMEM Diagnostic Plots

DV=Observed concentration

Pred=Predicted concentration

RES=Residual

WRES=Weighted Residual

with the criteria for transdermal therapeutic systems (TTS) (Table 5). Most of the known physicochemical properties of DES satisfied the criteria for TTS, except the partition coefficient. The partition coefficient of DES is 5.6, while that for TTS systems is in the range of 1 to 3. A high partition coefficient will cause the drug candidate to be retained more in the stratum corneum layer of the skin. Because most of the physicochemical properties of DES satisfied the TTS criteria, we proceeded with the in vitro permeation evaluation study.

4.3.1. Permeation Profile of DES Solution through Excised Mouse Skin

The data obtained by analyzing the samples from the transdermal permeation study were used to generate a permeation profile of DES from a solution in propylene glycol (Figure 22). The slope of the linear portion of the curve was determined using linear regression analysis. The lag time of DES from this formulation through excised mouse skin was derived, by extrapolating the linear portion of the curve to cross the time axis (x-axis). The point at which it crossed the x-axis is the lag time. The flux, J, of DES from a solution in propylene glycol was represented by the slope. The permeability coefficient, Kp of DES in propylene glycol was calculated by dividing the slope by the initial concentration of 2 mg/ml. The diffusion coefficient (D/h^2) and partition coefficient (Km.h) were calculated as explained in Section 3.2.4.4. The flux and permeability constant of DES from the solution were 3.6 ng/hr/cm^2 and 1.8 x 10^{-6} cm/hr, respectively. The lag

time (T_{lag}), diffusion coefficient (D/h^2), and partition coefficient (Km.h), both normalized by skin thickness were 6.9 hr, 2.4 x 10^{-2} h^{-1}, and 7.5 x 10^{-5} cm, respectively (Table 6).

Table 5. Comparison of Physicochemical Properties of DES and Criteria for Transdermal Therapeutic Systems

Property	Criteria for TTS*	DES property
Molecular Weight (Da)	< 1000	268
Melting point (^{o}C)	< 300	169℃ to 172℃
Partition coefficient (octanol/water)	1-3	5.6
Daily i.v dose	0.01-2	Not defined
Required plasma concentration (ng/ml)	< 100	1-4
Plasma half-life (hr)	short	1
Oral bioavailability	low	Not defined

*Shaw, 1981; Flyn and Stewart, 1988; Guy and Hadgraft, 1989

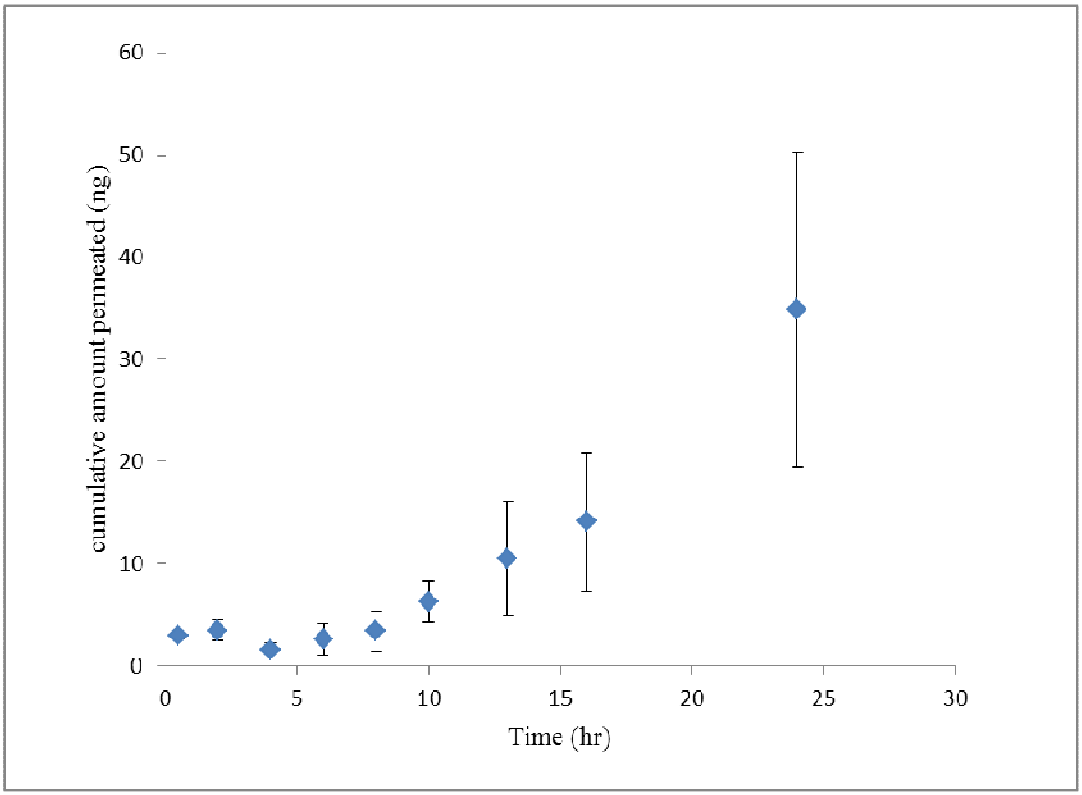


Figure 22. Transdermal Permeation Profile of DES Solution through Excised SCID Mouse Skin (n=3).

Data reported as Mean ± SD.

The formulation was applied on top of the mouse skin in the donor compartment of the Franz® diffusion cell.

Sample aliquots were collected up to 24 hours and equal volume of fresh media immediate replenished.

The cumulative amounts of DES permeated were plotted against time.

Table 6. Permeation Parameters of DES in Propylene Glycol (PG) through Excised SCID Nude Mice Skin (n=3).

Permeation Parameters	Study Values
Flux, J (ng /hr/cm^2)	3.6
Permeability Coefficient, K_p (cm/hr)	1.8 x 10^{-6}
Lag Time, T_{lag} (hr)	6.9
Diffusion Coefficient, D/h^2, (hr^{-1})	2.4 x 10^{-2}
Partition Coefficient, K_m.h, (cm)	7.5 x 10^{-5}

$$Kp = J/Cs \qquad \text{Eq. 1}$$

$$D/h2 = 1/6Tlag \qquad \text{Eq. 2}$$

$$Km.h = Kp / (D/h2) \qquad \text{Eq. 3}$$

4.4. DES Content Uniformity in ODF-DES

Before starting the PK studies we evaluated the uniformity and DES content of the ODF-DES film to ascertain the manufacturer's claims. The reported target weight and size of each film according to the manufacture is approximately 50 mg and 2 x 2 cm, respectively. The target DES content is 1 mg with uniform distribution of DES per film. The observed average weight of the film from 2 batches was 53.90 ± 1.12 mg. Each film was divided into four portions and each portion analyzed separately for DES content. The film portions were compared on mg/mg bases to determine the uniformity of DES distribution in each portion. The average DES content of the ODF films from 2 batches (n=4) was 0.96 ± 0.07 mg (range of 0.922 to 1.062 mg). The average DES content in each film portion was 0.240 ± 0.023 mg (n=16, range of 0.202 to 0.294 mg) and 0.018 ± 0.00 mg/mg (n=16, range of 0.016 to 0.022 mg/mg) when normalized by weight (Table 7). The manufacturer's claim of film weight (50 mg) and DES content (1 mg) are both within 8 % of our observed values and considered acceptable. The variability of DES content within each film portion on mg/mg bases was also less than 8 %, and the uniformity is considered acceptable.

Table 7. DES Content Uniformity in ODF-DES

Film Code	Wt(mg)		DES Content	DES Content	
	Full Film	1/4 Film	(µg)	1/4 Film*, (mg/mg)	Full Film** (mg)
1	55.4				**1.062**
1A		13.4	294	0.022	
1B		14.8	275	0.019	
1C		14.2	266	0.019	
1D		12.9	227	0.018	
Mean		**13.83**	**265.5**	**0.019**	
S.D. (% CV)		0.84 (6.09)	28.2 (10.62)	0.002 (9.82)	
2	53.1				**0.947**
2A		12.5	228	0.018	
2B		13.8	243	0.018	
2C		13.9	257	0.018	
2D		13	219	0.017	
Mean		**13.30**	**236.8**	**0.018**	
S.D. (% CV)		0.67 (5.03)	16.7 (7.07)	0.001 (4.12)	
3	54.1				**0.912**
3A		13.6	231	0.017	
3B		14	243	0.017	
3C		14.5	236	0.016	
3D		12.4	202	0.016	
Mean		**13.63**	**228.0**	**0.017**	
S.D. (% CV)		0.90 (6.57)	18.0 (7.90)	0.001 (3.20)	

Table 7. DES Content Uniformity in ODF-DES (Continued)

Film Code	Wt(mg)		DES Content	DES Content	
	Full Film	1/4 Film	(µg)	1/4 Film*, (mg/mg)	Full Film** (mg)
4 [a]	53				**0.922**
4A		12.4	221	0.018	
4B		13.1	232	0.018	
4C		13	223	0.017	
4D		14.5	246	0.017	
Mean		**13.25**	**230.5**	**0.017**	
S.D. (% CV)		0.89 (6.71)	11.4 (4.94)	0.000 (2.40)	
Overall					
Mean	**53.90**	**13.50**	**240.2**	**0.018**	**0.96**
S.D (% CV)	**1.12 (2.07)**	**0.78 (5.78)**	**23.3 (9.72)**	**0.00 (7.52)**	**0.07 (7.19)**

a, Batch 2, used for the multiple dosing experiments.

A, B, C, and D are different film portions.

*DES content in ¼ film (mg/mg) = DES content (µg)/[(weight of ¼ film in mg)*1000]

**DES content in full film (mg) = ∑[(DES content in ¼ film) X weight of ¼ film]

4.5. Preclinical Pharmacokinetic Studies

In order to evaluate the comparative pharmacokinetics of ODF-DES and oral DES, we studied the PK parameters of DES from ODF-DES film and an oral suspension of DES in Sprague-Dawley rats. We employed a parallel study model with two groups of rats. One group of rats was applied half of an ODF-DES film topically to the inside of each cheek (total of 1 mg of DES per rat) and another group was administered a 1 ml suspension of 1 mg/ml DES by oral gavage as described in Sections 3.2.6.4. Plasma samples were collected through the tail vein at predetermined time points up to 30 hours post dose for the single dose study administration. For the multiple dose studies, plasma samples were collected from the 5th day up to the 8th day of the study. The plasma samples were analyzed using the validated LC/MS/MS assay method.

4.5.1. Comparative Pharmacokinetics of ODF-DES and Oral Suspension of DES

The data from the ODF and oral formulations of DES were modeled using PK models. The data were first analyzed using the noncompartmental PK model (Figure 23). We also fitted the data to one and two oral compartment models using the WinNoLin version 3.3. The best fit of the models was selected based on the comparison of the correlation coefficients (R^2) and on visual inspection of the agreement between the observed and predicted profiles. Using these criteria, the two compartment oral model had a better fit with R^2 of 0.91 and 0.89 for the ODF and oral formulations versus 0.88 and 0.87 with the one compartment model, respectively. The plasma level- time profiles for one and two

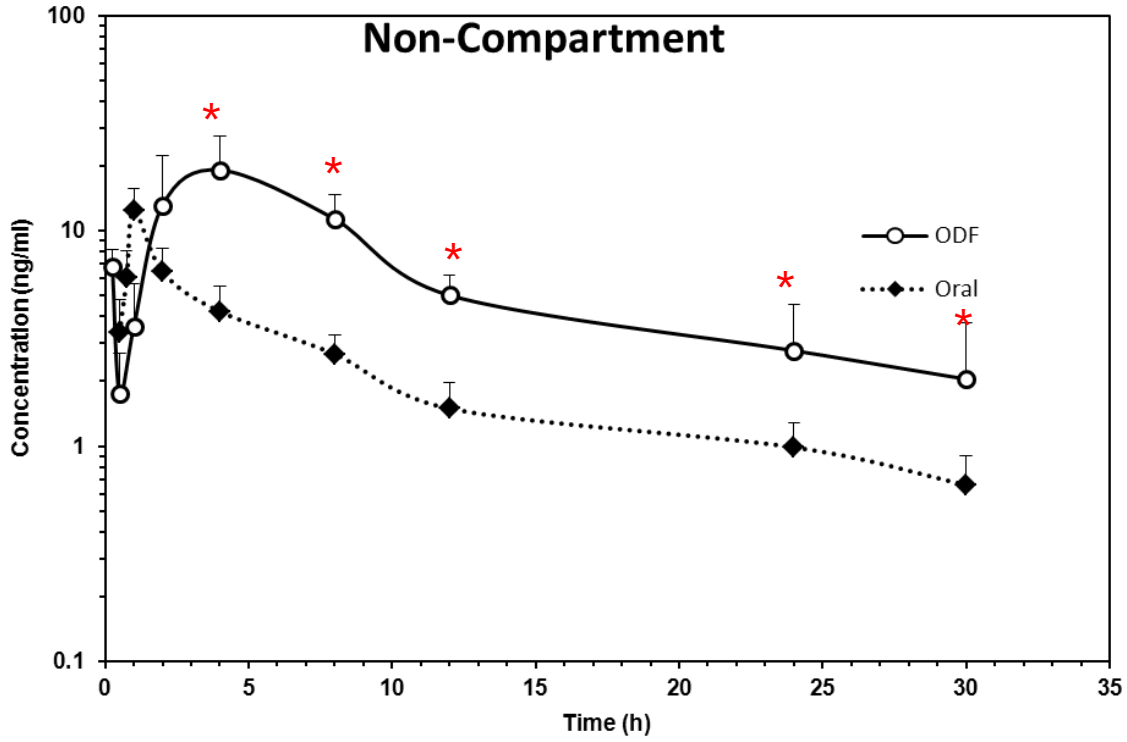


Figure 23. Plasma Level – Time Profiles for the Non-compartment Model Analysis after Single Dose (1 mg) of ODF-DES and Oral Suspension (n=4 each)

Data reported as mean ± SD

*Significant difference (Student's t-test, $p<0.05$)

compartment models are shown in Figure 24 and Figure 25. The parameters derived from the two compartment model are summarized in Table 8. The comparative PK analysis of the ODF-DES and oral suspension formulation was thus performed using the better fitted model, which is the two compartment model.

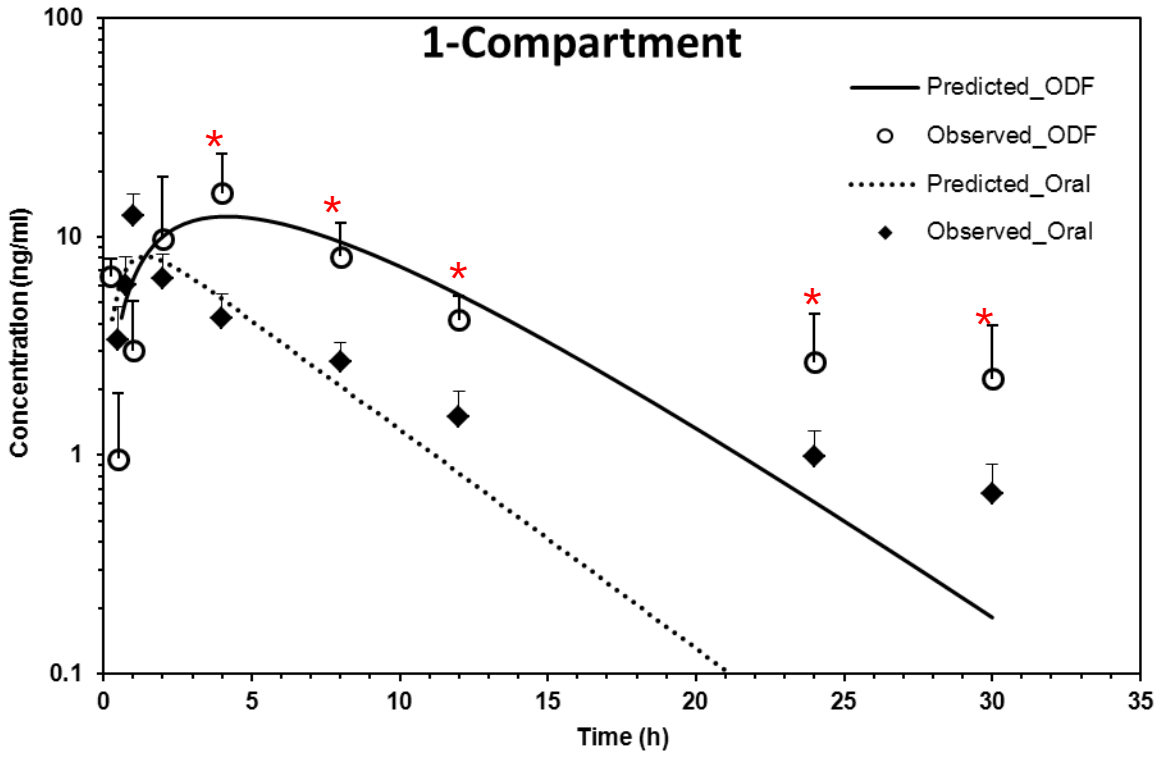


Figure 24. Plasma Level – Time Profiles for the One-compartment Model Analysis after Single Dose (1 mg) of ODF-DES and Oral Suspension (n=4 each)

Data reported as mean ± SD

*Significant difference (Student's t-test, $p<0.05$)

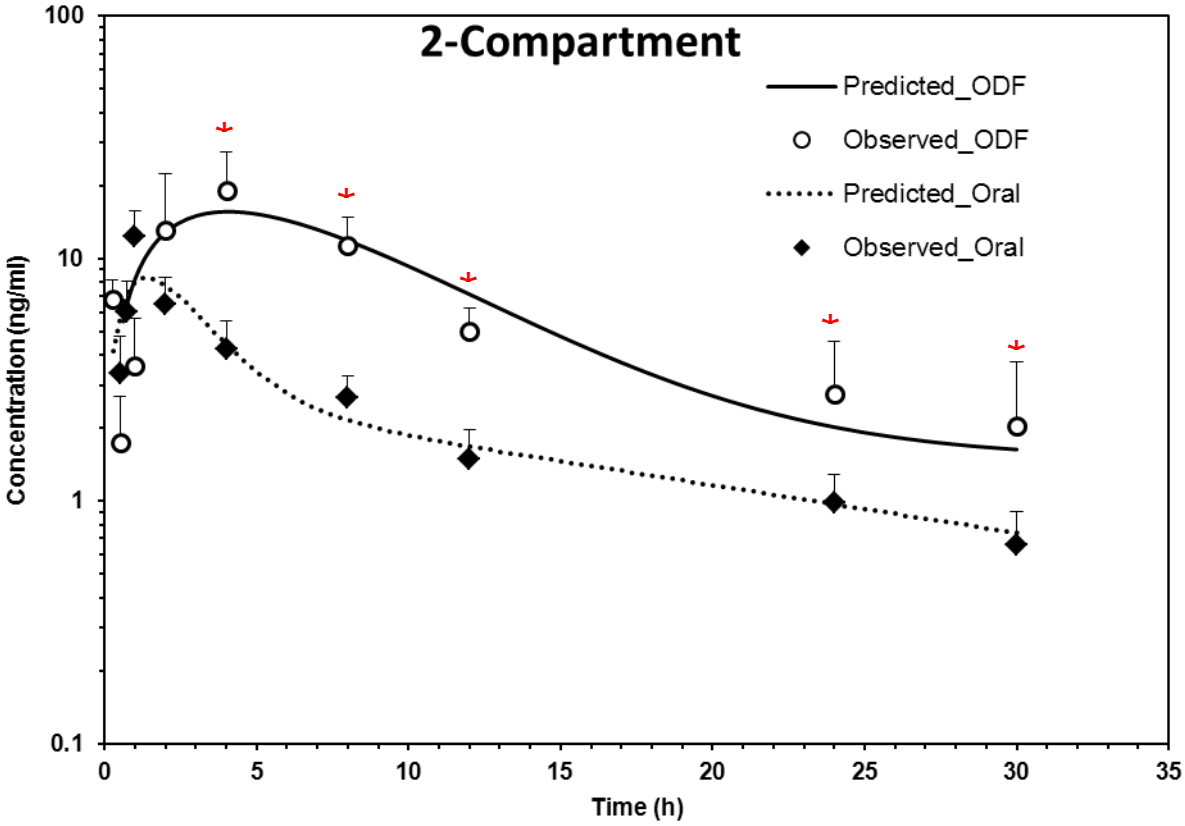


Figure 25. Plasma Level – Time Profiles for the Two-compartment Model Analysis after Single Dose (1 mg) of ODF-DES and Oral Suspension (n=4 each).

Data reported as mean ± SD

*Significant difference (Student's t-test, $p<0.05$)

Table 8. PK Parameters from ODF and Oral Suspension at Dose of 1 mg/day Using WinNonLin 2-Compartmental Model.

Parameters	Units	*ODF-DES*	*Oral Solution*
		2-compt	
Tmax	h	3.79±0.90*	1.36±0.18
Cmax	ng/ml	15.25±5.35	8.41±2.38
AUC $_{0\text{-}30h}$	ng /ml*h	179.07±69.50*	65.40±18.25
AUC 0-inf	ng /ml*h	197.16±58.94*	82.90±23.87
Ka	1/h	0.33±0.12*	0.88±0.15
$T_{1/2}$ β	h	12.66±7.82	16.11±3.50
V/F	L	0.033±0.02	0.052±0.016
CL/F	L/h	0.007±0.003*	0.013±0.003
V2/F	L	0.030±0.036*	0.170±0.062
CL2/F	L/h	0.002±0.003*	0.026±0.007

*****signifies a significant difference between the ODF and oral suspension groups at $p<0.05$ using student's t-test.

n=4 for each group.

There is a significant difference in peak time, the time to reach peak plasma concentration (T_{max}) between the two formulations. The T_{max} of 3.79 h from ODF-DES is approximately three times that of the oral suspension formulation (1.36 h). There are no statistically significant differences in the peak concentration (C_{max}), 15.25 ng/ml versus 8.41ng/ml and the terminal elimination half- life ($T_{1/2}\beta$), 12.66 h versus 16.11 h between the ODF and oral suspension of DES. The absorption rate constant of DES from ODF (0.33 h^{-1}) was significantly slower (~2.7 times less) than that from the oral suspension (0.88 h^{-1}).

The area under the curve (AUC) of DES from ODF is significantly greater than that from the oral suspension, with a more than 2.7 fold increase (179.07 ng/ml*h for ODF versus 65.40 ng/ml*h for oral suspension). Significant differences in the volume of distribution in the peripheral compartment (V2/F) and clearance (CL/F and CL2/F) were also observed between ODF and oral suspension. The V2/F, CL/F and CL2/F were 0.030 L, 0.007 and 0.002 L/h, respectively for ODF versus 0.170 L, 0.013 and 0.026 L/h for oral suspension (Table 8). The greater exposure (AUC) of DES from ODF may be explained, at least in part, by the significant reduction in the clearance of DES from the ODF formulation.

The bioavailability of ODF-DES relative to the oral suspension was calculated using the mean AUC with the equation of F = AUC (ODF) / AUC (Suspension). The bioavailability of ODF-DES relative to oral formulation suspension is 2.74.

4.5.2. DES Concentration in the Liver after Multiple Doses of ODF and Oral DES Suspension

The concentrations of DES in the liver of study rats (n=4 for each group) were analyzed. The animals were administered 1 mg/day dose of either ODF or oral suspension of DES for 7 days. On the 8th day, the animals were sacrificed to harvest the liver tissue for DES quantification. Before harvesting the liver tissue, the liver was perfused through the aorta with normal saline to remove any blood present in the organ.

DES showed a slightly higher accumulation in the liver of the rats administered the oral suspension 11.10 ± 7.50 ng/g than in the liver of those in the ODF-DES group 9.66 ± 4.63 ng/g, but the finding was not statistically significant (Table 9). This observation led us to the conclusion that ODF-DES significantly increases plasma exposure of DES without increasing the liver accumulation of DES, compared to the oral suspension formulation. This finding is significant owing to the fact that blood clotting proteins are synthesized in the liver and their synthesis is affected by estrogens like DES.

4.6. PK/PD Studies of DES from ODF and Oral Suspension

4.6.1. Relationship of Fibrinogen and AT-III Concentrations and Changes after Single Dose of ODF and Oral DES Suspension

DES plasma concentrations from ODF-DES were significantly greater than those from the oral suspension and it was necessary to evaluate if the higher concentrations and AUC of DES from ODF had effects on fibrinogen and AT-III levels. Higher levels of fibrinogen may indicate a tilt in the coagulation cascade towards more coagulation, while higher levels of AT-III may indicate an increase in fibrinolysis or a decrease in

Table 9. Concentration of DES in Liver of Rats after Administration of 1 mg/day DES Dose for 7 Days (n = 4)

DES Concentration (ng/g)		
Animal Code	***ODF***	***Oral Suspension***
1	15.80	12.13
2	6.46	4.97
3	10.63	5.99
4	5.74	21.30
Mean ± SD	**9.66 ± 4.63**	**11.10 ± 7.50**

coagulation. Thus higher levels of fibrinogen will be considered detrimental, while higher levels of AT-III are considered beneficial for the potential increase of clotting risk. Fibrinogen and AT-III levels were quantified using rat fibrinogen and AT-III ELISA Kits.

Fibrinogen levels from ODF-DES treatment ranged between 1.06 to 4.24 mg/ml, while those from the oral suspension ranged between 1.56 to 3.26 mg/ml. Fifty percent of fibrinogen levels from ODF-DES were below 3.26 mg/ml and the other fifty percent were above. The fibrinogen levels were not statistically different at all time points for the ODF and oral DES suspension, except at the 24 h time point (Figure 26). To understand the significance of this difference in levels between the two formulations, we calculated and plotted the fibrinogen change profiles (fibrinogen levels from the study animals being subtracted from the sham controls) (Figure 27). The changes in fibrinogen from ODF-DES and the oral suspension were similar between the two formulations up to the 12 h mark, after which there was a statistically significant increase in fibrinogen levels from the ODF-DES formulation. These apparent significant increases in fibrinogen levels at the 24 and 30 h time points were investigated further using a multiple dosing protocol lasting 7 days for both the ODF-DES and oral DES formulations.

We also compared the AT-III concentration profiles between the ODF-DES and oral DES suspension. The concentrations of AT-III from ODF-DES ranged between 384.87 to 886.46 µg/ml with 88 % of the levels (7 out of 8) below 600 µg/ml, and levels from the oral suspension ranged between 453.91 to 597.67 µg/ml (Figure 28). As could be seen, there is no statistically significant difference between the levels of AT-III from ODF and oral suspension of DES. We further plotted the change in AT-III levels relative to sham

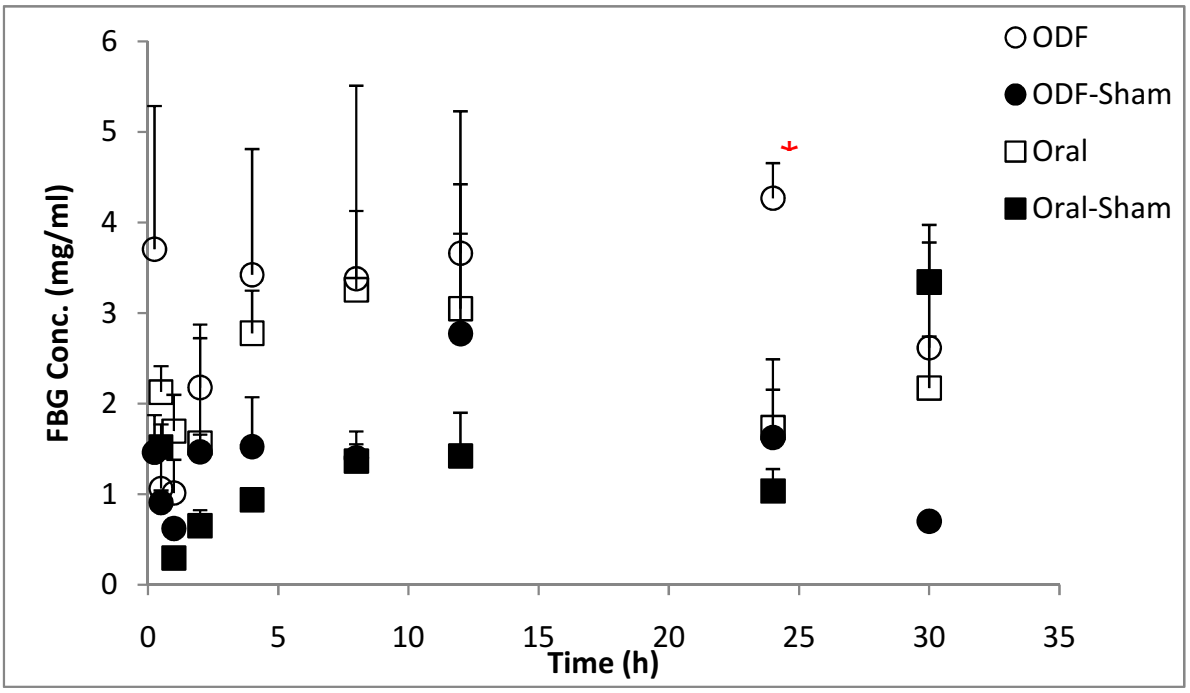


Figure 26. Fibrinogen Profiles after Single Dose (1 mg) of ODF-DES and Oral Suspension of DES in Rats (n=4 each).

* Significant difference between FBG concentration from ODF-DES and oral DES suspension (Student's t-test, $p<0.05$).

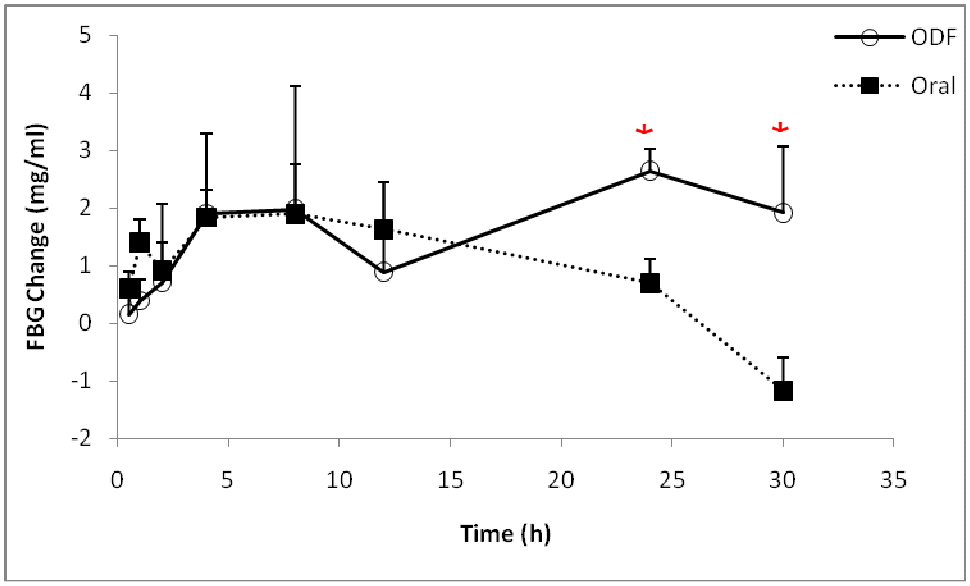


Figure 27. Fibrinogen Change Profiles after Single Dose (1 mg) of ODF and Oral Suspension DES in Rats (n=4 each).

Data reported as mean ± SD

The fibrinogen levels for the study animals were compared to the sham controls.

FBG=Fibrinogen

*Significant difference (Student's t-test, $p<0.05$)

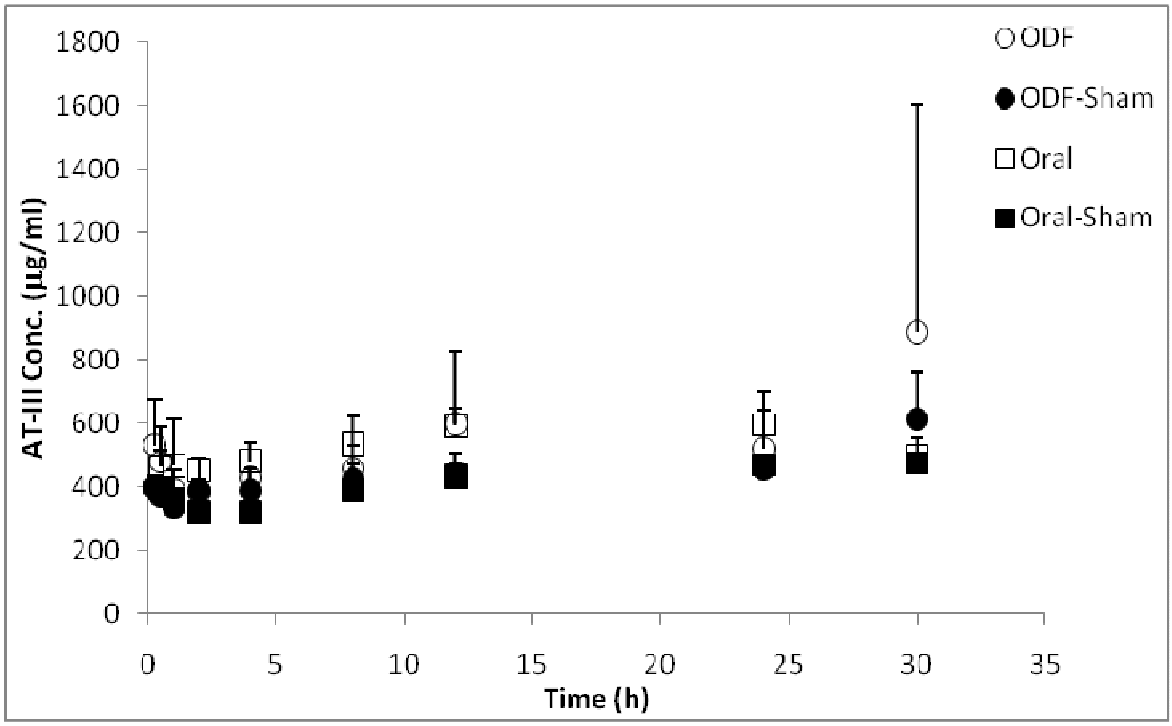


Figure 28. AT-III Profiles after Single Dose (1 mg) of ODF and Oral Suspension DES in Rats (n=4 each).

Data reported as mean ± SD

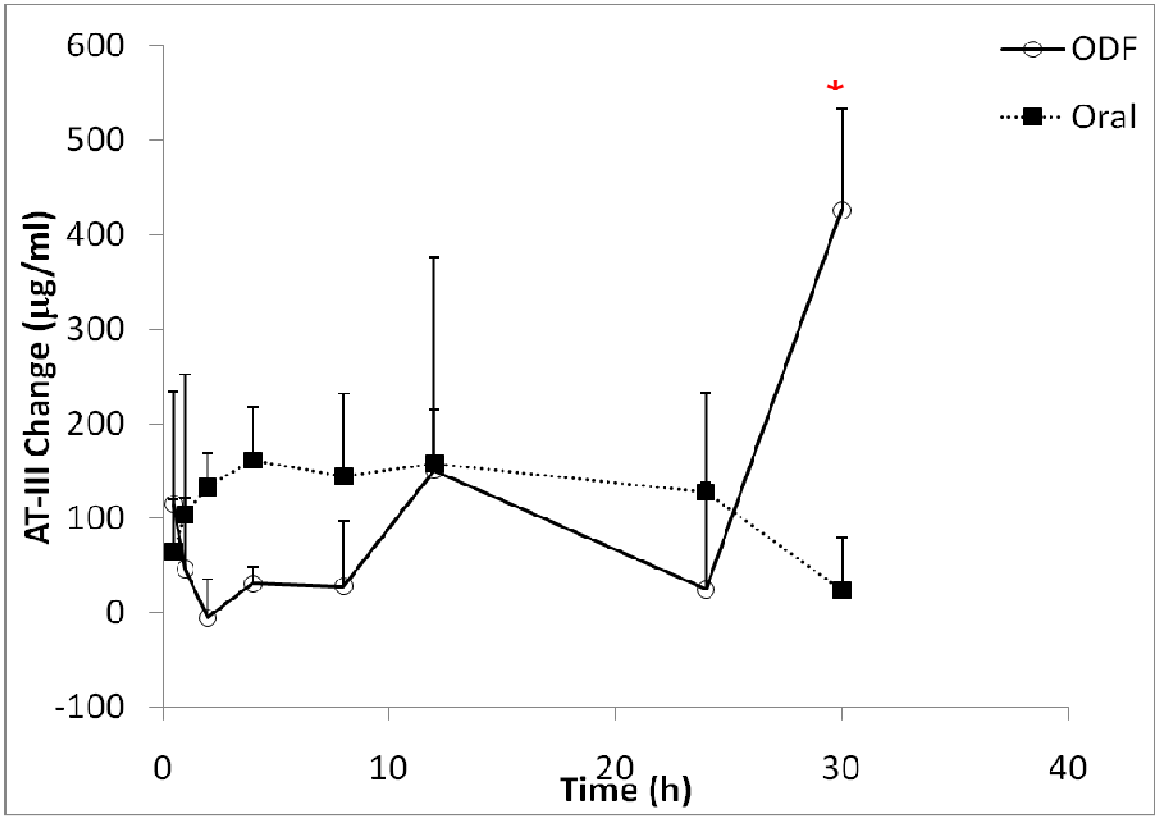


Figure 29. AT-III Change Profiles after Single Dose (1 mg) of ODF and Oral Suspension DES in Rats (n=4 each).

Data reported as mean ± SD

The AT-III levels for the study animals were subtracted from the sham controls.

*Significant difference (Student's t-test, $p<0.05$)

control after the administration of both formulations (Figure 29). The analysis of these plots did not reveal any statistical significant increase in AT-III levels from the ODF-DES formulation compared to the oral DES suspension, except at the 30 h time point. Whether this value at the 30 h time point was an outlier or not was investigated further with the multiple dosing study covering 7 days.

4.6.2. Relationsip of Fibrinogen and AT-III Concentrations after Multiple Doses of ODF and Oral Suspension

The effect of multiple doses of DES from ODF and oral suspension on fibrinogen and AT-III levels were investigated. Two groups of rats (n=4 for each group) were dosed the two formulations, respectively, for 7 days and plasma samples were collected for the quantification of fibrinogen and AT-III levels starting from the 5th to the 8th days.

Fibrinogen levels from this study were in the range of 0.30 to 1.45 mg/ml and 0.24 to 0.47 mg/ml for the ODF and oral suspension of DES, respectively (Table 10 and Figure 30). All fibrinogen levels were within the same range between the two formulations, except on day 5 when levels from ODF-DES were greater than for oral suspension, but the finding was not statistically significant ($p > 0.12$). Profiles depicting changes (relative to sham control) (Figure 31), absolute changes (Figure 32), and absolute percent changes (Figure 33) in levels did not show any significant difference in fibrinogen levels between the two formulations all through the 5 days that samples were collected.

AT-III levels between ODF-DES and oral DES suspension ranged from 417.10 to 478.69 µg/ml and 396.06 to 439.72 µg/ml for ODF and oral suspension, respectively (Table 11 and Figure 34). Levels of AT-III for ODF-DES were slightly higher for all the

study days, as compared to those from oral suspension, but the differences were not statistically significant ($p>0.25$). The profiles for changes relative to sham control (Figure 35), absolute changes (Figure 36), and percent absolute changes (Figure 37) in levels did not show significant differences in levels between the two formulations.

Table 10. Fibrinogen Concentrations and Changes Following Multiple Dosing of ODF and Oral Suspension DES in Rats (n=4 each)

Days	FBG Levels (mg/ml)			Absolute Change[a]		% Absolute Change	
	Sham Control	ODF	Oral Susp.	ODF	Oral Susp.	ODF	Oral Susp.
5	0.45 ± 0.39	1.45 ±1.05[b]	0.45 ±0.33[b]	-0.10±1.13	-1.11±0.35	-9.19±102.78	-100.84±32.19
6	0.75 ± 0.25	0.44 ± 0.39	0.47 ± 0.12	-1.41±0.42	-1.37±0.13	-127.88±37.89	-124.97±11.35
7	0.33 ± 0.09	0.30 ± 0.07	0.24 ± 0.12	-1.13±0.07	-1.20±0.14	-103.03±6.62	-108.67±12.22
8	0.64 ± 0.14	0.37 ± 0.20	0.44 ± 0.77	-1.37±0.21	-1.3±0.83	-124.11±19.36	-118.27±75.31

Data reported as mean ± SD

The rats were dosed with either ODF-DES or oral DES suspension for 7 days and on the 5th day plasma samples (once daily) up to the 8th day were collected for quantifications of fibrinogen and AT-III levels.

No significant difference in the levels of fibrinogen between the two groups.

a Change relative to day zero (1.10 ± 0.56 mg/ml),

b Signifies no significant change, p> 0.12

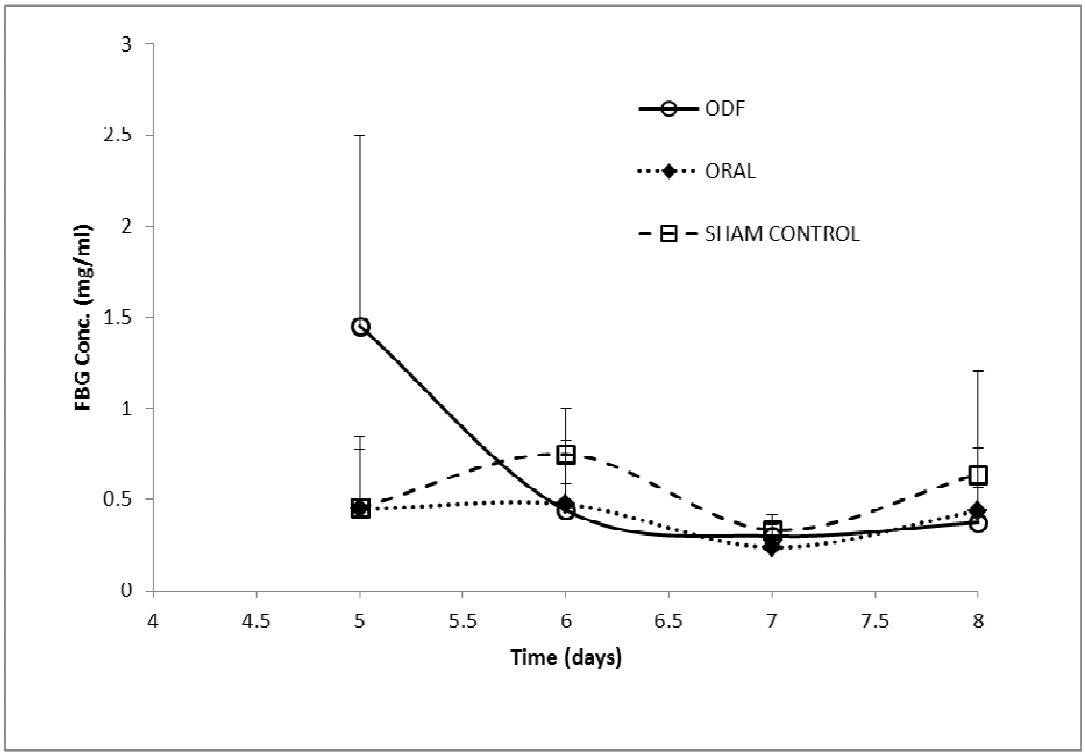


Figure 30. Fibrinogen Profiles Following Multiple Dosing (1 mg/day for 7 days) ODF or Oral Suspension of DES in Rats (n=4 each).

Data reported as mean ± SD

The rats were dosed daily with either ODF-DES or oral DES suspension for 7 days and on the 5th day plasma samples (once daily) up to day 8th day were collected for quantifications of fibrinogen and AT-III levels.

No significant differences in the levels of fibrinogen between the two groups. Fibrinogen levels were greater for the ODF-DES formulation on Day 5, but the differences were not statistically significant (p> 0.12)

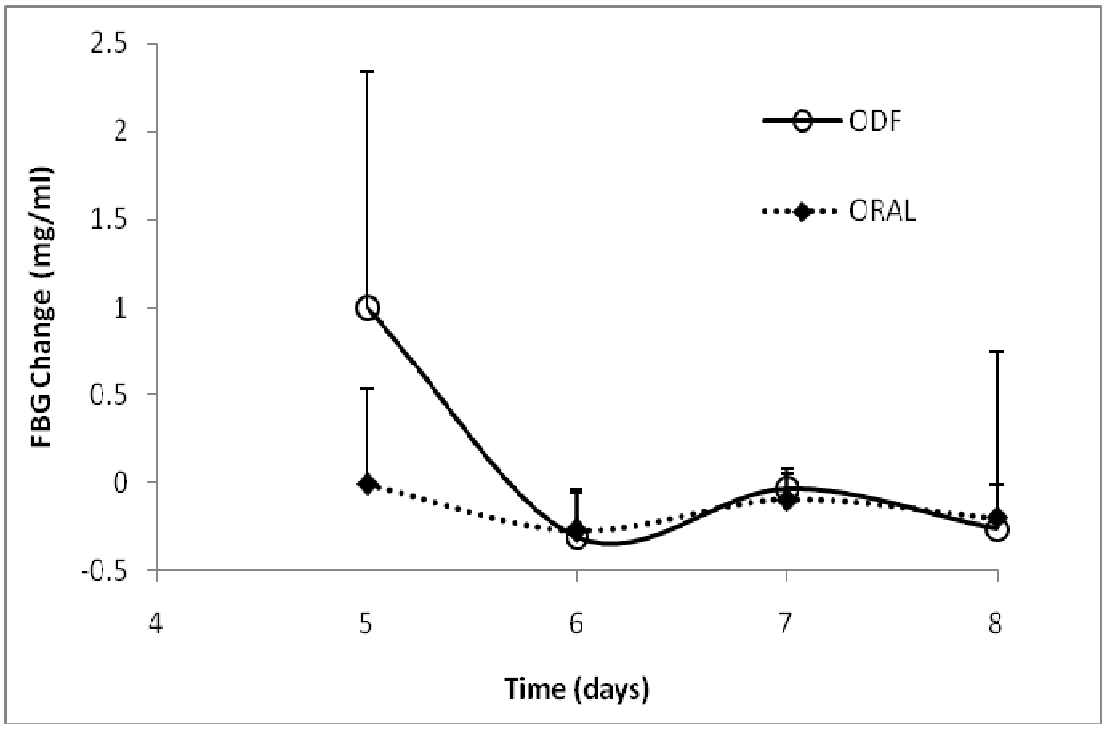


Figure 31. Profiles of Changes Relative to Sham Control in Fibrinogen Following Multiple Dosing (1 mg/day for 7 days) of Rats with ODF and Oral Suspension of DES (n=4 each)

Data reported as mean ± SD

The rats were dosed daily with either ODF-DES or oral DES suspension for 7 days, and on the 5th day plasma samples (once daily) up to the 8th day were collected for quantifications of fibrinogen and AT-III levels.

No significant difference in the levels of fibrinogen between the two groups. Fibrinogen levels were greater for the ODF-DES formulation on Day 5, but the differences were not statistically significant ($p > 0.12$)

FBG = fibrinogen

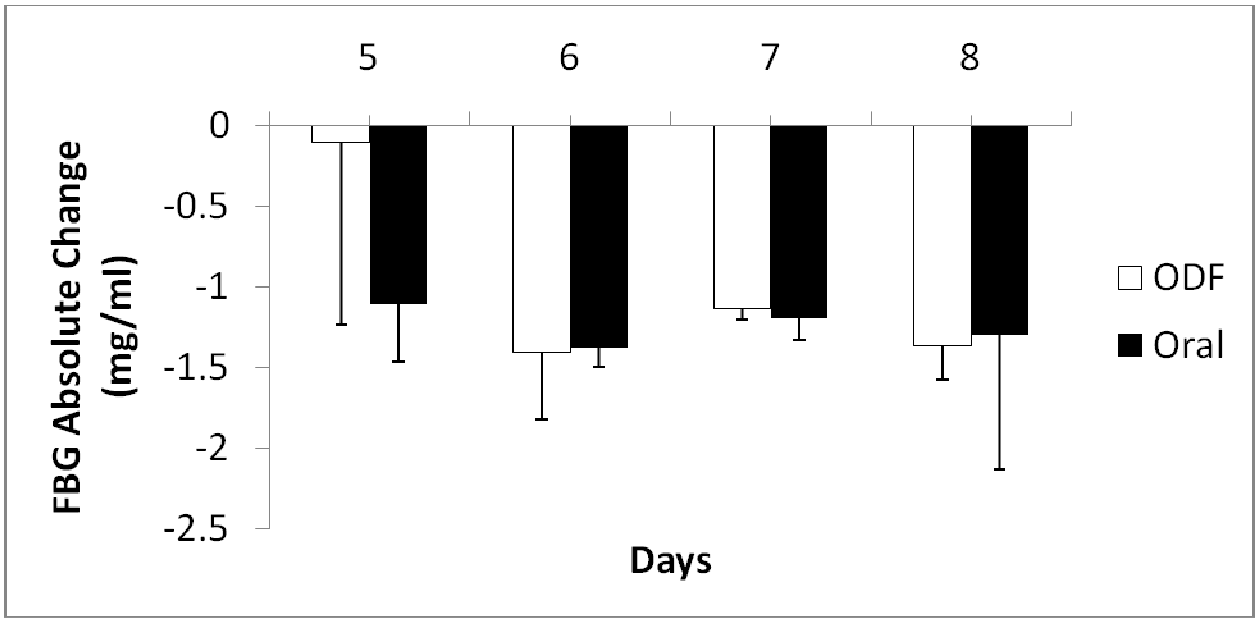


Figure 32. Absolute Changes Relative to Day Zero (Baseline) in Fibrinogen Profiles Following Multiple Dosing (1 mg/day for 7 days) of Rats with ODF and Oral Suspension of DES (n=4 each).

Data reported as mean ± SD

The rats were dosed daily with either ODF-DES or oral DES suspension for 7 days and on the 5th day plasma samples (once daily) up to the eighth day were collected for quantification of fibrinogen.

No significant difference was observed in fibrinogen levels between the ODF and oral DES suspension.

FBG = fibrinogen

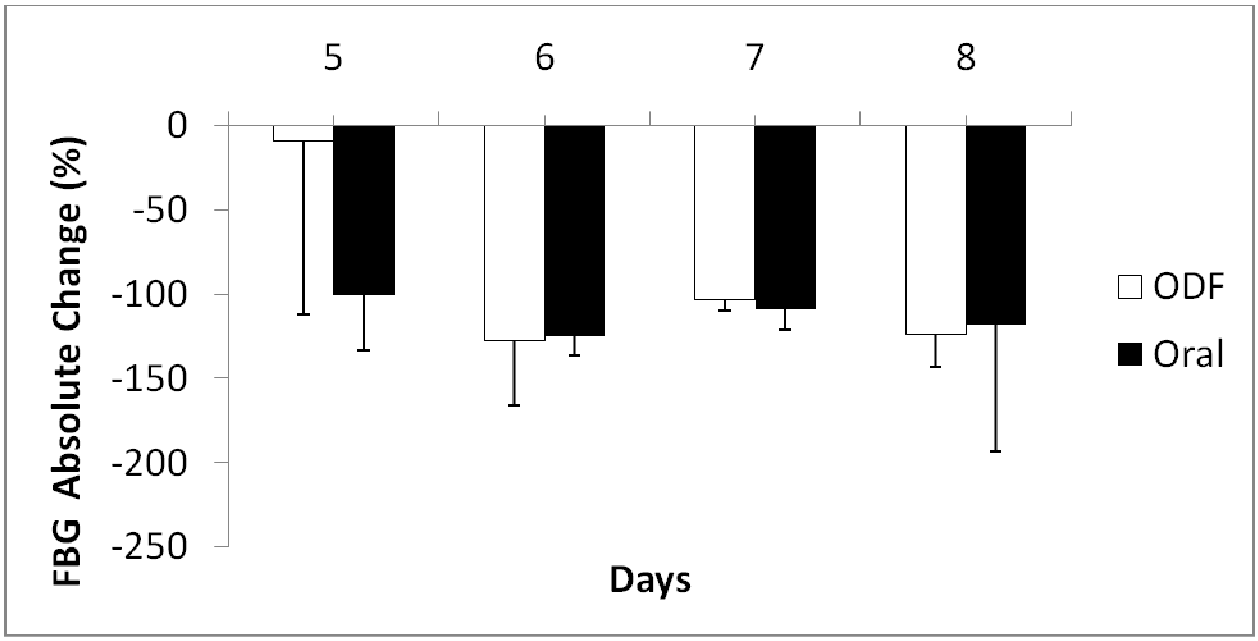


Figure 33. Percent Absolute Changes Relative to Day Zero baseline in Fibrinogen Profiles following Multiple Dosing (1 mg/day for 7 days) of Rats with ODF or Oral DES Suspension (n=4 each)

Data reported as mean ± SD

The rats were dosed daily with either ODF-DES or oral DES suspension for 7 days and on the 5th day plasma samples (once daily) up to the 8th day were collected for quantifications of fibrinogen.

No significant difference was observed in fibrinogen levels between the ODF and oral DES suspension.

FBG = fibrinogen

Table 11. AT-III Concentrations and Changes Following Multiple Dosing of ODF and Oral Suspension of DES in Rats (n=4 each)

Days	AT-III Levels (µg/ml)			Absolute Change [a]		% Absolute Change	
	Sham Control	ODF	Oral Susp.	ODF	Oral Susp.	ODF	Oral Susp.
5	359.38 ± 83.01	417.1±15.19	396.1±30.11	-346.87±14.85	-322.69±27.56	-85.73±3.67	-89.79±7.67
6	418.56 ± 34.98	478.7 ± 80.36	439.7 ± 97.71	-344.46±84.17	-397.39±97.58	-85.14±20.80	-94.94±23.31
7	410.29 ± 49.81	446.4 ± 29.70	398.0 ± 53.36	-368.45±16.75	-422.57±53.86	-91.07±4.14	-102.99-13.13
8	461.32 ± 160.76	430.0 ± 50.36	410.1 ± 74.43	-435.94±51.23	-512.53±74.60	-107.74±12.66	-111.10±16.17

Data reported as mean ± SD

The rats were dosed with either ODF-DES or oral DES suspension for 7 days and on the 5th day plasma samples (once daily) up to the 8th day were collected for quantifications of fibrinogen and AT-III levels.

No significant difference in the levels of fibrinogen between the two groups.

a Absolute Change from day zero (404.60 ± 24.66 µg/ml).

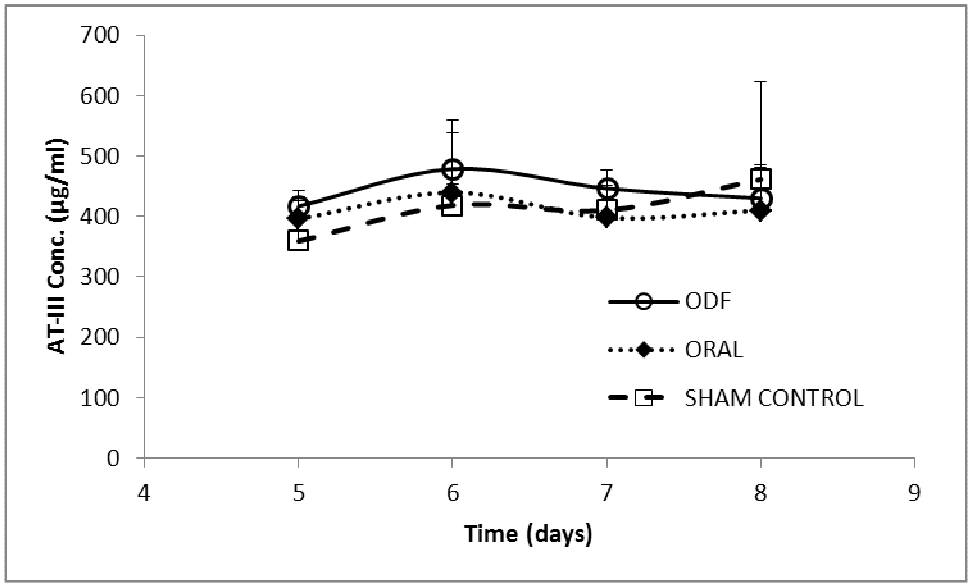


Figure 34. AT-III Profiles Following Multiple Dosing (1 mg/day for 7 days) of Rats with ODF and Oral Suspension of DES (n=4 each).

Data reported as mean ± SD

The rats were dosed daily ODF-DES or oral DES suspension for 7 days and on the 5th day plasma samples (once daily) up to the 8th day were collected for quantifications of AT-III levels.

No significant differences in AT-III levels between the ODF and oral suspension of DES groups.

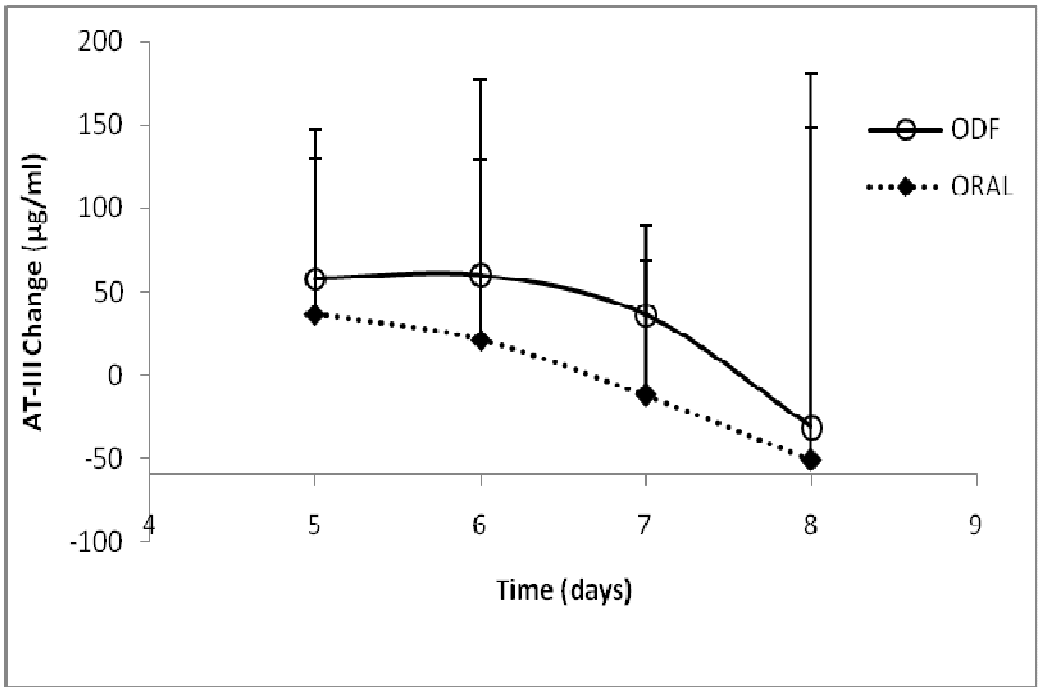


Figure 35. Profiles of Changes Relative to Sham Control in AT-III Following Multiple Dosing (1 mg/day for 7 days) of Rats with ODF or Oral Suspension of DES (n=4 each).

Data reported as mean ± SD

The rats were dosed daily with either ODF-DES or oral suspension of DES for 7 days and on the 5th day plasma samples (once daily) up to the 8th day were collected for quantifications of AT-III levels.

No significant differences in AT-III levels between the ODF and oral suspension of DES groups.

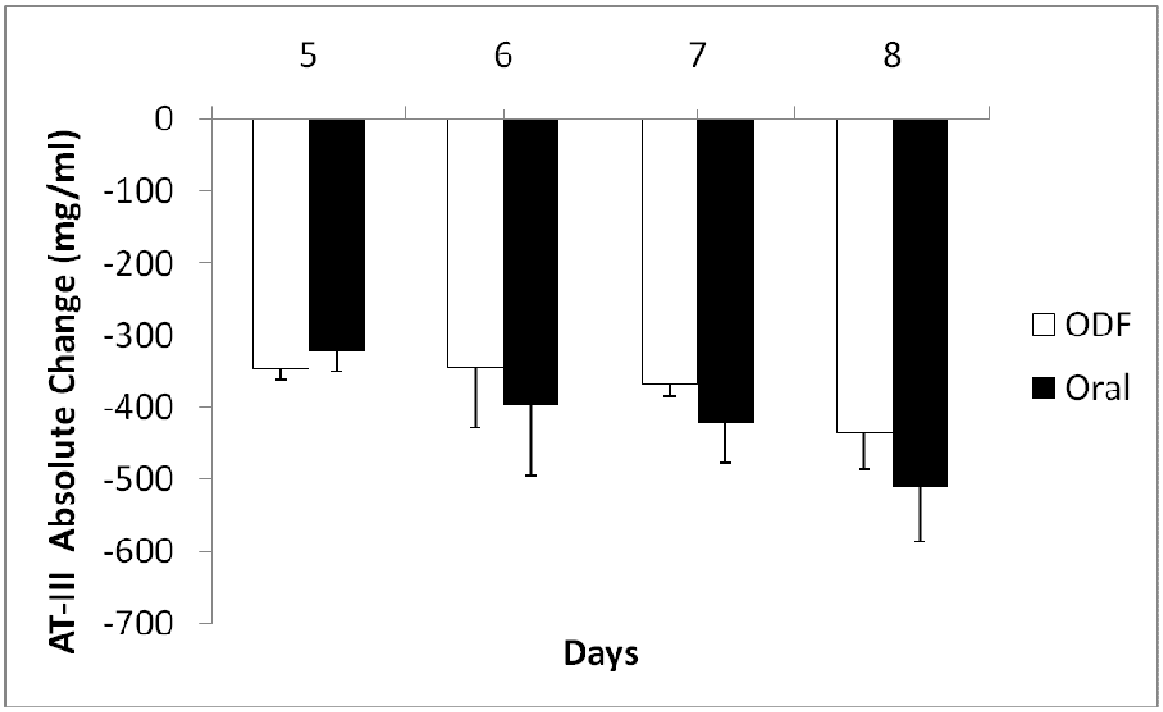


Figure 36. Absolute Changes Relative to Day Zero Baseline in AT-III Profiles Following Multiple Dosing (1 mg/day for 7 days) of Rats with ODF and Oral Suspension of DES (n=4 each).

Data reported as mean ± SD

The rats were dosed daily with either ODF-DES or oral suspension of DES for 7 days and on the 5th day plasma samples (once daily) up to the 8th day were collected for quantifications of AT-III levels.

No significant differences in AT-III levels between the ODF and oral suspension of DES groups.

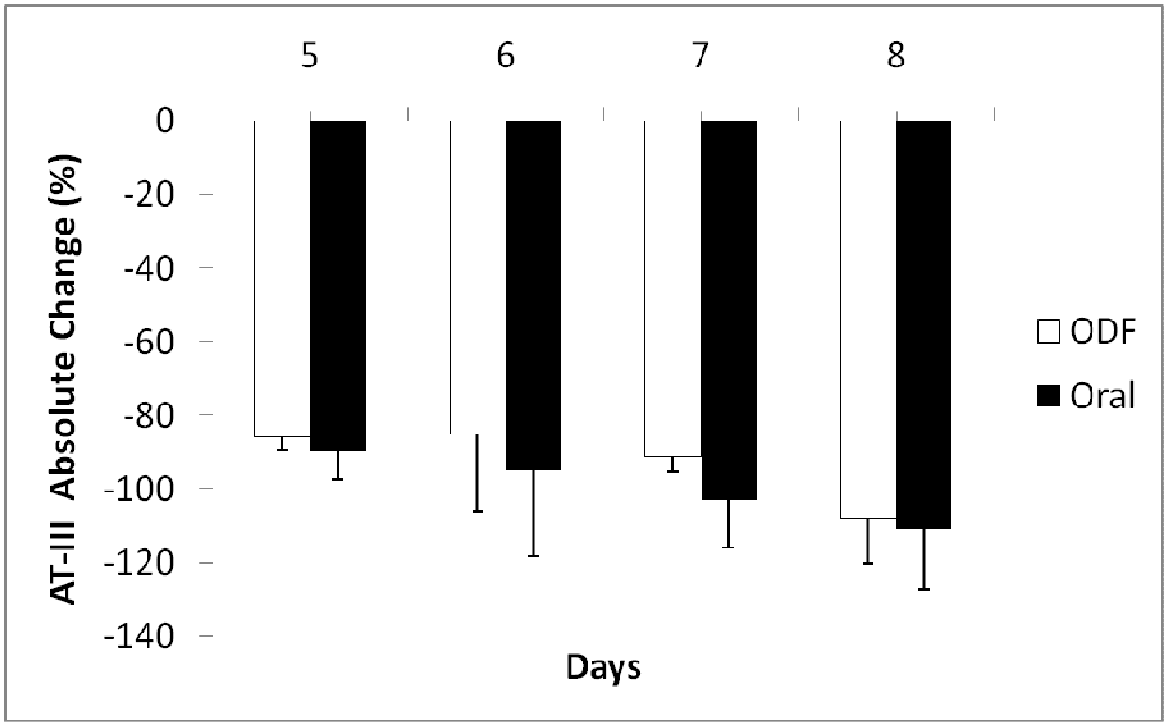


Figure 37. Percent Absolute Changes Relative to Day Zero Baseline in AT-III Profiles Following Multiple Dosing (1 mg/day for 7 days) of Rats with ODF or Oral Suspension of DES (n=4 each).

Data reported as mean ± SD

The rats were dosed daily with either ODF-DES or oral suspension of DES for 7 days and on the 5th day plasma samples (once daily) up to the 8th day were collected for quantifications of AT-III levels.

No significant differences in AT-III levels between the ODF and oral suspension of DES groups.

If one combines the data from the single dosing studies and the multiple dosing studies covering 7 days, it would be safe to conclude that ODF-DES will significantly increase the concentrations and exposures of DES (AUC) without resulting in additional risk for clotting as measured by fibrinogen and AT-III levels, as well as the changes in this levels.

4.6.3. Correlation of Mean Absolute Percent Changes in Fibrinogen and AT-III with DES AUCs after Single Dose (1 mg) of ODF and Oral Suspension of DES

In the PK studies, it was observed that the AUC of DES from ODF was more than 2.7 times of the AUC from the oral suspension. We wanted to know if there was an effect on the fibrinogen levels of the increased AUC from ODF-DES. Although this question was investigated above, we wanted to investigate further the relationship of DES AUCs and fibrinogen and AT-III levels. We proceeded by correlating the mean absolute percent changes in fibrinogen for each animal with the corresponding AUC (Figure 38). From the figures, it is clear that the significant increases in AUCs of DES from ODF as compared to the oral DES suspension did not result in differences in the mean absolute percent changes in fibrinogen between the two formulations.

A similar observation was made for the AT-III levels between the two formulations, where although the AUCs of ODF-DES were significantly greater for the ODF-DES formulation, no overall difference was observed in the mean absolute percent changes in AT-III levels between the two formulations (Figure 39). This observation reinforces our claim that ODF-DES will increase the bioavailability of DES compared to oral formulation without significantly increasing clotting risk as measured by fibrinogen and AT-III.

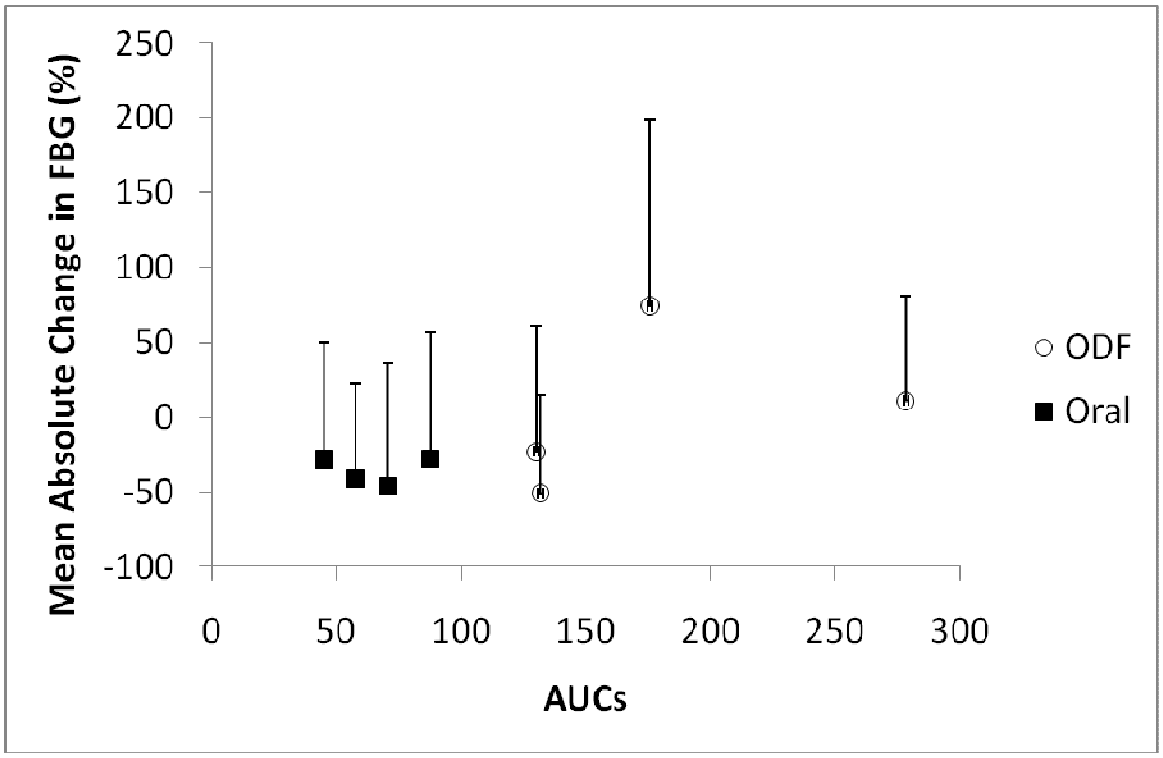


Figure 38. Correlation of Absolute Mean Percent Changes in Fibrinogen with DES AUCs in Rats Treated with ODF and Oral Suspension of DES (n=4 each).

Mean Absolute % change data reported as mean ± SD

AUCs from ODF-DES were greater than those from oral suspension of DES, but the mean absolute percent changes in fibrinogen were similar between the two formulations. FBG=Fibrinogen

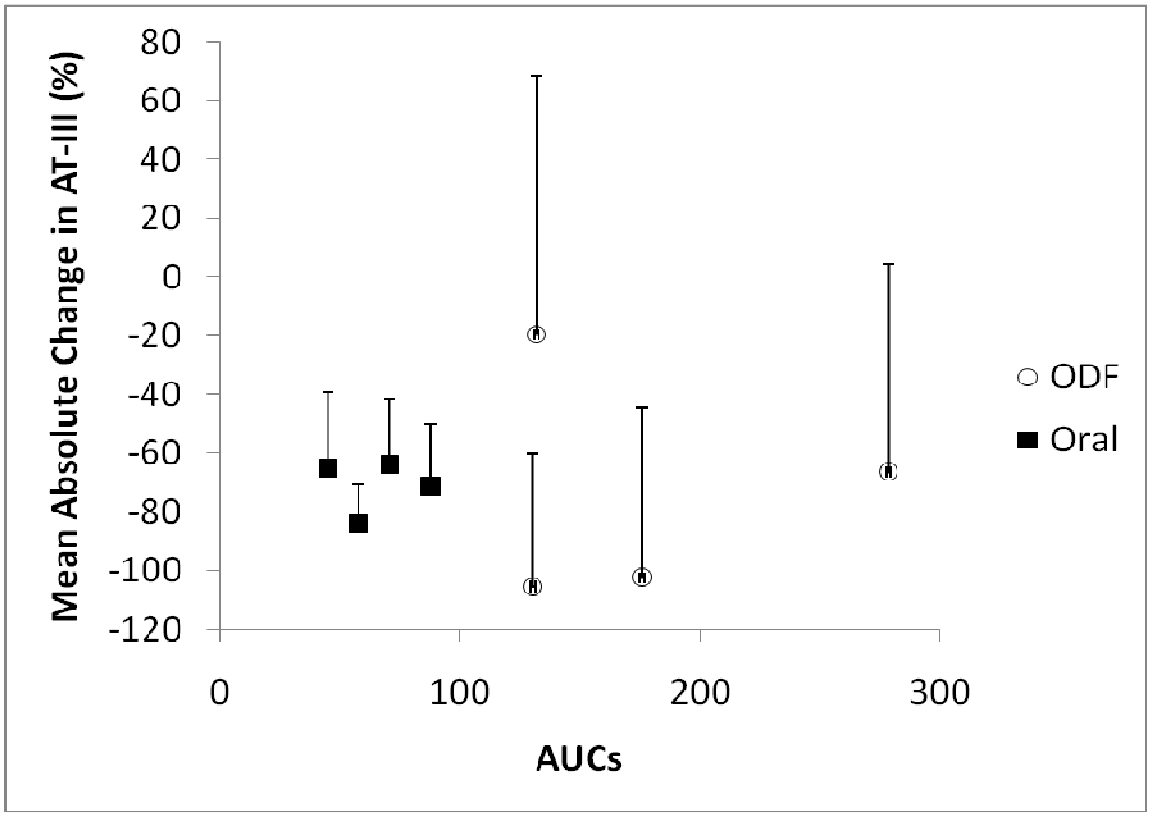


Figure 39. Correlation of Mean Absolute Percent Changes in AT-III with AUCs in Rats from Treated with ODF and Oral Suspension of DES (n=4 each).

Mean Absolute % change data reported as mean ± SD

AUCs of DES from ODF-DES were greater than those from the oral suspension, but the mean absolute percent changes in AT-III were similar between the two formulations.

4.6.4. Correlation between Fibrinogen and AT-III Absolute Percent Changes after Single Dose of ODF and Oral Suspension of DES

Fibrinogen and AT-III are both proteins synthesized in the liver; we investigated any correlation between their levels. To achieve this goal, we correlated the absolute percent changes in fibrinogen and AT-III after the administration of 1 mg of DES from either ODF or oral suspension of DES in rats. We observed a strong positive correlation between fibrinogen and AT-III changes after a single dose of oral suspension (R^2= 0.78), but did not observe one between fibrinogen and AT-III after ODF-DES dosing (Figure 40).

4.6.5. Exposures of DES, Fibrinogen and AT-III after Single Dose (1 mg) of ODF-DES and Oral Suspension of DES

The exposures (AUCs) of DES, fibrinogen and AT-III after ODF and oral suspension of DES dosing were evaluated and compared. The AUC gives the total exposure of the animal to each of the clotting factors and DES after administrations of both formulations. From Section 4.5.1, the AUC of DES from ODF is significantly greater (> 2.7 times), than the AUC from oral suspension; we investigated whether this difference in AUCs had an effect on the exposures (AUCs) due to fibrinogen and AT-III. The AUCs of fibrinogen (Oral: 74.24 mg/ml*h, ODF: 106.45 mg/ml*h) and AT-III (Oral: 16579.80 µg/ml*h, ODF: 16498.30 µg/ml*h) after ODF-DES and oral DES suspension were not significantly different (p> 0.09 for fibrinogen and p> 0.98 for AT-III) (Figure 41). This observation strengthens our earlier observation that levels of fibrinogen and AT-III after ODF and oral suspension of DES dosing were not significantly different between the two formulations,

even though AUCs of DES from ODF were 2.7 times greater compared to oral DES suspension.

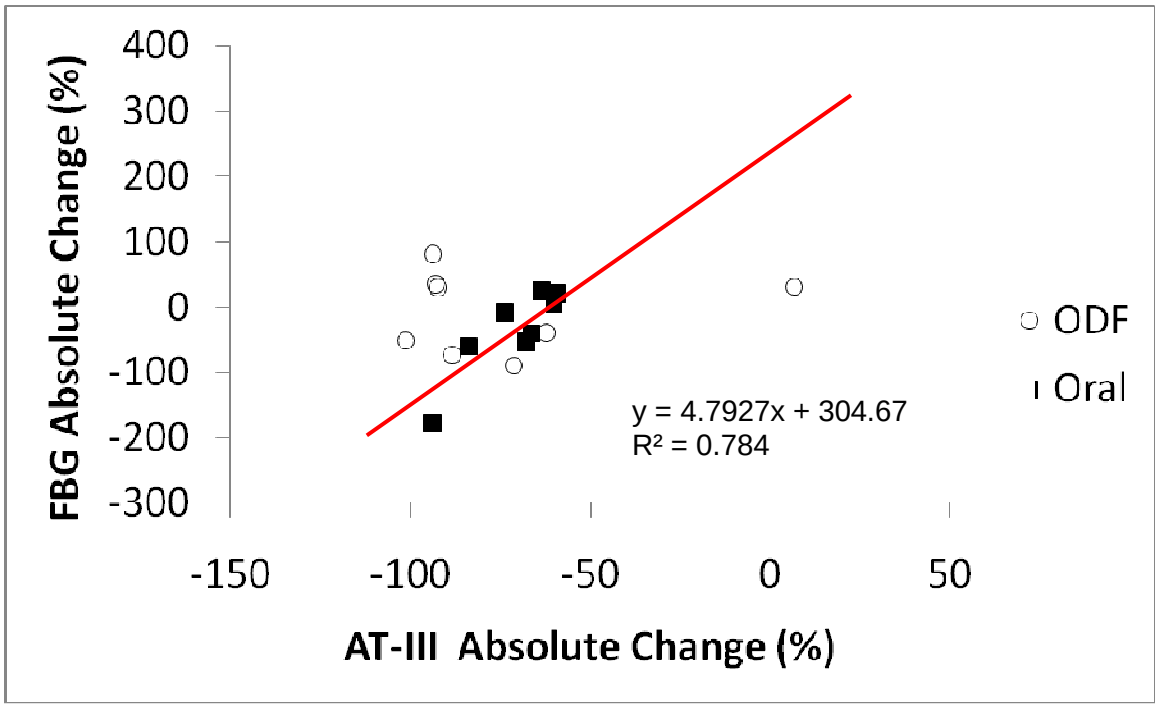


Figure 40. Correlation between FBG and AT-III Absolute Percent Changes after Administration of Single Dose of ODF and Oral Suspension of DES in Rats (n=4 each).

Data reported as mean ± SD

There is a strong positive correlation between changes in fibrinogen and AT-III for the oral formulation, but not for the ODF-DES formulation.

FBG=Fibrinogen.

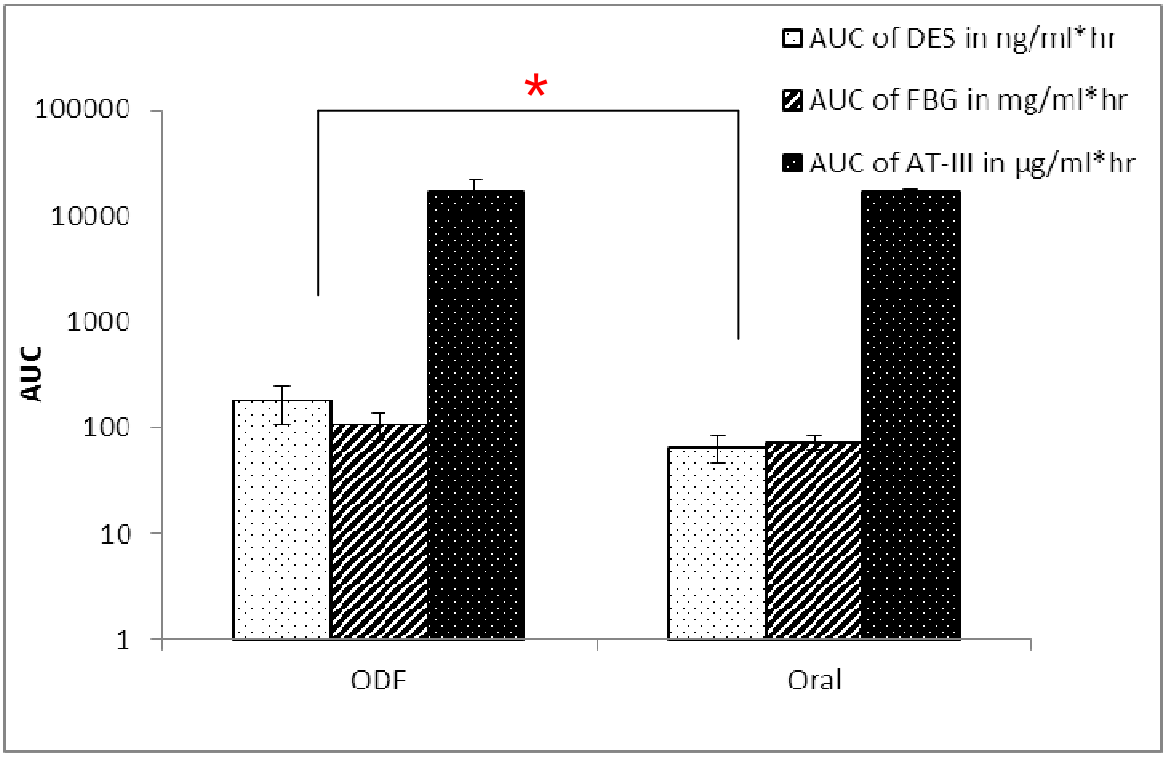


Figure 41. AUCs of DES, Fibrinogen and AT-III after Administrations of ODF and Oral Suspension DES in Rats (n=4 each).

Data reported as mean ± SD

AUC of DES from ODF is significantly greater than that from oral formulation, but AUCs of fibrinogen and AT-III were not significantly different between the two formulations.

* Significant difference between the two formulations, $p < 0.05$.

Chapter 5. Discussion

Oral DES is an effective agent for the treatment of prostate cancer. It induces castrate serum testosterone levels in most patients with prostate cancer. There is evidence suggesting that DES may improve the survival of patients with advanced prostate cancer compared with orchiectomy and luteinizing hormone releasing hormone (LHRH) agonist (Haapiainen et al, 1986; Osborne et al, 1990). This important advantage over the other therapies is attributed in part to the favorable effects of DES on the bone by reducing osteoporosis and retarding the development of bone metastases (Jilka et al, 1992). DES has also been shown to have superior clinical outcome by targeting both androgen-dependent and -independent prostate cancer cells (Smith et al, 1998; Nesbit et al, 1950; Robertson et al, 1996). Unfortunately, oral DES is neither ideal nor appealing because it may potentially exacerbate thromboembolic complications. The thromboembolic complications of oral DES include stroke, heart attack, deep vein thrombosis, pulmonary embolism as well as stable and unstable angina. Because of these complications, oral DES therapy for prostate cancer was replaced by LHRH agonist in the 1980s. In the leuprolide study conducted in the 1980s, oral DES therapy at 3 mg/day regimen was compared to leuprolide (a LHRH agonist) at 1 mg subcutaneously to evaluate the safety and efficacy of both treatments. The study concluded that oral DES therapy was as effective as leuprolide in halting disease progression, 85 % versus 86 % for leuprolide, but DES had excess side effects, 13 % versus 3 % for leuprolide (The Leuprolide Study Group, 1984). Leuprolide and their analogues replaced DES after the publication of this

study as the mainstay therapy for the treatment of advanced prostate cancer. DES was therefore withdrawn from the US market, and many clinicians have been reluctant to prescribe DES for prostate cancer, even it is still available through compounding pharmacies.

DES therapy for prostate cancer has received renewed a interest because of: (A) the evidence of DES effectiveness in the treatment of androgen-independent prostate cancer, (B) the high cost of LHRH agonist compared to oral DES, (C) the discovery of a new estrogen receptor β which is believed to play a protective role in the pathogenesis of prostate cancer and DES is a substrate of the receptor, and (D) an appreciation now of the side effects of LHRH agonist, especially in causing anemia, hot flashes and osteoporosis, while DES is known to modulate increase in bone mineral density, reverses hot flashes in castrated men and may stimulate erythropoiesis . Other estrogens and estrogen formulations have been studied for the treatment of prostate cancer, but none has been shown to be superior to DES in efficacy. Parenteral (Hedlund et al, 2002) and transdermal (Bland et al, 2005) estrogens have been used to treat prostate cancer with varying degrees of success; both have been shown to have fewer thromboembolic side effects compared to oral estrogens. The thromboembolic side effects of oral DES are thought to result from the first pass metabolism of DES in the liver. This liver metabolism induces the production of pro-coagulation factors in the liver, which may tilt a patient's coagulation balance towards more coagulation (De Linieres et al, 1986; Caine et al, 1992).

Because of the observation that other formulations of estrogens have fewer side effects than oral estrogen, we started looking at other formulations of DES that will potentially bypass liver metabolism and hence potentially decrease the thromboembolic side effects of oral DES. We are interested in investigating the transmucosal delivery of DES through the buccal cavity using an oral dissolving film (ODF).

Oral dissolving film preparation is a novel drug delivery system that can be used to deliver drugs to patients with swallowing difficulty, and it does not require water for administration. The film is placed in the buccal mucosa and the contents are dissolved by saliva and absorbed through the buccal cavity. This form of drug administration also has the advantage of potentially bypassing the first-pass liver metabolism (Shojaei, 1998), thus potentially increasing drug bioavailability. In the case of DES, avoiding the first pass metabolism will potentially decrease the excessive exposure of drug to the liver and decrease the production of pro-coagulation proteins.

Our study is divided into two aspects of investigations: A clinical trial and a preclinical evaluation. In the clinical trial, 20 advanced prostate cancer patients were enrolled in a clinical study at the University of Texas MD Anderson Cancer Center in Houston, Texas. Each patient received a 1 mg twice daily dose of DES, except 3 patients who received a 1 mg/day dose (Patients # 3, 5 and 8) and two patients whose therapy was modified during the study (Patients # 2 and 13). Plasma samples were drawn from the patients 2-3 hours post-dose for DES, FVII and PSA quantifications. The ultimate goal of the clinical study is to optimize the dosing regimen of oral DES in patients with recurrent prostate cancer, so as to increase efficacy and decrease thrombogenic toxicity. The

preclinical studies involved the evaluation of other formulations and routes of administration of DES that will potentially decrease thrombogenic side effects and increase efficacy. We studied the transdermal permeation of DES solution in propylene glycol through excised nude mice skin, and the transmucosal delivery of DES through the buccal cavity of a rat model. The goals of the preclinical studies are to establish the feasibility of the transdermal delivery of DES, and the potential merits of ODF-DES over an oral suspension of DES, which was achieved by determining the relative bioavailability and the effect of ODF-DES on two key clotting factors (Fibrinogen and AT-III) compared to the oral suspension.

5.1. LC/MS/MS Assay

5.1.1. LC/MS/MS Assay for Quantification of DES

There is a need for a sensitive and reliable assay method to quantify DES from human as well as rat plasma and tissues which DES is distributed such as the liver. DES has been in human use since the 1940s, but it is surprising that an extensive literature search did not retrieve any LC-MS/MS assays methods for the quantification of DES in human plasma. Two papers published in 1981 (Kemp et al., 1981) and 1982 (Abramson and Miller, 1982), respectively, made reference to the quantification of DES in human plasma using the GC/MS instrument, but none actually referenced published assay papers. Published assays for measuring DES in plasma are radioimmunoassays (Kemp et al., 1981; Usui et al., 1984; Nakamura, 1986). Radioimmunoassays for DES quantifications in plasma have the major disadvantage of poor specificity, due to cross reactivity with other steroids and metabolites of DES in plasma (Economou et al., 1993).

DES undergoes extensive metabolism in the liver producing metabolites with similar structure as DES (Metzler, 1981), so any robust assay to quantify DES in plasma must have the ability to discriminate between DES and its metabolites. LC/MS/MS assay methods make use of molecular weight transitions to detect compounds, and for this reason are able to differentiate similar compounds by making use of their different molecular weights. Because of the renewed interest in oral DES therapy for prostate cancer and its inherent thrombogenic complications, it is crucial to monitor DES levels in patients on oral DES therapy and correlate the levels with efficacy and toxicity biomarkers, PSA and clotting factors, respectively. This correlation may guide the optimization of oral DES therapy for advanced or recurrent prostate cancer patients to potentially decrease thrombogenic toxicity and increase efficacy.

The LC/MS/MS assay developed was highly sensitive, reproducible and efficient in quantifying DES in plasma of humans and rats and in liver tissues of rats. The assay was linear in the range of 0.78 – 100 ng/ml and the recovery of DES and internal standard were 71 % and 94 %, 76.7% and 92.7%, and 68.8% and 86.6% for human plasma, rat plasma and rat liver tissue, respectively. The assay was validated with quality control samples with a within-day (n=6) and between-day (n=16) accuracy and precision of < 17 %. This accuracy and precision are below the guidelines stipulated by the FDA for bioanalytical method validation (Guidance for Industry. Bioanalytical Method Validation, accessed in June 2010). DES stock solution did not show any degradation, while DES in plasma showed a degradation of < 13 % during 12 h on the bench top. DES prepared for analysis, but left in the autosampler at room for 12 h degraded < 6 %.

DES samples stored in 4℃ for 73 h (three days), showed a degradation of 21 %. The duration of 12 h that DES stock solution and DES samples were left on the bench top is long enough to cover the time period needed to prepare plasma samples for injection. It usually takes us a maximum of 4 h to prepare samples for injection into the LC/MS/MS. Because of the possibility that the instrument may breakdown while the samples are running, the stability of prepared samples (for analysis) in autosampler for 12 h were studied. The 12 h period mimics the maximal duration of samples in the LC/MS/MS plus the time required to resolve any LC/MS/MS instrument problems encountered during the sample run. The stability of DES in 4 ℃ temperature for 3 days was studied because we anticipated receiving plasma samples from other parts of the U.S requiring shipment durations of 1 – 2 days. In the end all the plasma samples from the patients used for this study were obtained from The University of Texas MD Anderson Cancer Center in Houston, Texas, within 10 min walking distance from our building. In conclusion, **this is the first published LCMS/MS assay method for the quantification of DES in plasma of human, as well as rat plasma and liver tissue.** This method is highly sensitive, reproducible and efficient in quantifying DES. A manuscript has been written for publication.

5.2. Phase I Clinical Trial of Oral DES in in Recurrent Prostate Cancer Patients

The human plasma samples were obtained from 19 patients on oral DES therapy for recurrent prostate cancer, in a clinical phase I trial at the University of Texas MD Anderson Cancer center in Houston, Texas. All patients gave their informed consent

before enrolling to study. All patients received 1 mg twice daily dose of oral DES, except Patients # 3, 5 and 8, who received 1 mg/day dose and Patients # 2 and 13, whose therapies were modified from 1 mg/day to 2 mg/day (Patient #2) or from 2 mg/day to 1 mg/day dose (Patient # 13) during the course of the treatment. One patient (Patient # 17) dropped out of the study after receiving study medication for just 2 days and the patient was not included in the data analysis because no plasma samples were drawn. The samples were quantified using the validated LC/MS/MS assay method described in Section 4.1.

5.2.1. Patients DES Concentrations

All 19 patients in this study had quantifiable DES concentrations. The DES plasma levels ranged between 0.11 and 4.29 ng/ml, except one patient (#11) whose level was 16.27 ng/ml. The mean DES plasma levels for these patients was 1.57 ng/ml (and 1.19 ng/ml without Patient #11). Eighty seven percent of all the DES levels for this population were below 2 ng/ml and 97 % were below 4.30 ng/ml.

Two other studies published DES levels in patients on oral DES therapy. In the first study by Kemp and colleagues, the 6 patients in the study were hospitalized as part of the study protocol to ensure compliance with study medication. The patients were administered 3 mg/day dose of oral DES and blood samples were collected for DES analysis. The DES plasma levels in these patients ranged between 0.15 to 6.0 ng/ml (Kemp et al., 1981). In the second study conducted a year later by Abramson and Miller (1982), 7 patients were administered on average 2 mg/day dose of oral DES therapy and randomly timed blood samples were collected from the patients for the quantification

of DES plasma levels. The study reported mean DES plasma concentration of 2.4 ± 0.4 ng/ml (range of 1 to 5 ng/ml). The DES plasma levels from our study ranged between 0.11 to 4.30 ng/ ml, with a mean concentration of 1.57 ng/ml for all but one of the patients. These DES plasma levels are within the range of the levels observed in the two studies. It is safe to conclude that at oral DES doses of 1 to 3 mg/day, observed plasma DES levels will be ≤ 6 ng/ml.

Patient # 11 was the only patient with plasma DES levels > 4.30 ng/ml and he was the only patient also reported to take two herbal supplements, prostasol and PC-SPES. Both supplements are believed to have strong estrogenic activity and are marketed as a mixture of herbs. Their exact mechanisms of actions in lowering testosterone and PSA are not known, but they are both believed to exert their effects through phytoestrogens in the herbal mixture (Clement and Bubley, 2008; Pirani, 2001). Additionaly, some batches of PC-SPES were shown to be contaminated with DES and other prescription drugs, leading to the withdrawal of the supplement from the US market in 2002 (White, 2002; Ko and Wilson, 2003; Oh et al., 2004). The presence of estrogenic compounds in these herbal supplements may result in competition with DES for metabolism in the liver, thereby influencing the clearance of DES (O'Connell, 1995). It is possible that this competition may result in the saturation of the enzymes capacities responsible for estrogen metabolism and hence inhibit the metabolism of DES. The presence of DES in PC-SPES will increase plasma levels of DES in a way dependent on the amount of DES in the supplement. Assuming that all or any of the above scenarios are true, the plasma levels of DES is expected to increase in Patient #11. Because the DES plasma

concentrations from our study are all < 4.30 ng/ml, but the DES level in patient # 11 is 16.27 ng/ml, > 4 times the level of the next highest patient, 4.29 in Patient 1. It will be reasonable to assume the involvement of other factors than DES dose alone to affect his levels. One of those factors was probably due to his herbal supplement regimen.

In this study, DES also showed considerable within and between patient variability. The two studies by Kemp and colleagues (1981) and Abramson and Miller (1982) reported no variability in DES levels between patients. For the Kemp and colleagues study, plasma samples were collected from the patients within a period of 8 h (5 patients) and 16 h (1 patient) with an indwelling catheter, indicating that the patients may have been hospitalized. Only one patient had his DES levels studied for a period longer than one day. In the Abramson and Miller study, all the patients were hospitalized which should assure compliance to study medication administration. None of the two studies reported if any of the patients was on concurrent medications that may influence their DES plasma levels. There may be three main reasons for DES variability within and between the study patients in this present study: (A) this patient population represented very sick patients who had previously been on multiple therapies. These multiple therapies present or past may affect the capacity of the liver to metabolize drugs and hence the clearance of DES; (B) compliance and therapy adherence is another reason. Because these patients were not hospitalized, it was difficult to monitor compliance and adherence to the study protocol as it concerns the adherence to DES therapy. Some patients reported self-modifying or discontinuing DES therapy due to the presence of painful gynacomastia and resuming the therapy when this side effect subsides. At least

one patient (Patient # 16) reported running out of study medication for a week before reporting for office visit. In these situations, DES levels will fluctuate with dose modification or discontinuation; and (C) some patients reported taking herbal supplements with estrogenic activity. This supplement will affect DES levels, especially, if the doses of the supplements are not standardized. It is also quite possible that other factors like polymorphism in estrogen metabolizing enzymes may also play a role in the variability within and between patients of oral DES. In such a scenario, poor metabolizers will have higher DES concentrations compared to fast metabolizers with low DES concentrations.

5.2.2. Response to DES Therapy by PSA Levels

All patients with documented entry PSA levels reported responses to the DES treatment as measured by PSA reductions of 17 % to 99 %. Sixty three percent of all the patients had a significant response, defined as PSA decline of ≥ 50 % (The Prostate Specific Antigen Working Group, 1999) and the average DES concentration for this response range was 1.23 ng/ml. Many studies have evaluated the response to DES therapy by PSA decline in patients with recurrent prostate cancer**, but this is the most recent study and it is the first study to correlate DES plasma levels to PSA response**. In a 2009 study conducted by Serrate and colleagues (2009), with 20 patients on an initial 1 mg/day regimen of DES therapy for advanced prostate cancer, 15 % had a significant response (≥50 %) and 25 % of the patients had a response ≥ 30 %. The median overall survival for this study was 20.7 months (range of 13.4 to 28 months). Other studies conducted mostly in the 1990s or early 2000s have also reported varying PSA response

Table 12. Effects of Diethylstibestrol in Androgen-Independent Prostate Cancer

Study (year)	Patient No.	Drug Regimen	>50% PSA Decline	Median Duration of Response (months)	% Cardiovascular Toxicity
Serrate (2009)	20	1 mg/day	15 %	3.7	NR
Smith (1998)	21	1 mg/day	43 %	NR	5 %
Rosenbaum (2000)	18	3 to 4 mg/day	66 %	7.5	0
Shahidi (2011)	127 115	1 mg/day 3 mg/day	26 % 32 %	3.4	11 %*
Farrugia (2000)	34	1 mg/day	72 %	6	20.2 %
Our Study	19	1 to 2 mg/day	63 %	3.8	5 %

NR= Not Reported

*Cardiovascular toxicity reported for both groups

levels. Smith et al. (1998) reported a significant response in 43 % of 21 patients on 1 mg/day DES dose. Rosenbaum et al. (2000) evaluated the effects of DES at 3 to 4 mg on 18 patients with androgen independent prostate cancer, and reported an overall response rate of 66 %. The median duration of the response was 7.5 months. Shahidi and colleagues (2001) reported overall response rates of 26 % and 32 % from patients treated with 1 mg and 3 mg/day regimen of DES, respectively. The mean duration of the response was the same for both groups at 3.4 months. In another study by Farrugia and colleagues (2000), 34 patients on 1 mg/day dose of oral DES therapy reported 72 % response (PSA decline ≥ 50 %). The median duration of symptom response was 6 (2 to 21) months. The 63 % response rate (PSA decline ≥ 50 %) observed in our study is in the top half of the published studies (Table 12). The median time to maximum PSA reduction was 114.5 days (3.8 months), with a range of between 1 to 8.3 months. The average DES concentration for this PSA response was 1.23 ng/ml and at DES concentrations of ≤ 2 ng/ml, 56 % of the patients had a significant PSA response.

We also found a correlation between the PSA reduction and the duration on DES therapy (R^2 = 0.23). When Patient #13 was not included in the correlation analysis, there was a stronger correlation (R^2 = 0.63) between the duration on DES therapy and percent reductions in PSA levels. This correlation reinforces our claim for the need of safe and effective DES dosing regimens and/or formulations that will enhance patient compliance by decreasing thrombogenic toxicities.

From the data, it can be suggested that at DES concentration ≤ 2 ng/ml more than 50 % of patients shall be expected to have a significant response to therapy as measured by

PSA reduction ≥ 50 %. Therefore, DES levels of 2 ± 1 ng/ml may be suggested as a therapeutic goal for majority of patients on oral DES therapy for recurrent prostate cancer. This suggestion comes from the understanding that the average DES concentration for patients with significant PSA response in this study is 1.23 ng/ml and also that at DES levels of ≤ 2.0 ng/ml, 56 % of patients had a significant response.

In conclusion, **this is the first study to evaluate the PSA response and correlate the response to patient plasma DES levels.** We also suggest therapeutic drug monitoring of DES concentration in patients on DES therapy to maintain plasma levels in the therapeutic range and enhance compliance to therapy.

5.2.3. Correlation of DES Levels to FVII Levels and Toxicity

There is evidence suggesting that elevated FVII activity is a significant predictor of ischemic heart disease and cardiovascular death (Meade et al., 1990; Huntlin, 1991). DES toxicity in this study was measured by quantifying FVII levels in plasma of all patients in the clinical study and also by observing for any clinical signs of overt thromboembolic complications (HA, stroke, pulmonary embolism, deep vein thrombosis and angina). Manifestation of any of these thromboembolic complications was attributed to DES therapy because patients were screened before the study entry. One study of 32 patients on 2 to 3 mg of DES for recurrent prostate cancer quantified FVII levels in a subset of 7 patients and reported elevated levels of FVII in all of the patients, despite the addition of warfarin (Klotz et al., 1999). The study reported thrombogenic side effects in 28 % of the patients. Other studies have looked at FVII levels in women taking oral estrogens for hormone replacement therapy and the results have been inconclusive with

some studies showing elevated levels (Kroon et al., 1994; Linberg et al., 1989), other studies reported a decrease in FVII activity (The Writing Group for Estradiol Clotting Factor, 1996), and still some studies showed no changes (Boschetti et al., 1991; Conard et al, 1995). Factor VII levels in our study were within the normal range, except one patient (Patient # 1) with a range of 1562 to 1617 ng/ml at effective DES levels of 1.38 (41 days) and 0.40 ng/ml (42 days) after commencing therapy. The FVII level for Patient # 1 did not seem to relate to the patient's DES level.

Other studies using 1 to 3 mg/day dose of oral DES have reported thromboembolic complications following oral DES therapy. In the study by Smith and colleagues (1998), 5 % (one patient) had a deep vein thrombosis; and Shahidi and colleagues (2001) reported 11 % of the patients experienced thromboembolic complications, without specifying the difference in events between the 1 mg and 3 mg doses. Klotz and colleagues (1999), in their study combined 1 mg of warfarin (an anticoagulant) to reduce the risk of thrombogenic complications, but still 28 % of the patients had a thrombogenic event. Ferrugia and colleagues (2000) added low dose aspirin (75 mg) and hydrocortisone at 40 mg (to reduce thrombogenic side effects) to oral DES therapy and 20.2 % of the study participants were diagnosed with thrombogenic toxicity. One patient (Patient #8) in our study with FVII level of 155.31 ng/ml had a thromboembolic event. Although his FVII levels were within the normal range, it is widely accepted that the risk for thrombosis may be related to the baseline levels even though the patients FVII was within normal. If patient's baseline level was very low, any significant increase in FVII levels from the baseline may elevate their clotting risk (Norris and Bonnar, 1997). This

patient's baseline FVII level was unavailable, so we could not confirm if the rationale was valid in this patient. Moreover, multiple clotting factors other than FVII are involved in modulating the clotting cascade. It requires better understanding of the levels of various clotting factors in the patient to explain the episode.

The most frequent side effect reported by most of the patients in this study is painful gynacomastia, and this was especially true for Patient # 11 with abnormally high DES plasma levels. Patient 11 reported voluntarily stopping his DES therapy in the presence of painful gynacomastia and resuming when the side effect subsides. Stopping his DES therapy will also cause his plasma DES levels to decrease easing his pain.

In this study, the 1 – 2 mg/day regimen of DES appears to be safe with all but one patient having FVII level > 500 ng/ml of normal level, and only one patient with a thromboembolic event attributable to the oral DES therapy.

5.2.4. Population Pharmacokinetic Analysis of Clinical Data

We employed the NONMEM software which has the ability to integrate sparse clinical data (Graves and Chang, 1990) to derive the population PK parameters of oral DES in our clinical study population. There is no information in the literature concerning the population PK parameters of DES in humans, so in order to derive the parameters for our study population; we estimated entry parameters for the NONMEM software. The population PK parameters of elimination rate constant (k), volume of distribution (V), and absorption rate constant (k_a) were derived by fitting the one-compartment model into the clinical data, because we did not have enough data time points to use other models that

require many more data time points. We also constructed diagnostic plots for the model used, and ideally the data should be equally distributed along the line of identity. The plots of residual versus predicted and weighted residual versus predicted values seemed to suggest that the estimated values from our model are reasonable. While the plots may attest to the viability of model, the plots cannot tell if the estimated PK parameters are the optimal for the population. This is partly because the estimated PK parameters are based on the entry values we input into the NONMEM database for the patients. **Nevertheless, this is the first attempt to our knowledge to derive the population PK parameters of DES in advanced prostate cancer patients on oral DES therapy**.

5.3. In Vitro Permeation of DES from Propylene Glycol Solution.

5.3.1. In Vitro Permeation of DES Solution through Excised Mouse Skin

We are interested in studying other routes of delivering DES that will potentially bypass the first pass liver metabolism and potentially decrease thrombogenic toxicities. Other estrogens like estradiol have been successfully delivered transdermally for systemic purposes, so we evaluated the transdermal permeation of DES solution (2 mg/ml) in propylene glycol through excised nude mouse skin. The permeability constant of DES in the solution was 1.8×10^{-6} cm/hr, which is comparable to two commercially available steroids cortisone (3.0×10^{-6} cm/h) and cortisol (1.0×10^{-5} cm/h), respectively (Barry, 1983).

This is the first report of the transdermal permeation of DES in a solution of propylene glycol through excised mouse skin. Knowledge of the delivery of a drug through excised skin, preferably human skin is very crucial for the development of transdermal therapeutic systems (Baker and Kochinke, 1989), so the significance of the data from this experiment cannot be overemphasized. Due to the difficulty in obtaining human skin, we employed the mouse skin which although more permeable than human skin, has been widely used to estimate the transdermal permeation of drugs (Huq et al., 1986).

In conclusion, we have established the permeability parameters of DES in a solution of propylene glycol through excised mouse skin. The transdermal delivery of DES is feasible based on the K_p of DES from the solution in propylene glycol which compares favorably with other formulations in the market; cortisone and cortisol. It is therefore feasible to deliver DES transdermally.

5.3.2. DES Content Uniformity in ODF-DES

The DES content of the ODF-DES film was analyzed for the amount and uniformity of DES. We wanted to validate the manufacturer's claim of weight, DES content potency and uniformity before proceeding to use the film in the animal PK studies. Drug content potency of 90 % above claimed value is widely considered accepted (Connors et al., 1986; Carstensen, 1990). The DES content of the films from our studies averaged 0.96 ± 0.07 mg with a range of 0.912 to 1.062 mg and is considered acceptable. The manufacturer's claim of film target weight of 50 mg and DES uniformly distributed in the film were also confirmed with variability of < 8 %.

5.4. Preclinical Pharmacokinetic Studies

The second hypothesis of this project is that the routes of administration of DES that bypasses liver metabolism may potentially increase DES bioavailability and decrease toxicities. To test this hypothesis, we studied the comparative PK and derived PK parameters of DES from ODF and oral suspension of DES in Sprague-Dawley rats.

5.4.1. Comparative Pharmacokinetics of ODF and Oral Suspension of DES

The two compartment model was the best fitted to the data, and hence was selected for the derivation of PK parameters. The plasma profiles comparing DES from ODF and oral suspension demonstrated that the AUC of DES from ODF is 2.7 times greater than that from the oral suspension of DES. The absorption rate constant (K_a), the clearance (CL) and volume of distribution (V2/F) were significantly different between the two formulations. The K_a and CL were significantly slower and the V2/F was significantly smaller for DES from ODF compared to the oral suspension. The significantly longer time to reach peak plasma concentration (T_{max}) of DES from ODF could be explained by the slow absorption rate (2.7 times slower) of DES from ODF as compared to the oral suspension of DES. No significant differences were observed in peak plasma concentration (C_{max}) and the terminal half-life (T_{β}) between the two formulations.

The slow absorption and thus longer T_{max} of DES from the ODF may be explained by a number of factors: (a) the excipient make-up of the film. The excipient making up the ODF will play a crucial role on how and when the film will dissolve to release DES embedded in the film matrix (b) the physical nature of the buccal membrane, the

presence of saliva (or liquid) will facilitate the dissolution of the film and subsequent release of DES, and (c) other factors like the permeability difference between the GI tract and the buccal cavity can be modified by adjusting the composition ODF. The increase in DES bioavailability (2.7 times) from ODF relative to the oral suspension can be attributed to the decreased clearance of DES from ODF-DES. The decreased clearance is due to the decrease in hepatic metabolism of DES from ODF. DES and other estrogens are extensively metabolized in the liver after oral administration, and this metabolism has the consequence of deactivating the drug and decreasing the bioavailability of estrogens (Shifren et al., 2008).

There are currently no available studies describing the complete PK parameters of DES in animals including rats, but a few studies have looked at some aspects of the disposition of DES in other animal species. Mroszczak and Riegelman (1975) evaluated the disposition of DES after intravenous and duodenal administration in the rhesus monkey. Their conclusion among other statements included that (a) the two compartment model was the best fit to describe the disposition of DES, (b) bioavailability of DES after duodenal administration was very low, and (c) that DES was rapidly metabolized and excreted after IV administration (Mroszczak and Riegelman, 1975). In another study (Abramson and Miller, 1982) to evaluate oral DES disposition in dogs, the authors also concluded that a two compartment model was the best fit to describe DES pharmacokinetics in dogs. The study reported two PK parameters of DES in dogs, an initial distributive half-life of just less than 1 hour and a terminal elimination half-life of 24 h dependent on enterohepatic recycling. The terminal half-life was said to decrease in

their study when the dogs were feed cholestyramine to interrupt enterohepatic recycling. The authors concluded that DES undergoes enterohepatic recycling and that the terminal half-life of DES is dependent on the amount of drug in the enterohepatic "compartment" for recycling. None of the above studies was carried out in a way to permit direct comparison with our work. It is usually difficult to compare PK parameters across different animal species because the disposition of drugs will be different from species to species. In our study, the two compartment model fitted better to the data compared to the one compartment model. The T_{β} from ODF and oral suspension of DES (12.66 ± 7.82 h versus 16.11 ± 3.50 h) did not show any statistical significant difference.

Oral dissolving film is a new, novel delivery system, which is still being perfected to deliver systemic drugs through the mucosa lining of the oral cavity. Most of the available studies on ODF are in the evaluating stages, studying the in vitro performance of polymers and optimal conditions to design the best film (Gohel et al., 2009; Kunte and Tandale, 2010; Garsuch and Breitkreutz, 2010).Two studies by the same group have designed and compared the PK of ODF and oral administration of drugs in rats. In the first study by Nishimura and colleagues, the PK parameters of ODF-prochlorperazine and oral solution of prochlorperazine were compared. They reported no statistically significant difference between the two formulations (Nishimura et al., 2009). The second study Shimoda and colleagues also reported no statistically significant difference between oral thin film of dexamethasone and oral dexamethasone suspension (Shimoda et al., 2009). Because of the different excepient make-up of the ODF films, the different conditions that the films were applied to the buccal cavity (instilling water or not when

applying film), and the different disposition profiles of DES, dexamethasone and prochloperazine, a direct comparison of the three studies would not be rational. The dissolution and subsequent absorption of drug from ODF depend on the excepient that make-up the film and the conditions under which the films are applied to the oral cavity. While the excepient for both studies are reported, the excepient used in the present study has not been released due to pending patents. The manufacturer of the ODF-DES has coded the excepients (ODF Carrier-611, Bio-FX-200, Bio-FX-115, Bio-FX-215, Bio-FX-243, and Bio-FX-301), making it difficult to compare with other excepients. Also, before the administration of ODF in both studies, water was instilled in the oral cavity of the rats to ease the dissolution and release of the drugs from the film, which is not the case with our study. We are interested in delivering DES using ODF-DES to bypass the extensive metabolism of DES in the liver (Metzler, 1981). This extensive metabolism, leading to the formation of many metabolites including DES monoglucuronide has the potential of decreasing DES bioavailability and increasing toxicity. We showed that the bioavailability of ODF-DES was 2.7 times greater than that from the oral suspension. We believe this increase bioavailability can be explained by the significant decrease in CL and V2/F of DES from ODF-DES compared to oral DES suspension. The decrease CL may be as a result of the reduced exposure of DES from ODF-DES for liver metabolism. The significant implication of this increase in bioavailability is that less DES from ODF-DES is needed to attain the same level of DES exposure as from the oral suspension. The dose reduction, while maintaining the therapeutic concentration is desirable because less DES dose may potentially result in decreased thrombogenic side effects.

In conclusion, **this is the first study to determine all the parameters of oral DES in any animal species**, and moreover, **it is the first study comparing the PK of DES from ODF-DES (a novel DES formulation) and oral DES suspension**. The study clearly establishes the merit of ODF-DES over oral DES suspension by establishing the increased bioavailability of DES from ODF-DES compared to the oral suspension.

5.4.2. DES Concentration in the Liver after Multiple Doses of ODF and Oral DES Suspension

The concentrations of DES in the liver after the administrations of DES from ODF and oral suspension of DES for 7 days were evaluated. This step was necessary in order to determine if the increased bioavailability of DES (2.7 times) from ODF-DES resulted in significant accumulation of DES in the liver of the study rats as compared to the oral suspension.

The liver has been identified as the main site for DES disposition after DES administration (Engel et al., 1976; Huber et al., 1972). Shah and McLachlan (1976) reported significant accumulation of DES in the liver of pregnant mice after the IV administration of DES. The present study did not find any significant difference in the concentrations of DES in the liver of rats after the administration of DES from ODF and oral suspension for 7 days. Two possible reasons may explain this finding: (a) DES in the liver is converted to DES conjugated products. This suggestion is supported by the findings of Shah and McLachlan, (1976) which reported that about 80 % of DES in the liver is converted to DES conjugated products, and (b) significant amount of DES from

ODF-DES avoids the liver first pass metabolism, which will decrease DES exposure in the liver despite the high plasma concentrations.

In conclusion, ODF-DES increases the bioavailability of DES without selectively increasing the concentration of DES in the liver of study rats, compared to the oral DES suspension.

5.5. PK/PD Studies of DES from ODF and Oral Suspension of DES

5.5.1. Relationship of Fibrinogen and AT-III Concentrations and Changes after Single Dose of ODF and Oral DES Suspension

The concentrations and AUC of DES from ODF were 2.7 times greater than those from the oral DES suspension, and we wanted to evaluate if the increased AUC of DES from ODF had impacts on the two key clotting proteins, fibrinogen and AT-III. Increase levels of fibrinogen may indicate potential increased risk of clotting, while increase levels of AT-III may indicate increased fibrinolysis or decreased clotting. DES like other estrogens has been shown to have profound effects on clotting proteins in humans. In rats, DES has also been shown to affect AT-III and fibrinogen levels in the plasma. In a study conducted by Owens and Cimino (1985), DES pellets (5 mg of DES) were implanted subcutaneously in rats and plasma samples were collected for fibrinogen and AT-III quantifications. They reported a significant decrease in the levels of AT-III, but no significant changes in the fibrinogen levels. This study even though did not compare two formulations of DES; it gives an idea of the effect of DES on clotting factors. Our study which compared levels of fibrinogen after the administration of DES from ODF and oral

suspension, did not find any significant difference in fibrinogen levels between the two formulations, except at the 24 h time point, when there was a significant increase in fibrinogen levels from the treatment with ODF-DES formulation. With respect to AT-III levels, there was no significant difference between the ODF and oral DES suspension. Changes in fibrinogen and AT-III between the two formulations showed significant increases in fibrinogen at the 24 h and 30 h and in AT-III at the 30 h time-points, respectively. These observed increases in fibrinogen and AT-III levels from the ODF-DES formulation occurring towards the end of the study was investigated further with a multiple dosing protocol for both ODF and oral DES suspension lasting 7 days.

The observation that both the change in fibrinogen and AT-III levels from ODF-DES increased significantly at the 30 h time point is interesting, due to the fact that increase fibrinogen may potentially increase clotting risk, while increased AT-III decreases clotting risk. This observation led us to suggest that at the 30 h time point, any potential increase risk of clotting from increased fibrinogen may be mitigated by the increase in AT-III levels.

5.5.2. Relationship of Fibrinogen and AT-III Levels after Multiple Doses of ODF and Oral Suspension of DES

Following the single dose protocol for ODF and oral suspension of DES, significantly increased fibrinogen and AT-III levels seemed to occur in the later sampling times (24 h and 30 h for fibrinogen, and 30 h for AT-III) of the ODF-DES formulation, compared to the oral DES suspension. The multiple dose protocol was designed to evaluate if the observation will carry over to long term dosing. Data suggest that the increased in levels

of fibrinogen (at 24 and 30 h) and AT-III (at 30 h) observed for from the single dosing study did not carry over to the multiple dosing study lasting 7 days. There was no significant difference in levels of fibrinogen and AT-III between days 5 to 8 that samples were collected between the ODF and oral suspension of DES.

Owens and Cimino (1985) reported statistically significant decrease in AT-III levels on day 15^{th} in the plasma of rats treated with subcutaneous DES pellet for 14 days, but observed no changes in fibrinogen levels. Another study, using estradiol injected to the tail vein of rabbits did not find any significant difference between levels of fibrinogen before and Day 10 of the estradiol administration. Both of these studies collected samples only on the last day of the studies, while our study collected samples for 4 of the 8 days that the study lasted. Collecting samples for multiple days will give a better idea of and trends in the levels of the study proteins. This study did not find a significant difference in both AT-III and fibrinogen levels in rats between the ODF and oral suspension of DES administration, compared to the controls.

In conclusion, ODF-DES significantly increased DES concentrations and AUC (2.7 times), but no significant difference in the levels of the two key clotting proteins (fibrinogen and AT-III) were observed, as compared to the oral DES suspension formulation. An estimate of clotting risk using these key proteins as biomarkers suggested that there will be no difference in clotting risk between the ODF and oral suspension of DES in rats.

5.5.3. Correlation of Mean Absolute Percent Changes in Fibrinogen and AT-III with DES AUCs after Single Dose (1 mg) of ODF and Oral Suspension of DES.

The finding that ODF-DES significantly increased systemic exposure of DES compared to the oral formulation prompted us to correlate the DES exposure with mean absolute percent changes of AT-III and fibrinogen for each animal. Data from this study revealed that there was no significant difference in the mean absolute percent changes in AT-III and fibrinogen between the treatment of ODF-DES and oral suspension of DES on a same dose basis. There are presently no studies correlating systemic estrogen exposure and changes in AT-III and fibrinogen. Therefore, this study contributed to the knowledge that increased systemic exposure of DES from ODF-DES resulted in no corresponding increase of percent absolute changes in AT-III and fibrinogen.

This observation that the mean percent changes in fibrinogen and AT-III between the two formulations are not different reinforces our suggestion that, ODF-DES increases the exposure of DES in systemic circulation without posing additional increased risk of potential clotting as compared to oral DES suspension.

5.5.4. Correlation between Fibrinogen and AT-III Absolute Percent Changes after Single Dose of ODF and Oral Suspension of DES

The goal here was to determine if a relationship between the plasma levels of fibrinogen and AT-III exist after the administration of a single dose of ODF and oral suspension of DES. To achieve this goal, we correlated the absolute percent changes in fibrinogen with AT-III after the administration of both formulations to rats. We found a strong positive correlation (R^2 = 0.78) between fibrinogen and AT-III levels after the administration of the

oral suspension of DES, but not with ODF-DES formulation. While it is clear that both formulations have impacts on fibrinogen and AT-III levels, the significance of this correlation with oral suspension only, but not with ODF-DES needs further investigations. It is worth of a note that one additional advantage than increasing DES exposure of ODF-DES is the decreased of DES exposure in the liver. Both fibrinogen and AT-III are synthesized in the liver; therefore the decreased exposure of DES from ODF-DES to the liver may partly explain the lack increase of AT-III corresponding to the high concentration and exposure in plasma.

5.5.5. Exposures of DES, Fibrinogen and AT-III after Single Dose (1 mg) of ODF-DES and Oral Suspension of DES

We had shown that levels of fibrinogen and AT-III were not significantly different after the administration of DES from ODF and the oral suspension. The significance of this study was to further investigate if the increased bioavailability of DES from ODF-DES will result in significant differences in the AUCs of fibrinogen and AT-III from ODF-DES formulation as compared to the oral DES suspension. A comparison of the AUCs of fibrinogen and AT-III did not reveal any significant difference between the two formulations, despite the increase (2.7 times) in the AUC of DES from ODF-DES compared to the oral suspension.

There has been no studies comparing the systemic exposure (AUC) of fibrinogen and AT-III after the administration of DES, and more especially, there are no studies comparing the AUCs of the two proteins with DES from ODF and oral suspension of DES. This study further establishes the merits of ODF-DES and reinforces our claim that

ODF-DES increases exposure of DES in systemic circulation, but with no additional potential risk to clotting as measured by fibrinogen and AT-III levels. The significance of this finding is two-fold as follows: (a) a lower dose of DES from ODF-DES may be tailored with ODF to achieve a comparable concentration from a higher dose of oral DES, and (b) the lower dose of DES from ODF-DES may well decrease potential side effects of DES experience from a higher dose of oral DES.

Chapter 6. Summary

6.1. LC/MS/MS Assay

Oral DES has been around since the 1940s, but surprisingly there are no reliable and robust commercial assays available to quantify DES in human plasma. A highly sensitive, reproducible and efficient LC/MS/M assay method to quantify DES in human, and rat plasma and rat liver tissue was developed and validated. The assay was linear in the range of 0.78 – 100 ng/ml, and with a within and between day variability below the FDA recommended limits. The assay was employed to quantify DES in human, as well as rat plasma and liver tissues.

6.2. Phase II Clinical Trial of DES in Recurrent Prostate Cancer Patients

The human plasma samples used for this study were obtained from 19 patients on oral DES therapy for recurrent prostate cancer, in a clinical phase II trial at the University of Texas MD Anderson Cancer Center in Houston, TX. Patients received 1 – 2 mg/day dose of oral DES capsules, as the study medication.

6.2.1. Patient DES Concentrations

DES levels were detected in all the patients in this clinical study. DES plasma levels for the study ranged between 0.11 and 4.29 ng/ml, with a mean concentration of 1.57 ng/ml for all but one patient, (Patient # 11) whose levels were 16.27 ng/ml. This patient was on

two herbal supplements with estrogenic activity, which competed for hepatic metabolizing enzyme for DES and may have caused the abnormal increase in his DES plasma levels. DES showed considerable variability within and between patients in this clinical trial. They may be 3 reasons for this variability: (a) this patient population represented very sick patients, (b) compliance to study medication could not be verified, and (c) some patients were on multiple medications including herbal supplements.

6.2.2. Response to DES Therapy by PSA Levels

All patients in this study for which baseline PSA levels were available (16 patients) had a response of reducing PSA to oral DES therapy (response defined as difference in PSA level from baseline to PSA nadir). The response ranged from 17 % to 99 % and the average DES concentration for this response range is 1.21 ng/ml. In this study with 2 mg/day regimen, 63 % of patients had a significant PSA reduction of 50 % or greater and the mean DES concentration for this response is 1.23 ng/ml. We suggested therapeutic drug monitoring for patients on DES therapy for recurrent prostate cancer with a DES therapeutic goal of 2 ± 1 ng/ml.

6.2.3. Correlation of DES Concentrations and FVII Levels

DES toxicity was evaluated by quantifying FVII levels for the 19 patients in this clinical trial. The FVII levels in this study population were within the normal range (< 500 ng/ml), except for Patient #1 whose levels were between 1562 to 1617 ng/ml. One patient (6 %) was diagnosed with unstable angina attributable to his DES therapy. Patient #11 with abnormally high levels of DES did not have any thrombogenic event, but complained of frequent painful gynacomastia.

6.2.4. Population Pharmacokinetic Analysis of Clinical Data

The population PK parameters of oral DES were derived for this study population using NONMEM software version 7. The population K, V and K_a of DES from the clinical study population are 1.49 $h^{-}1$, 795 L and 9.97 h^{-1}. The population clearance (CL) and $t_{1/2}$ were calculated using the PK equations; CL = KV and t ½ = 0.693/k, giving 1,184.55 L/h and 0.47 h, respectively. This is the first study to derive the population PK parameters of DES.

6.3. In Vitro Permeation of DES from Propylene Glycol Solution

6.3.1. In Vitro Permeation of DES from Solution through Excised Mouse Skin

We successfully demonstrated that the transdermal delivery of DES is feasible, by deriving the permeation parameters of DES in a solution of propylene glycol. The Kp of DES in this solution (1.8 x 10^{-6} cm/hr) was comparable to the Kp (3.0 x 10^{-6} cm/h and 1.0x10-5 cm/h) of cortisone and cortisol, respectively, two steroids that are in the market.

6.3.2. DES Content Uniformity in ODF-DES

We validated the manufacturer's claim of DES film target weight of 50 mg/film and drug content potency of 1 mg with uniform distribution. The drug content potency of the ODF-DES film was 96 % of the manufacturer's claim and considered acceptable.

6.4. Preclinical Pharmacokinetic Studies

6.4.1. Comparative Pharmacokinetics of ODF and Oral Suspension of DES

The two compartment model was the best fit to the data, and hence was selected to derive the PK parameters of DES from ODF and oral suspension of DES. The relative bioavailability of DES from ODF-DES was 2.7 times compared to the oral suspension of DES. The study also determined the pharmacokinetics parameters of DES from ODF-DES and oral suspension of DES. The significant increase in DES bioavailability with ODF is due to the reduction in overall clearance (CL), and peripheral volume of distribution, V2/F. The study clearly established the merit of ODF-DES over oral DES suspension by establishing the increased bioavailability of DES from ODF-DES compared to the oral suspension.

6.4.2. DES Concentration in the Liver after Multiple Doses of ODF and Oral DES Suspension

There was no significant difference in the concentrations of DES in the liver of study rats from ODF (9.66 ± 4.63 ng/ml) and oral suspension (11.10 ± 7.50 ng/ml). This study confirmed that the 2.7 fold increase in the bioavailability of DES from ODF did not result in significantly higher DES levels in the liver of ODF-DES animals.

6.5. PK/PD Studies from ODF and Oral Suspension of DES

6.5.1. Relationship of Fibrinogen and AT-III Concentrations and Changes after Single Dose of ODF and Oral Suspension

Two key clotting proteins, AT-III and fibrinogen were quantified after the administration of 1 mg of DES from ODF and oral suspension formulations. The levels were compared between the formulations and there was no significant difference in the levels of fibrinogen except at the 24 h time point when levels from the ODF-DES formulation were significantly greater compared to the oral DES suspension formulation. We observed a similar trend for AT-III levels, which were significantly greater at the 30 h time point with ODF-DES. This late increase in the levels of both proteins for the ODF-DES formulation was investigated further with a multiple dosing protocol for ODF and oral suspension of DES.

6.5.2. Relationship of Fibrinogen and AT-III Levels after Multiple Doses of ODF and Oral DES Suspension

The multiple dosing protocols were initiated to investigate if the late increase in levels of AT-III and fibrinogen from the single dose study with ODF-DES will carry over when DES was dosed for multiple days. Fibrinogen and AT-III levels from ODF-DES and oral suspension of DES did not show any difference after the multiple dosing study lasting 7 days. We concluded that ODF-DES increases significantly the concentration and AUC of DES, compared to the oral formulation without resulting in additional increase risk of thrombosis as measured by fibrinogen and AT-III.

6.5.3. Correlation of Mean absolute Percent Changes in Fibrinogen and AT-III with DES AUCs after Single Dose (1 mg) of ODF and Oral Suspension of DES.

There was no difference in percent changes in fibrinogen and AT-III, despite the increase AUCs of DES from ODF-DES, as compared to the oral solution of DES.

6.5.4. Correlation between Fibrinogen and AT-III Absolute Percent Changes after Single Dose of ODF and Oral Suspension of DES

We found a positive correlation (R^2 = 0.78) between absolute percent changes of fibrinogen and AT-III after the administration of the oral suspension, but not with ODF-DES formulation. While it is clear that both formulations have impacts on fibrinogen and AT-III production, the significance of this correlation between the two proteins with oral suspension, but lack of such a correlation with ODF-DES needs further investigations.

6.5.5. Exposures of DES, Fibrinogen and AT-III after Single Dose (1 mg) of ODF-DES and Oral Suspension of DES

We investigated the relationship between the significantly higher AUC of DES from ODF-DES and its effects on AUCs of fibrinogen and AT-III, as compared to the oral suspension of DES.

No significant differences in the AUCs of fibrinogen and AT-III between the two formulations ($p > 0.09$ for fibrinogen AUCs, and $p > 0.98$ for AT-III AUCs) were observed.

This finding reinforces our claim that ODF-DES increases exposure of DES in systemic circulation, but with no additional potential risk to clotting as measured by fibrinogen and AT-III levels.

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