Diethylstilbestrol Nanosuspensions for Prostate Cancer: Subcutaneous Sustained Release Formulations and Pre-clinical PK/PD Evaluations

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Dedicated to my Grandparents and Parents

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Abstract

Prostate cancer is the most common type of cancer and the second leading cause of cancer related deaths in men in the United States. According to the National Cancer Institute (NCI), 238,590 new cases with deaths of 29,720 from prostate cancer have been estimated for 2013.

Diethylstilbestrol (DES) was mainstay treatment for metastatic and castrate resistant prostate cancer for half a century. In the mid-1980s its use was drastically reduced because of its severe cardiovascular toxicity and thromboembolic complications such as deep vein thrombosis, pulmonary embolism and heart attack. At the same time, luteinizing hormone releasing hormone (LHRH) agonists were found to have similar efficacy profile to that of DES but with significantly lower toxicities. Thus LHRH agonists replaced DES. But, there has been a renewed interest in the treatment with DES since it has been shown to be efficacious in both androgen dependent prostate cancer and castrate resistant prostate cancer. Also, LHRH agonists with extensive use have started to show side effects.

We are interested in applying pharmaceutical formulation approach to reduce the complications of DES while sustaining its systemic exposure. This would be a significant advancement for the DES therapy against prostate cancer. The side effects of DES have been associated with its hepatic exposure which is

hypothesized to alter the coagulation cascade. The conventional oral DES because of its first pass effect led to a high hepatic exposure and significant toxicities. Thus, our aim was to develop a subcutaneous sustained release nanosuspension formulation of DES, which would circumvent the first pass effect of DES, potentially reducing the thromboembolic and cardiovascular complications and maintaining or improving the efficacy of DES by sustaining its release.

Subcutaneous nanosuspension formulations of various particle sizes of 160 nm (NS-160), 300 nm (NS-300) and 500 nm (NS-500) were successfully formulated and extensively characterized. The *in vitro* release study demonstrated a sustained release of DES from the nanosuspensions as compared to that of the DES co-solvent preparation. Also, the *in vitro* release of DES was inversely dependent on the particle size of the nanosuspensions. The pharmacokinetic studies in the Sprague Dawley rats demonstrated that as compared to the oral DES suspension and subcutaneous co-solvent (solution), the DES nanosuspensions significantly sustained the release of DES and prolonged the circulation of DES. In addition, the DES nanosuspensions showed a longer elimination half life and slower clearance than the oral DES suspension. The DES nanosuspensions, with higher systemic exposure, showed a 20 times reduction of the hepatic exposure as compared to the oral DES suspension on the same dose basis. The subcutaneous DES nanosuspensions showed

significantly lesser changes in the levels of the coagulation factors such as fibrinogen and anti-thrombin III and the rat blood clotting time as compared to those of oral DES suspension in the short term and long term toxicity studies. These effects could potentially decrease the cardiovascular toxicity and thromboembolic complications of DES. In the efficacy study in a prostate cancer tumor mouse model, the DES nanosuspension (NS-160), exhibited significant tumor suppression as compared to the oral DES suspension on the same dose basis. Also, the prostate specific antigen (PSA) levels elevated significantly with the progression of the tumors for treatment with oral DES but were maintained stable for the NS-160 treatment group over a period of 28 days.

In conclusion, this study for the first time successfully demonstrated the viability of formulating a subcutaneous nanosuspension formulation of DES that achieved sustained release of DES, low hepatic exposures and potential reduction of oral DES complication with enhanced efficacy. We anticipate that the DES nanosuspension could be a lead candidate for further clinical trials on DES for its use against prostate cancer.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ACN	Acetonitrile
ADPCa	Androgen dependent prostate cancer
ANOVA	Analysis of Variance
ATIII	Antithrombin III
AUC	Area under the curve
CRPC	Castrate resistant prostate cancer
DES	Diethylstilbestrol
DMSO	Dimethyl sulfoxide
FBG	Fibrinogen
IACUC	Institutional Animal Care and Use Committee
IS	Internal standard
LLOQ	Lower limit of quantification
MT	17-α-methyl testosterone
NS	Nanosuspension formulation
NS-160	Nanosuspension with particle size of 160 nm

NS-300	Nanosuspension with particle size of 300 nm
NS-500	Nanosuspension with particle size of 500 nm
PBS	Phosphate buffered saline
PEG 400	Polyethylene Glycol 400
PK	Pharmacokinetics
PD	Pharmacodynamics
PSA	Prostate Specific Antigen
RES	Reticuloendothelial system
SCID	Severe combined immunodeficiency

Chapter 1 Literature Review

1.1 Introduction to Prostate Cancer

Prostate cancer is defined as the cancer that forms in the tissues of the prostate which is a gland located below the bladder and in front of the rectum in men. It is the most commonly diagnosed cancer in men in the US (1). According to the 2013 statistics from the National Cancer Institute, new cases of 238,000 have been estimated for prostate cancer making it the most common type in men in the US. It has a risk of about 1 in 6 with total estimated deaths of about 30,000. Prostate cancer is considered as one of the most enigmatic cancers. It grows slowly with the risk of developing clinically detectable cancer is about 8%; lifetime risk of prostate cancers deaths is approximately 3%. Even after such an impact on the society on account of prostate cancer, little is known about the fundamental causes. The main risk factors (2) for prostate cancer are age, race/ethnicity and family history. The other factors under consideration are international immigration (3), diet containing high saturated fats and prostate cancer (4), endogenous hormones in relation to levels of testosterone (5) and insulin-like growth (IGF-1) in the circulation (6), genetic polymorphism, low exposure to sunlight and other environmental factors.

1.2 Stages of Prostate Cancer and Treatment Options

1.2.1 Localized (Stages I and II) Prostate Cancer

Localized prostate cancer is the most commonly diagnosed type (7) of prostatic carcinomas. The T1-T2, NxM0 or Stage 1-2, localized prostate cancer is believed to be confined to the prostate gland. T1 tumors are typically those with a normal Digital Rectal Examination (DRE) which are typically detected by changes in the prostate specific antigen (PSA) levels and surgical biopsy. T1a and T1b are defined as histologic findings of less than and greater than 5 percent of prostate tissue resected during transurethral resection of the prostate (TURP), respectively. T1c is defined as non-palpable tumor detected by elevated PSA levels. The T2 stage which is divided into T2a and T2b is divided into one half of the lobe and more than one half of the lobe but less than one lobe, respectively. But due to the limited sensitivity of pretreatment evaluations, the disease can progress out of the prostate gland. The measure of aggressiveness of the prostate cancer is the Gleason Histologic Score which ranges from 2-10. In the Gleason score measurement, prostate tumors with a score of less than 6 are considered potentially indolent, score of 7 are less aggressive and a score of 8-10 is considered to be the most aggressive of the tumors (8). One of the currently recommended risk classification is, low risk: PSA ≤ 10 ng/mL, Gleason score ≤ 6, and clinical stage T1c and T2a; intermediate risk: 10 < PSA ≤ 20 ng/mL, or

Gleason score 7, or clinical stage T2b; high risk: PSA > 20 ng/mL or Gleason score 8-10 or clinical stage T2c. On an average, PSA levels of between 4 - 10 ng/mL and in some cases between 2.5 – 4 ng/mL indicate high risk of prostate cancer. However, some of the recent studies have shown that some people with PSA levels < 4.0 ng/mL have prostate cancer and many men with high levels of PSA do not have prostate cancer (9). However, according to the National Cancer Institute, PSA is still a widely used test to detect the chances of developing prostate cancer.

There are many treatment options for prostate cancer each having its pros and cons. Some of them include:

Radical retropubic or perineal prostectomy (RP): Complete surgical removal of prostate gland with seminal vesicles and sometimes pelvic lymph nodes.

External-beam radiation (EBRT): Multiple doses of radiation from an external source applied for several weeks. Proton therapy is also used.

Brachytherapy: Radioactive implants are injected using radioactive guidance. EBRT or androgen deprivation therapy is sometimes used in conjunction with brachytherapy.

Active surveillance (watchful waiting): It involves active plan to postpone intervention. It may involve monitoring of the prostate tumor based on DRE and PSA levels followed by biopsy.

Androgen Deprivation Therapy: Medications or surgical removal of testis in order to block or lower circulating androgens.

The other therapies used are cryoablation, Laproscopic (LRP) and Robotic Assisted Radical Prostatectomy (RLRP) and High-Intensity focused ultrasound therapy (HIFU).

1.2.2 Castrate Resistant Prostate Cancer (CRPC)

In most tumor progressions, genetic modification is the main factor. It is true even in case of the development of Castrate Resistant Prostate Cancer (10). Cher et al., also suggested that ‘untreated metastatic tumors contain the bulk of chromosomal aberrations necessary for recurrence to occur during androgen deprivation’ (11). The prostate tumor progression is shown in Figure 1.

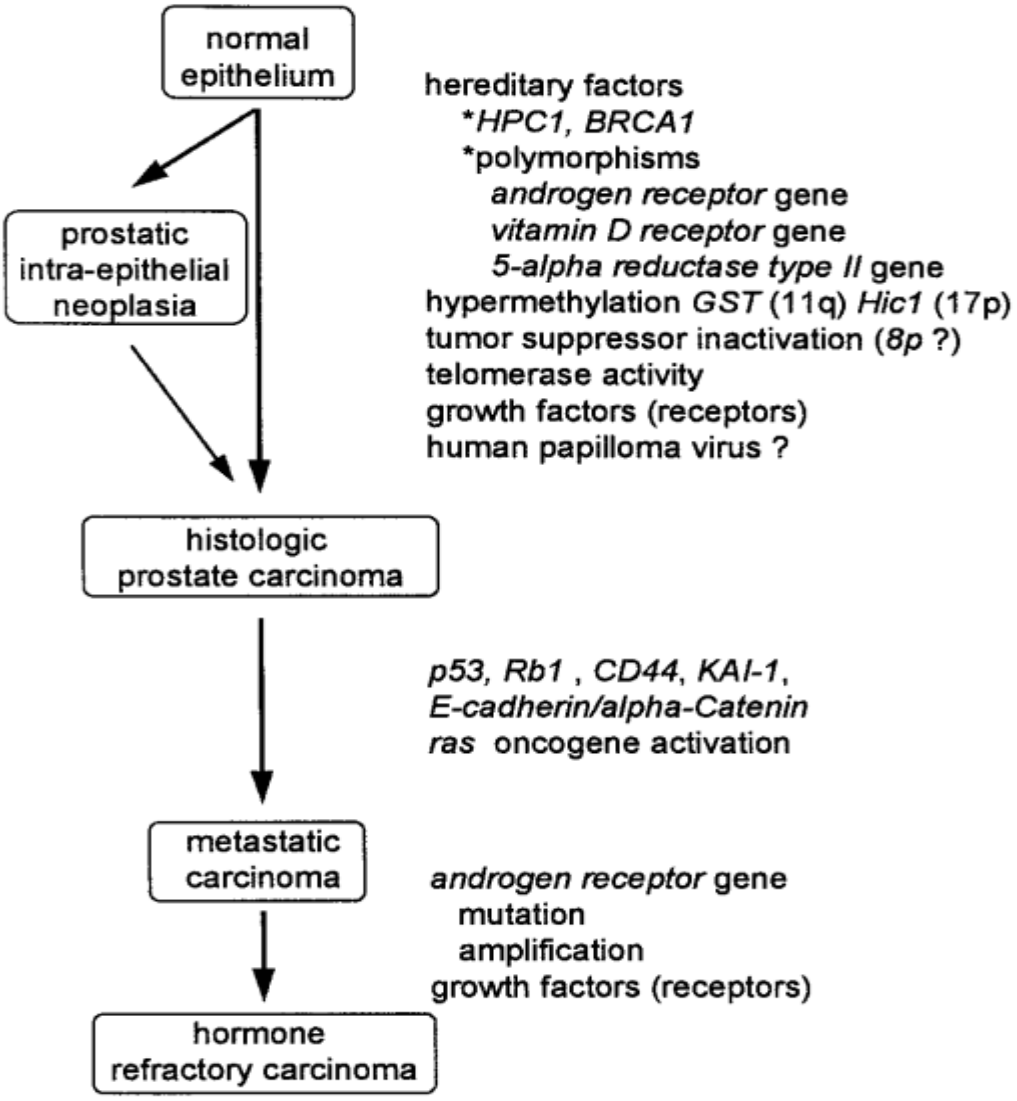


Figure 1: Summary of Factors Potentially Involved in the Subsequent Stages of Prostate Carcinoma Progression (10)

Some of the mechanisms (Figure 2) that have been proposed leading to CRPC include (12):

a) Type 1, Hypersensitivity Pathway:

Prostate cancers that use this mechanism are not totally androgen independent. Their responses still depend on androgen receptor (AR) and androgen, but they have lower threshold for androgens. This pathway maybe caused by:

AR amplification: Tumor cell proliferation occurs because of expression of AR itself. Approximately 30% of tumors that become androgen independent after ablation therapy have amplified the AR gene, resulting in increased AR expression. On the contrary, none of the primary tumors before androgen ablation had increased levels of AR gene amplification (13) (14).

Increased AR sensitivity: This is the second hypersensitive mechanism which results in a high level expression of the AR, increased stability and enhanced nuclear localization of AR in the recurrent prostate carcinoma (12). This particular mechanism was first shown in animal models where the androgen-dependent prostate cancer was transitioned into CRPC (15).

Increased Androgen Levels: The third proposed mechanism of hypersensitivity involves prostate cancer cells themselves producing androgens (16). This mostly occurs by increased rate of conversion of testosterone to dihydrotestosterone

(DHT) by increasing the 5-alpha reductase activity (12). Supporting this mechanism it has been found that after total androgen ablation, the decrease in the levels of testosterone is by 95 % but the levels of DHT decrease only by 60% in the prostate tissue (17). Also, in certain ethnic groups higher levels of 5-alpha reductase have led to higher incidence of prostate cancer (18).

b) Type 2, The Promiscuous Pathway:

This pathway involves mutations that allow to circumvent the normal growth regulation by the androgens. These mutations allow the AR to be activated by non-androgenic steroid molecules in circulation as well as anti-androgens (19) (20) (21).

c) Type 3, The Outlaw Pathway:

'Outlaw receptors' are the steroid hormone receptors that are activated by ligand independent mechanisms (22). There is no androgen control over the growth and proliferation of tumor cells. This type of activation has been termed as outlaw activation (23).

The different types of outlaw pathway include: a) Growth-factor activated outlaw pathways (24), b) Receptor-tyrosine-kinase-activated outlaw pathways (25) and c) The AKT pathway (26).

d) Type 4, The Bypass Pathway

There is a mechanism by which the AR pathway is bypassed completely and majorly involves change or modulation of apoptosis and up-regulation of anti-apoptotic molecules that are often found in the CRPC tumor cells (27) (28). The bypass mechanism can also occur by neuroendocrine differentiation of the prostate cancer cells. The neuroendocrine cells secrete neuropeptides which can stimulate proliferation of the adjacent cancer cells (19).

Thus deep understanding of the various pathways, can lead to better evolutions of effective therapies against CRPC.

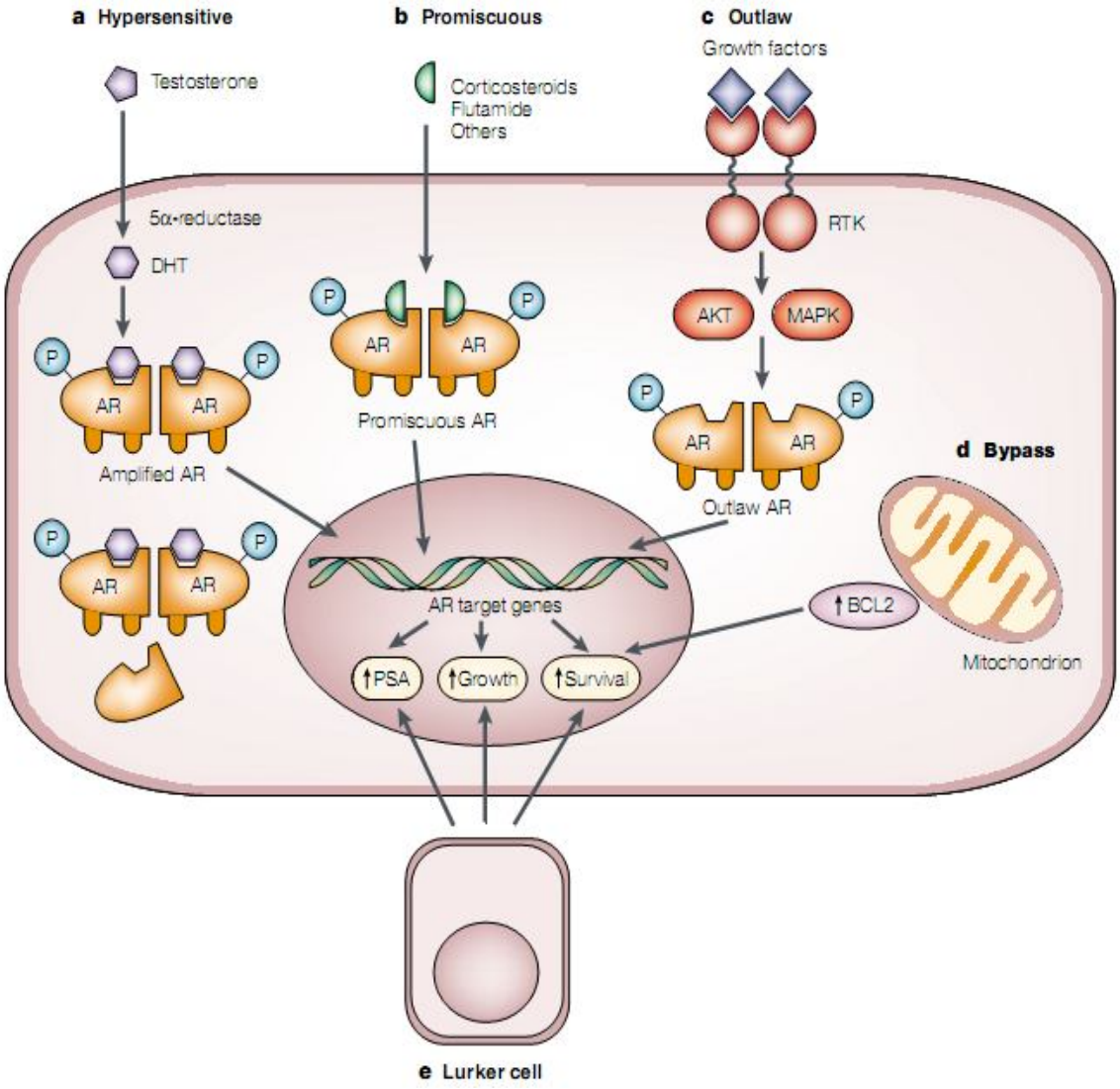


Figure 2: Possible pathways to Castrate Resistant Prostate Cancer (12).

1.3 Introduction to Diethylstilbestrol

1.3.1 History

Diethylstilbestrol (DES) is a synthetic nonsteroidal estrogen. It was first synthesized in 1938 at the University of Oxford. Its first report of synthesis was published in the Nature Journal (29) (30). In the 1940s, DES was approved by the US FDA for indications such as gonorrheal vaginitis, atrophic vaginitis, menopausal symptoms, and post partum lactation suppression to prevent breast engorgement (31) (32).

1.3.2 Use of Diethylstilbestrol for Prostate Cancer

Following the early investigative efforts of Huggins and Hodges in the early 1940s, DES became the mainstay in androgen deprivation therapy for prostate cancer (33). From the late 1950s to the early 1980s, estrogen therapy and mainly DES was most widely used chemo-hormonal agent against prostate cancer (34). In the series of experiments conducted by Huggins et al, it was demonstrated that castration and estrogen administration reduced the levels of acid phosphatase in patients with metastatic prostate cancer and dramatically improved the clinical effect in majority of the patients. It was also shown by Huggins et al., that DES was also effective against localized prostate cancer (33) (35). These studies cemented the place of DES and estrogens in the treatment of

prostate cancer. Bilateral orchiectomy and estrogen therapy remained the standard treatment of advanced prostate cancer for more than four decades (36). A clinical advantage of endocrine therapy was also noted when a study was conducted for survival rate of 47 patients treated with estrogens or bilateral orchiectomy were compared to the survival of many patients from the pre-endocrine time period (37).

In 1950, an important study conducted by Nesbit and Baum on 1818 prostate cancer patients, comparing retrospectively 947 patients treated with hormonal therapy and followed for three years to 795 followed in the pre-endocrine period concluded that patients who respond to endocrine therapy live longer and comfortably as compared to untreated patients. The hormonal treatment groups in the study were bilateral orchiectomy, DES at doses of 1 to 5 mg. orally, DES plus bilateral orchiectomy, sequential DES followed by bilateral orchiectomy and sequential bilateral orchiectomy followed by DES (38). Thus hormonal therapy with DES became the mainstay in the treatment for prostate cancer.

1.3.3 Decline of Diethylstilbestrol

In 1967, The Veterans Administration Cooperative Urological Research Group (VACURG), conducted studies on all patients with different stages of prostate cancer with 5 mg DES and placebo (39). There were two major conclusions from the studies: 1) hormonal therapy should be withheld until symptoms of metastatic

prostate cancer appear and 2) DES at 5 mg orally per day was associated with an excessive high cardiovascular mortality rate. This started a cascade of various studies which dealt various blows to the status of DES as the primary treatment of prostate cancer. In another study, three doses, 0.2, 1 and 5 mg, of DES were compared to placebo. It was found that 1 mg of DES had comparative efficacy to that of 5 mg in controlling stages T3 and M+ (Stages III and IV) of prostate cancer but 5 mg has excessive cardiovascular toxicity (40). Thus from these studies, the discrepancy between disease specific survival and overall survival was evaluated and recognized (41). The estrogen therapy achieved clinical responses and delayed the disease progression in 80 % of the patients but failed to improve the overall survival. The studies also revealed significantly higher risk of about 35 % of cardiovascular toxicity and thromboembolism in about 15 % of the patients. Thus the overall survival might not have been improved because of the toxicity of orally administered DES.

The European Organization for Research and Treatment of Cancer (EORTC) genitourinary group clinical trials indicated that a dose of 3 mg of DES was associated with significant cardiovascular toxicity. The continuation of the Veterans Administration Cooperative Urological Group (VACURG) studies concluded that 1 mg of oral DES had similar efficacy to that of the 5 mg daily dose of DES but was relatively safer (42). In this research, the authors commented that no form of endocrine therapy had proven to be better than the 1

mg daily dose of DES. Many clinical trials over a period of time indicated that a dose of 3 mg of DES is needed to obtain castrate levels of testosterone which in turn lead to the toxicity of DES. A proper dose escalation study should have been done initially to figure out that 1 mg of DES causes the same level of decrease in the testosterone levels but lower complications (36). But this was not done and DES and estrogen therapy was blacklisted.

A landmark study was published in 1984 which dealt a severe blow to the DES therapy for prostate cancer. The Leuprolide Study Group documented the results of 3 mg of oral DES versus leuprolide, which is a Luteinizing Hormone Releasing Hormone (LHRH) agonist (43). There was no significant difference in the suppression of testosterone, overall response rates and overall survival rates. But the patients receiving DES had higher thromboembolism, painful gynecomastia, nausea, vomiting and edema as compared to the LHRH agonist group. The authors thus inferred that leuprolide had similar efficacy to that of DES but lower cardiovascular toxicity and thromboembolic complications. This form of medical treatment revolutionalized and altered the trends in the treatment of prostate cancer (44).

Thus in summary, there was a decline in the use of DES because of the consistent cardiovascular toxicity and thromboembolic complications, and DES

was replaced by the LHRH analogues which is an alternative form of medical castration.

1.3.4 Thromboembolic Complications and Oral Diethylstilbestrol

Estrogen mediated coagulation has been ascribed to multiple abnormalities because of the orally administered DES. These abnormalities arise because of the hepatic exposure of DES. The liver is the main organ that is responsible for the synthesis of the proteins that are needed in the coagulation cascade. Thus the exposure of DES in the liver leads to various changes in the coagulation proteins thus leading to thrombosis and cardiovascular complications. Some of the effects include increases in factor VIII activity, induction of prothrombin activation, decrease in fibrinogen, increased resistance to activated protein C (APC) and decrease in the antithrombin III levels (45) (46). It is well known that exogenous estrogens greatly affect the liver metabolism which is directly or indirectly linked to cardiovascular disorders (47). It is found that only synthetic estrogens or high doses of natural estrogens given orally are capable of saturating the metabolizing capacity of the liver and result in a pharmacologic response (48) (49). This response leads to changes in the synthesis of liver derived proteins such as high and low density lipoproteins, various coagulation factors such as fibrinogen, Factor VIII and anticoagulants such as antithrombin III which may lead to elevated risk of hypertension, hypercoagulation and hyperlipidemia.

1.3.4.1 Coagulation Cascade

In the newer coagulation cascade, the thrombin is considered as the locus of the coagulation cascade. Both the intrinsic and extrinsic pathways are combined into one pathway. The figure 3 gives a schematic representation of the coagulation cascade. The coagulation cascade begins with the activation of tissue factor which binds to factor VII or VIIa. Factor VIIa activates Factor X which on activation forms factor Xa that converts prothrombin to thrombin. This generation of a small amount thrombin is sufficient to kick-start the coagulation cascade. The other factors get activated and in turn generate more thrombin. Thrombin plays a very important role in the activation of fibrinogen to form fibrin and hence the clot. Along with this, it also has platelet aggregation, protein C activation and endothelial effects amongst others (50). Antithrombin III is another protein that is synthesized by the liver and works as an anti-coagulant. It helps to dissolve thrombin and directly inhibits the coagulation cascade.

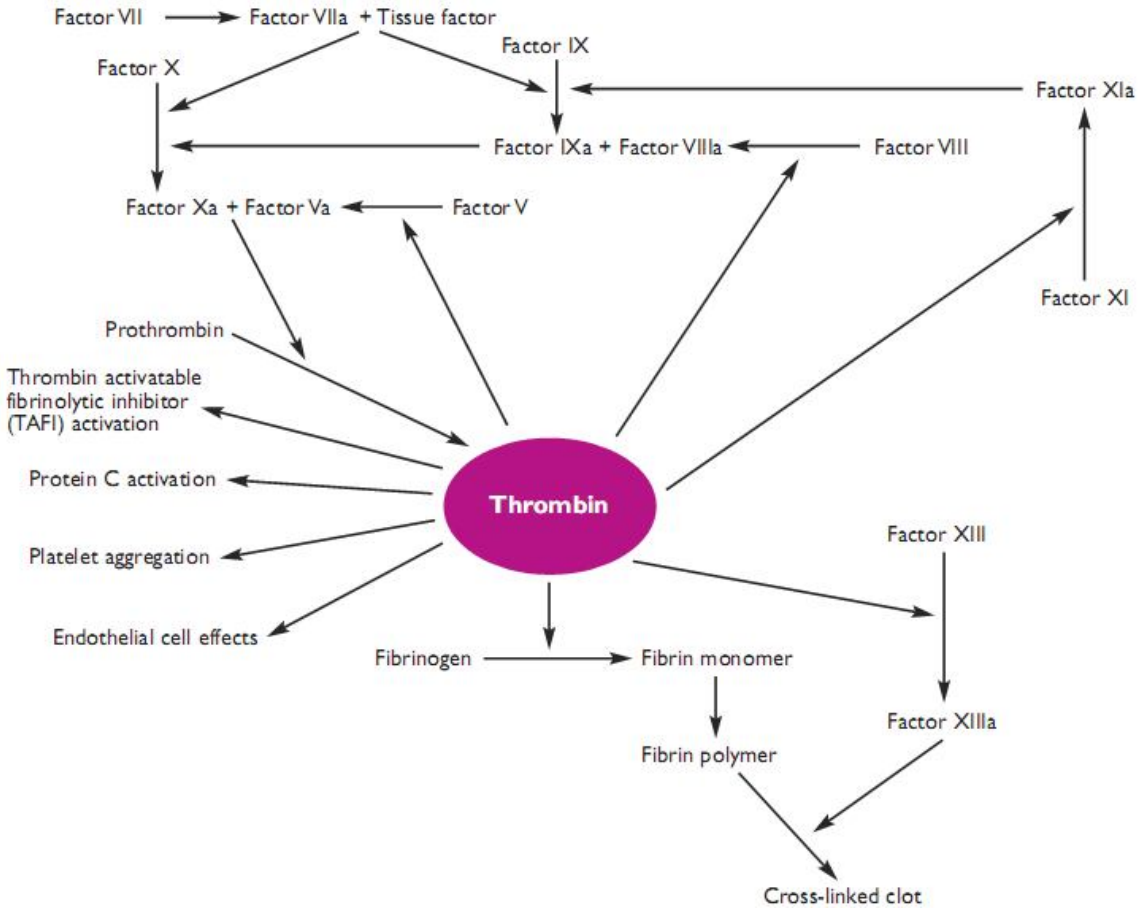


Figure 3: Newer Model of Coagulation Cascade (50).

1.3.4.2 Fibrinogen

Fibrinogen is called as the ‘ultimate substrate protein’ (50) in the coagulation cascade since it leads to the formation of fibrin and hence the clot. This is synthesized by the liver and circulates in the plasma at levels of 2-5 g/l. High levels of fibrinogen have been associated with cardiovascular disease including the deep vein thrombosis and pulmonary embolism. At another end, the fibrinogen levels have been found to be reduced during the disseminated intravascular coagulation (DIC) (51, 52). DIC itself is the most common coagulation complication in the prostate cancer (53) (54). During DIC, there is an elevated production of thrombin which leads to the excessive formation of fibrin and decrease in the levels of fibrinogen. Also, there is simultaneous suppression of the anti-coagulation mechanism and delayed removal of fibrin because of impaired fibrinolysis (55). Thus, this combination of increased formation of fibrin and inadequate removal of fibrin results in dissemination intravascular thrombosis. These thromboembolic complications could in turn lead to cardiovascular toxicity. There are many studies with the administration of estrogen which have shown various outcomes, a decrease (56), constant (57) or even an increase (58) in the levels of fibrinogen. These variations may be due to the differences in the study designs, the type of estrogens used and the duration of the study.

1.3.4.3 Antithrombin III

Antithrombin III is an important anticoagulant in the coagulation cascade. It is a potent inhibitor of many pro-coagulation pathways. It majorly inhibits or dissolves thrombin. It also acts directly on the conversion of Factor X to Factor Xa and Factor IX to Factor IXa. It is a protein that is synthesized by the liver. Antithrombin III is the most important protein inhibitor of the activated coagulation factors (59). Orally delivered estrogens are known to reduce the levels of antithrombin III and these changes are similar in nature to the changes occurring prior to the cardiovascular deaths (60) (61).

1.3.5 Mechanism of Action of Diethylstilbestrol

Diethylstilbestrol action on prostate cancer is multipronged. The first important research on DES against metastatic prostate cancer conducted by Huggins and Hodges in 1941 has several proposed mechanisms of action, including the ability of estrogen to reduce acid phosphatase levels in prostate cancer patients, including a “direct action on the prostatic epithelium, inactivation of androgens, depression of gonadotropic agents of the anterior pituitary and depression of interstitial cells of the testis” (36). There is not only a direct cytotoxic and apoptotic effect of DES on prostate tumor cells *in vitro* (62) (63), but also an indirect evidence that this effect is present *in vivo* (34) (64). Geier et al., also demonstrated that DES inhibited the telomerase activity and gene expression of

prostate cancer cells (65). In this study, it was shown that DES is a potent inhibitor of telomerase bringing its activity down to 44 %. This inhibitory effect of DES on telomerase is independent from the enhancing effect of androgens. The most common and familiar mechanism of DES action is the primary steroidal effect of estrogens. This is the primary negative feedback mechanism on the hypothalamus and the anterior pituitary gland leading to a down-regulation of luteinizing hormone (LH) and decrease in testosterone to castrate levels (36). It has also been evaluated that estrogens can increase the levels of the sex hormone binding globulin, increase pituitary prolactin secretion and decrease testosterone production in the testis (66). The effect of DES in CRPC is based on its ability to decrease the dehydroepiandrosterone sulphate (DHEA-S) and increase the serum cortisol levels (67). In hormone refractory prostate cancer, the levels of testosterone required to increase growth of the tumor cells is maintained by the adrenal gland cortex. A decrease in the DHEA-S and increase in the serum cortisol levels help significantly decrease in the levels of testosterone in CRPC. These non-steroidal non-hormonal mechanisms that distinguish therapy of DES from non-estrogenic forms of ADT (34).

1.3.6 Comeback of Diethylstilbestrol

Even though LHRH agonists have been primarily used as androgen deprivation therapy against recurrent and metastatic prostate cancer for the past 20 years, recent developments have brought to the forefront many side-effects. Hence its

position as the premier treatment against prostate cancer is undergoing a change of outlook. The most serious side effects of LHRH agonists are osteoporosis (68), anemia (69), hot flushes (70), depression (71), loss of muscle mass (72), decreased cognitive abilities, fatigue and vascular stiffness and also the growing rate of castrate resistant prostate cancer (34). These are under prime consideration since prostate cancer is majorly found in the elderly patients. Degeneration of the daily functions has a serious effect on the overall health of the elderly. Hence DES has gained a renewed interest in the treatment for prostate cancer.

DES is effective in the treatment of both androgen dependent prostate cancer and CRPC (63, 64). DES is also efficacious in metastatic prostate cancer even after the failure of the first-line hormonal therapy (64). The levels of prostate specific antigen (PSA) levels have been found to decline by more than 50 % in 21 – 86 % of patients treated in phase II clinical trials of CRPC patients (73-75). Apart from the direct effect of DES on the prostate cancer cells, the estrogen metabolite 2-methoxyestradiol has significant anti-angiogenic and pro-apoptotic effects (76) (77). The rates of bone resorption and osteoporosis are also much less with estrogen treatment than the LHRH treatment (78) (79). Estrogens also have beneficial effect on the cognitive functions (80) (81). Thus androgen deprivation without estrogen deprivation certainly warrants further attention in the field of treatments of recurrent prostate cancer (34). Recurrent prostate cancer

therapy with estrogens is much cheaper than the therapy with LHRH agonists. Yearly cost analysis of therapy with LHRH agonists (goserelin, 10.8 mg every 3 months) is around $4995 whereas for DES (1 mg daily), it is only around $36 per patient (82). Thus, our goal of treating recurrent prostate cancer with DES with a subcutaneous nanosuspension will not only overcome the stigma of cardiovascular toxicity but also prove to be cost effective.

The recent clinical trials suggest that circumventing the first pass effect via the parenteral route for the synthetic estrogens may significantly reduce their cardiovascular toxicity. In a phase I clinical trial with intramuscular polyestradiol phosphate did not cause any cardiovascular or thromboembolic complications in patients with newly diagnosed prostate cancer treated for a period of 12.9 months (83). Ockrim et al., studied the transdermal estradiol in 20 patients. There was no reported cardiovascular toxicity and thromboembolic complication but castrate levels of testosterone was found with tumor response in all the patients (84). In another transdermal estrogen phase II clinical trial on the CRPC patients, there were no thromboembolic complications observed. The decrease in the PSA levels was more than 25% in most of the patients (85).

However, it is unclear if all these beneficial effects can be achieved with DES by sustaining its action without the cardiovascular toxicity. The contribution of the proposed research will be to develop subcutaneous sustained release

nanosuspension formulations of DES for reducing hepatic exposure of DES by circumventing the first pass metabolism while maintaining its efficacy by sustaining the plasma DES concentrations. This contribution is significant because it will potentially bring back DES, with all its high efficacy and reduced toxicity, into the forefront of treatment against recurrent, Stage III and Stage IV prostate cancer. Thus developing novel formulations of DES will enable us to sustain the action of DES and eliminate the cardiovascular toxicity. Our approach will benefit a large number of patients suffering from recurrent and advanced prostate cancer. Also, a sustained release of DES would enable less frequent dosing improving patient compliance.

1.4 Nanosuspension Formulations

In the current state of the pharmaceutical industry, the number of drugs that are synthesized and poorly water soluble are significantly increasing (86). Approximately 60 % of the drugs have poor water solubility (87). The problem of formulating these drugs is exacerbated as they are poorly soluble in both aqueous and organic phase. Such drugs have erratic absorption profiles and a highly variable bioavailability (88). There have been many methods to formulate such drugs so as to increase the solubility and for more predictable absorption. These formulation approached include co-solvent solubilization, oily solutions, liposomes, micro-emulsions and solid dispersions. But there is still a growing

need for devising a unique strategy that can not only overcome the problem of formulating hydrophobic drugs but also be feasible to manufacture commercially to improve the pharmacoeconomics (89). Nanosuspension is considered an important choice in such circumstances.

Nanosuspensions are sub-micron colloidal dispersions of pure particles of drug, which are stabilized by surfactants (90). Nanosuspensions can be used for drugs or compounds that are water insoluble and have a high log P value (91). The nanosuspensions are stabilized by minimum amount of surfactants while the drugs remain in solid state. The formulations have low toxicity, high drug loading and a significantly higher stability as compared to drug solutions (86).

There are two basic technologies to prepare nanosuspensions: a) media milling and a) high pressure homogenization.

In media milling the system is charged with milling media which could include glass or zirconium beads, water, drug and surfactants. The high shear and attrition forces of the media mill lead to reduction in particle size. The advantages of this technique are that drugs which are poorly water and organic solvent soluble can be formulated into nanosuspensions, ease for scale up, achieving narrow size distribution and ability to formulate large quantities of drugs with varying concentrations. The disadvantage is the introduction of residues in the formulation (89).

1.5 Subcutaneous Drug Delivery

Subcutaneous route for drug delivery is frequently used to increase the bioavailability of the drugs, or to extend and sustain the drug release into the systemic circulation. It can also be used as an alternative administration route to avoid the first pass effects of orally administrated drug which is the case for DES. Hence, characterization of the impact of subcutaneous route of administration on the pharmacokinetics and bioavailability of DES is of prime importance.

Subcutaneous drug administration results in delivery to the interstitial area lying underneath the dermis of the skin (Figure 4). A fibrous collagen network supporting a gel-phase comprising of negatively charged glycoaminoglycans, salts and plasma derived proteins (92) (93). When a formulation is delivered subcutaneously, there is significant systemic and lymphatic absorption. The absorption into the lymphatic system leads to the drug absorption into the systemic circulation of the entire particles (Figure 5) (94). All the nanoparticles do not have direct access into the systemic circulation but are drained into the lymphatic capillaries. The particles that pass through the lymphatics without being captured by the lymph nodes reach the general circulation where they behave as administered by the intravenous route (95). The particle size of the nano-formulations has a profound effect on the absorption. The overall structure of the interstitial subcutaneous tissue mandates that larger particles (larger than

100 nm) will have more difficulty to pass through the interstitium and will remain at the injection site (95). The smaller particles less than 100 nm have a much better chance to migrate through the aqueous channels in the interstitium and hence have better access to the lymphatic circulation (96).

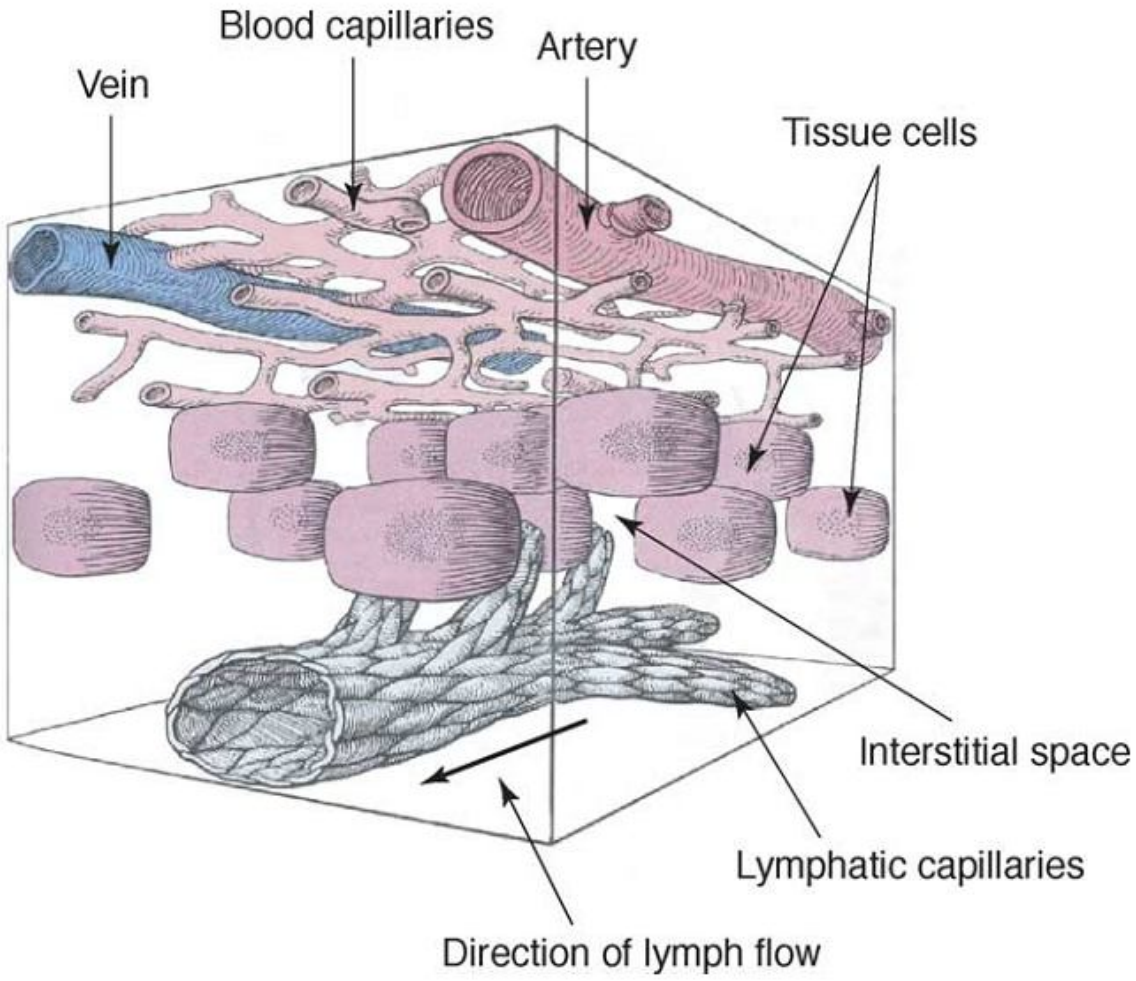


Figure 4: A Diagrammatic Representation of the Subcutaneous Site (97).

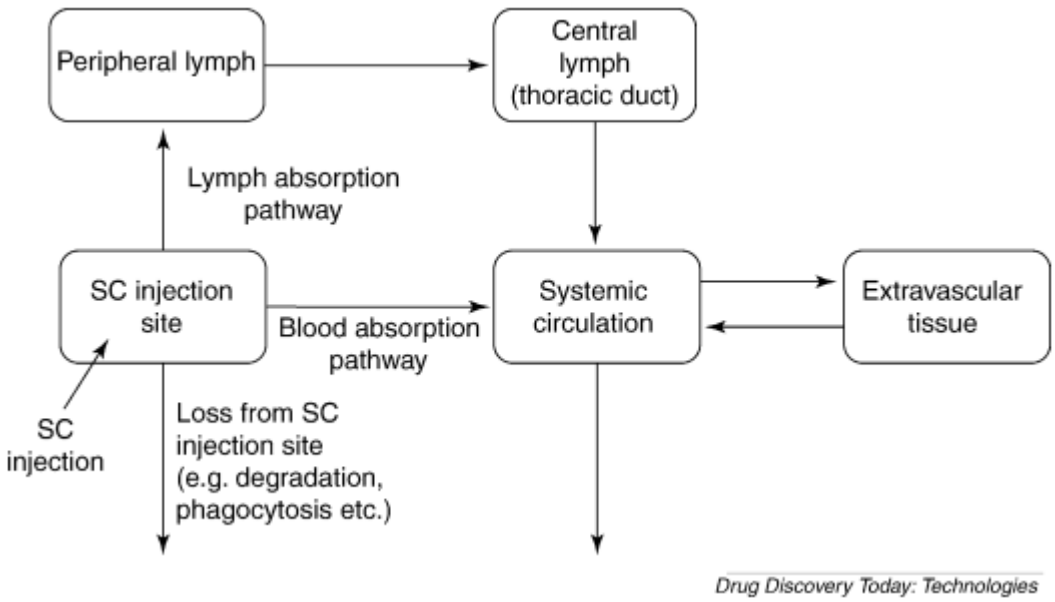


Figure 5: Generalized Schematic Representing SC Absorption via the Blood and Lymphatic Absorption Pathways into the Systemic Circulation (94).

Summary

This survey of the relevant current research findings illustrates that, Diethylstilbestrol which has lost its primary place for the treatment of prostate cancer because of its cardiovascular toxicity and thromboembolic complications, needs a serious consideration to be brought back to the forefront. Therefore, it was our hypothesis that the reduction of the toxicity profile and maintenance of the sustained release of the drug in the systemic circulation for its efficacy can be achieved by developing a sustained release subcutaneous nanosuspension formulation of DES.

Chapter 2 Hypotheses and Specific Aims

2.1 Central Hypothesis

The overall objective for this project is subcutaneous delivery of DES by developing a nanosuspension formulation. The central hypothesis is that compared to the oral route, the subcutaneous nanosuspension formulation will, by forming a depot, sustain the plasma DES concentration and minimize the hepatic exposure by circumventing the first pass effect. We propose that this change in the formulation and route of administration would potentially reduce the thromboembolic complications and cardiovascular toxicity.

2.2 Specific Aims

2.2.1 Aim I

To determine the feasibility of developing a DES nanosuspension formulation for subcutaneous delivery.

We hypothesize that DES with its physicochemical properties and chemistry can be successfully formulated as a nanosuspension formulation. The DES nanosuspension with different particle sizes can be developed. The nanosuspension formulations will be characterized based on particle size, zeta potential, polydispersity and *in vitro* release.

2.2.2 Aim II

To determine the pharmacokinetic parameters and the liver bio-distribution of the DES from nanosuspension formulations *in vivo*.

We hypothesize that DES nanosuspension formulation when delivered subcutaneously will sustain the plasma DES concentration, with good bioavailability and low hepatic exposure, by forming a depot *in vivo*.

2.2.3 Aim III

To evaluate the performance of the subcutaneous DES nanosuspension formulations based on toxicity and efficacy profiles.

We hypothesize that as compared to the oral DES, the subcutaneous sustained delivery of DES by the nanosuspension formulations will potentially decrease the changes in the coagulation factors while maintaining the efficacy.

This aim will be divided into two parts:

A) Pharmacodynamic evaluations: To evaluate the safety profiles of DES nanosuspensions with the cardiovascular toxicity biomarkers (Fibrinogen and Anti-thrombin III) and rat blood clotting time.

B) To evaluate the efficacy of nanosuspensions with the PCA 183 xenograft prostate tumor mouse model as compared to the conventional oral DES.

Chapter 3 Materials and Methods

3.1. Materials

3.1.1 Chemicals and Materials

- Acetopromazine (Phoenix, St. Joseph, MO, USA) in combination with ketamine and xylazine (Sigma Chemical Co., St. Louis MO, USA) as a anesthetic cocktail was used in the animal experiments.
- Acetonitrile HPLC grade (EMD, NJ, USA) was used in the preparation of mobile phase B for the LC/MS/MS assay, mobile phase of HPLC assay and solvent for extractions of DES from liver and plasma.
- Ammonium acetate HPLC grade (Sigma Chemical Co., USA) was used in the solution concentration of 2.5 mM as mobile phase A.
- Diadzein analytical grade (INDOFINE Chemical Company, Hillsborough, NJ, USA) as isoflavone was used as the internal standard for LC/MSMS assay.
- Diethylstilbestrol (DES) (a generous gift from NAL Pharmaceuticals, NJ, USA) was used in all the experiments.
- Glacial acetic acid (J.T. Baker Co, Phillipsburb NJ, USA) was used to adjust the pH of the LC/MS/MS mobile phase to 7.4.
- Glass beads (0.5 – 0.75, 0.75 – 1, 1 – 1.3 μm) purchased from Glenmills, Clifton, NJ, USA).

- Heparin sodium salt (Sigma Chemical Co., St. Loius MO, USA) was dissolved in normal saline (0.9% sodium salt) and used to coat the micro-centrifuge tubes used for blood collections in the pharmacokinetic studies.
- Methanol HPLC grade (EMD, NJ, USA) was used to prepare DES stock solutions.
- Phosphate buffer saline (PBS) containing 140 mM NaCl (Sigma Chemical Co., St. Loius, MO, USA) and 0.4 mM KH_2PO_4 (Sigma Chemicals Co., St. Louis, MO, USA) and 2 mM K_2HPO_4 (Fisher Scientific Co., Fair Lawn, NJ, USA), was used as the release medium for *in vitro* release study.
- Pluronic F108 (BASF Corporation, NJ, USA) was used to prepare the nanosuspension formulation.
- Polyethylene glycol 400 (PEG 400) (Merck, Darmstadt, Germany) was used as a component of cosolvent formulation of DES.
- Rat antithrombin III ELISA kit (GenWay Biotech, Inc. San Diego, CA, USA) was used to determine the levels of antithrombin III in rat plasma.
- Rat fibrinogen ELISA kit (GenWay Biotech, Inc. San Diego, CA, USA) was used to determine the levels of fibrinogen in rat plasma.
- Sodium chloride (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in double distilled water to prepare normal saline solution.
- Tween 80 was purchased from PCCA, Houston, Texas, USA. It was used in the formulation of the nanosuspension formulations.

3.1.2 Equipment and Apparatus

- Balance (digital, 0.0001-g sensitivity, Mettler AE100, Mettler Instrument Corp., Hightstown, NJ, USA) to weigh all chemicals and organs.
- Beckman Coulter Microfuge 22R Refrigerated Microcentrifuge was used to separate the plasma from blood, to extract DES from plasma and liver samples.
- Brookhaven ZetaSizer with Zeta Plus Particle Sizing software Ver.3.85 (Brookhaven Instrument Corporation, NY, USA) to characterize particle size and zeta potential of nanosuspension preparations.
- GraphPad Prism 5 was used for statistical data analysis including unpaired t-test, ANOVA, ANOVA with post-hoc analysis.
- HPLC system with A XTerra ® C18 column (5 µm, 150 x 4.6 mm i.d., Waters), Empower 2 chromatography software applied for peak area integration with this system (Waters Corp., Milford, MA, USA) and Waters 515 pump as the solvent delivery system with a photodiode array detector (Waters 2996) and an auto-sampler (Waters 717), was used to quantify DES for *in vitro* studies.
- LC/MS/MS 3200 Q trap mass spectrometer (Applied Biosystem/MDS SCIEX, Foster City, CA, USA) was used to quantify DES for *in vivo* pharmacokinetic studies.
- pH meter (IQ 240, Scientific Instrument, Carlsbad, USA) was used to measure the pH of the mobile phase.

- Pipettes (Eppendorf®, three sizes: 1-10 µl, 10-100 µl and 100-1000 µl) and pipette tips (disposable; 1-10 µL, 10-100 µL and 100-1000 µL) were used along with the pipettes for withdrawing liquid samples.
- Shaking water bath (model YB-521, American Scientific Products, Japan) was used for the *in vitro* release study.
- Vortex mixer (Vortex-Genie 2, Scientific Industries, Inc., NY, USA) was used for vortexing and mixing liquids.
- WinNonlin profession version 6.3 (Phoenix WinNonlin) (Pharsight Corporation, Mountainview, CA, USA) computer program was used for pharmacokinetic analysis and parameter calculation.

3.1.3 Surgical Instruments and Supplies

- Alcohol wipes (Webcol® Alcohol Preps, Kendall Healthcare Products Co., Mansfield, MA, USA) were used to disinfect animal skin prior to administering the anesthesia in animal surgery.
- Cotton swabs (Q-tips, 6 inch) (Sherwood Medical, St. Louis, MO, USA) were used during animal experiments in preclinical pharmacokinetic studies and for applying heparin to the micro-centrifuge tubes.
- Gastric gavage blunt needle (20-gauge, 2.5 inch, curved, ball-end) (Harvard Apparatus Inc., Holliston, MA, USA), attached to a 1 cc syringe, was used for oral dosing in rats and mice.

- Gloves (Latex) were used for handling chemicals and animals.
- Inserts (200 µL) (Chrom Tech, Inc, Apple Valley, MN, USA) were used for holding samples for LC-MS/MS analysis.
- Insulin syringes (1/2 cc, sterile syringes) (Becton Dickinson & Co., Rutherford, NJ, USA) were used to administer the anesthesia.
- Isothermal pad (model 39 DP, Braintree Scientific, Braintree, MA, USA) was used to heat to 37 ^{o}C maintaining body temperature of rats.
- Membrane filters (47 mm, 0.45 µm, hydrophilic polypropylene; Pall Corp., Ann Arbor, MI, USA) were used to filter mobile phases.
- Microcentrifuge tubes (1.5 mL, Axygen Scientific Inc., Union City, CA, USA) were used for collecting and storing blood and tissue samples from the pharmacokinetic, pharmacodynamic and liver bio-distribution studies.
- Surgical absorbent pads (Medline, Mundelein, IL, USA) were used during all animal experiments.

3.1.4 Animals

- Male Sprague Dawley rats (250-300 g), purchased from Harlan Laboratories (Houston,TX) were used for pharmacokinetic and organ distribution studies.
- Severe Combined Immunodeficiency (SCID) mice were purchased from the Jackson Laboratory and were used for the efficacy study.

3.2 Methods

3.2.1 Preparation of DES Nanosuspensions

A top down (milling) approach was used in the formulation of the nanosuspensions. The DES nanosuspension formulations were prepared by the wet milling technique (98) (99) as shown in Figure 6. A mixture of DES powder (20 mg), stabilizers 10% w/w Pluronic F108 (130 µL) and 10% w/w Tween 80 (30 µL) and double distilled water (200 µL) was added to a 7 mL amber colored scintillation vial pre-filled with a mixture of glass beads and magnetic stir bars. The glass beads added were in a definite ratio of 1:1:2 (0.8 g) for the sizes of 0.5 – 0.75 (S), 0.75 – 1 (M), 1 – 1.3 (L) µm, respectively. The mixture was stirred at 1,600 rpm. Intermittent sampling was performed to evaluate the particle size distribution of DES nanosuspension formulations by using Brookhaven ZetaSizer with Zeta Plus Particle Sizing software Ver.3.85 (Brookhaven Instrument Corporation, NY, USA).

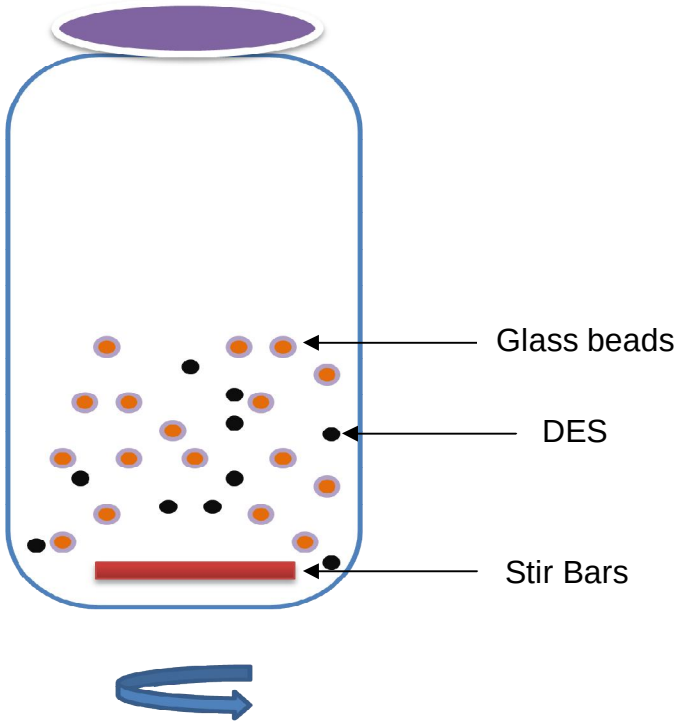


Figure 6: Nanosuspension Preparation by Wet Milling Technique.

3.2.2 Characterization of Nanosuspensions

3.2.2.1 Particle Size and Zeta Potential Measurements

Five µL of nanosuspension was used to measure the particle size and zeta potential by using the Brookhaven Zetasizer with Zeta Plus Particle Sizing software Ver.3.85 (Brookhaven Instrument Corporation, NY, USA). The nanosuspension was diluted (1:200) by double distilled water and placed in a cuvette for measurement.

3.2.2.2 *In vitro* Release of DES from Co-solvent and Nanosuspensions of Three Particle Sizes of 160 nm (NS-160), 300 nm (NS-300) and 500 nm (NS-500) in PBS.

The *in vitro* release experiments were performed by placing either the DES co-solvent (solution) or the nanosuspension formulations (NS-160, NS-300 and NS-500) in a dialysis bag (Molecular weight cut off: 6000 – 8000 Daltons) as shown in Figure 7. The dialysis bags were cut to approximately 4 cm each. The dialysis bags were immersed in plastic tubes containing 20 mL of PBS with 0.2% Tween 80 to maintain the sink condition. The temperature of the system was maintained at 37 ºC by placing it in a temperature controlled water bath and shaken at 100 rpm. A half mL of the solution was withdrawn at 0.02, 0.04, 0.08, 0.13, 0.17, 0.25, 0.31, 0.17, 0.25, 0.31, 0.42, 0.5, 1, 1.17, 1.33, 2, 3, 4, 5, 6, 8, 10, 12 and 14 days. As soon as the sample was withdrawn it was replaced by an equivalent volume

of half mL fresh medium. The samples collected were analyzed for DES by using the HPLC assay.

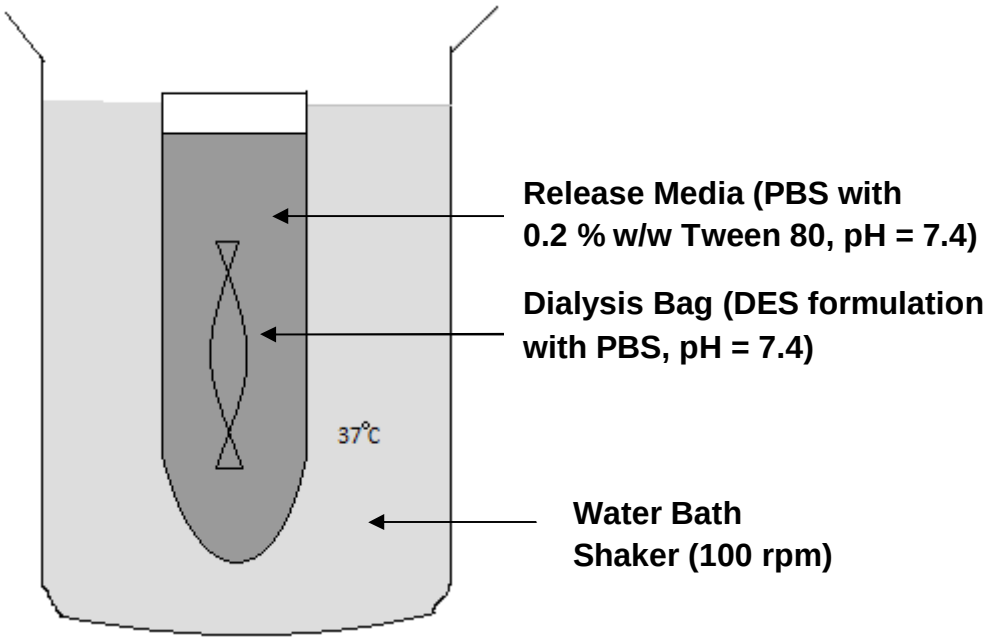


Figure 7: Dialysis Bag Diffusion Technique for *In Vitro* Release Study.

3.2.2.3 Release Kinetics of DES from DES Nanosuspensions

The release kinetics of the drug from the nanosuspensions was evaluated on the basis of various release kinetics models. The characterization of the release profiles was done by fitting the release from the nanosuspensions to zero order, first order, Higuchi and Weibull models. The goodness of fit and the applicability of the model were evaluated by comparing the Coefficient of Determination, R^2, of the model fits.

The kinetic models (100) are:

Zero order kinetics (Equation 1) where a relationship between drug - dissolved fraction versus time will be linear.

$$1 - (W_t/W_o) = K_o t \ldots (1)$$

Where W_o is the initial amount of drug in the pharmaceutical dosage form, W_t is the amount of drug remaining in the pharmaceutical dosage form at time t and K_o is the zero order release constant or apparent dissolution rate constant.

First order kinetics (Equation 2) where a plot of logarithm of the release amount of drug versus time will be linear.

$$\log Q_t = \log Q_o + K_1 t/2.303 \ldots (2)$$

Where Q_t is the amount of drug released in time t, Q_o is the initial amount drug and K_1 is the first order release constant.

Higuchi model (Equation 3) where the relationship between the amount of drug release versus square root of time will be linear.

$$Q_t = K_H t^{1/2} \ldots (3)$$

Where Q_t is the amount of drug released at time t and K_H is the Higuchi dissolution constant.

Weibull model (Equation 4) where a linear relationship will be obtained between a log-log plot of ln $\{1/(1 - Q_t)$ versus time.

$$\log [\ln \{1/(1 - Q_t)\}] = b \log (t - T_i) - \log a \ldots (4)$$

where Q_t is the amount of drug released at time t, T_i is the location parameter which represents the lag time before the onset of dissolution or release which is usually assumed to be zero, a is the time scale parameter of the process and b characterizes the curve of the release.

3.2.3 HPLC Assay for Quantification of DES in Aqueous Solution

3.2.3.1 Chromatographic Conditions

The DES and 17-α-methyl testosterone (MT) IS for the HPLC assay were separated with baseline resolution on a XTerra® C18 column (5 µm, 150 x 4.6 mm i.d.) at room temperature. The HPLC apparatus was Waters Model 515 pump, Waters Model 717 plus autosampler and Waters Model 2996 photodiode array detector. The mobile phase consisted of acetonitrile (ACN) : 22 mM tetraethyl ammonium acetate 60:40 (v/v) at pH 6.3. The flow rate was set at 1.0 mL/min and DES was detected at 254 nm.

3.2.1.2 Aqueous Calibration Curves

The stock solutions DES (1 mg/mL) and MT (100 µg/mL) (internal standard) were prepared in Methanol and Acetonitrile, respectively. The standard curves were prepared in 50 % Acetonitrile in the linear range of 100 µg/mL - 0.198 µg/mL by a serial dilution. MT (5 µL of 100 µg/mL) was added to 1 mL of the standard solutions to yield a concentration of 0.5 µg/mL. The peak area ratio of DES/MT was plotted against the corresponding DES concentrations. Linear regression was used to determine the slopes of the curves and the y-intercepts. The HPLC assay was repeated to establish within-day (n=3) and between day (n=6) variability.

3.2.4 Stability Study

The physical and chemical stability of the 160 nm and 500 nm nanosuspensions was evaluated at 4 ± 2 ºC and 20 ± 2 ºC. The nanosuspensions were prepared and divided into 8 batches (n=4 each) and kept at 4 ± 2 ºC and 20 ± 2 ºC, respectively. The changes in the particle size for the physical stability and chemical integrity for the chemical stability were monitored for a period of 4 months.

3.2.5 Pharmacokinetic and Liver Bio-distribution Studies of DES from DES Nanosuspensions in Sprague-Dawley Rats

3.2.5.1 Preparation of Dosing Formulations

DES cosolvent (Solution) (0.25 µg/mL) was prepared by dissolving DES in DMSO: Ethanol: PEG 400 (2:2:1) and was delivered subcutaneously. The nanosuspensions, 160 nm (NS-160) and 500 nm (NS-500) were prepared as mentioned in section 3.2.1. The nanosuspensions were diluted to the final concentration of 35 mg/mL using double distilled water. The oral suspension was prepared in the Oral Suspending Vehicle at a concentration of 14 mg/mL. The Oral Suspending Vehicle which contains purified water, lambda carageenan, simethicone, xanthan gum, microcrystalline cellulose/sodium CMC, sodium phosphate dibasic, citric acid, potassium sorbate, methyl paraben and propyl paraben was obtained from Professional Compounding Centers of America

(PCCA). The mixture was vortexed for 5 minutes and sonicated for 30 minutes at room temperature until no powder was visible. This was dosed in rats orally. The subcutaneous suspension (SubQ suspension) was prepared by triturating the DES powered in a mortar and pestle with Pluronic 108 and Tween 80 and double distilled water was added to give a final concentration of 14 mg/mL.

3.2.5.2 Rats Housing and Study Protocol

All animal experiments were conducted in accordance with the National Institutes of Health (NIH) Guideline for the Care and Use of Animal along with the approved protocol from the University of Houston Institutional Animal Care and Use Committee (IACUC). Male Sprague Dawley (SD) rats (300 – 350 g) were housed in individual cages for 7 days prior to the start of dosing and sampling regimen. The rats were randomly divided into different groups for the treatment with the different DES formulations mentioned in section 3.2.5.1. The rats were anesthetized by using anesthesia cocktail (Ketamine 50 mg/mL, Xylazine 3.3 mg/mL and Acetopromazine 3.3 mg/mL) prior to dosing. Blood samples were collected from the tail vein of the rats by clipping a small portion of the tail with a blade after dipping it in warm water for 30 seconds and keeping the tail warm under a lamp. At the end of the study the rats were euthanized using CO_2.

3.2.5.3 Pharmacokinetic Study

The rats were given a single dose of 0.125 mg of the DES solution and 7 mg each for NS-160, NS-500, oral suspension and subcutaneous suspension groups. Blood (250 µl) was sampled at 0.04, 0.08, 0.17, 0.33, 0.5, 1, 1.25, 1.5, 2, 2.25, 3, 4 and 6 days for all the tested groups. The blood was collected in pre heparinized micro-centrifuge tubes and immediately spun at 13,000 rpm for 20 minutes to separate the plasma from the blood cells. The plasma was transferred to a separate micro-centrifuge tube and stored at – 80 ºC until further drug analysis.

3.2.5.4 Liver Bio-distribution Study

The hepatic exposure of DES was evaluated in the NS-160, NS-500, oral suspension and solution groups. The SD rats were euthanized at 0.04, 0.17, 0.5, 1, 2, 4 and 6 days (n = 4 at each time point). The liver was immediately perfused by using saline solution and the liver was harvested for extraction and the DES extract samples were stored at – 80 ºC until further analysis. The liver/plasma ratio was calculated by using the equation (5).

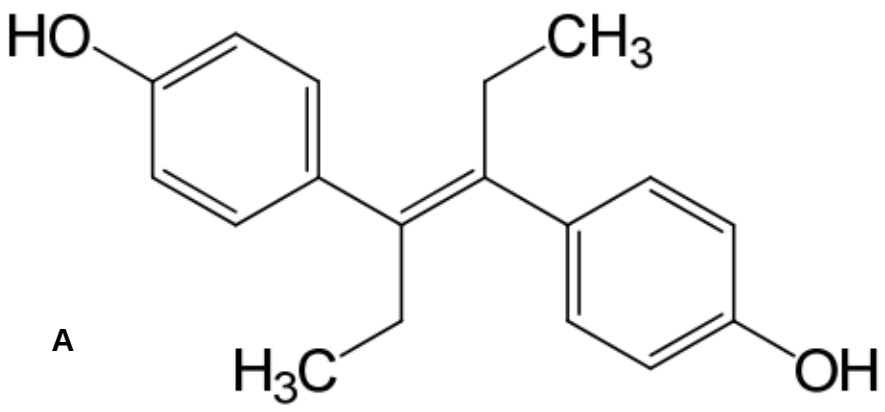
L/P ratio = $AUC_{0\text{-}t\ (liver)}$ / $AUC_{0\text{-}t\ (plasma)}$…. Equation (5)

This equation gives the extent of hepatic exposure as compared to the plasma exposure of DES.

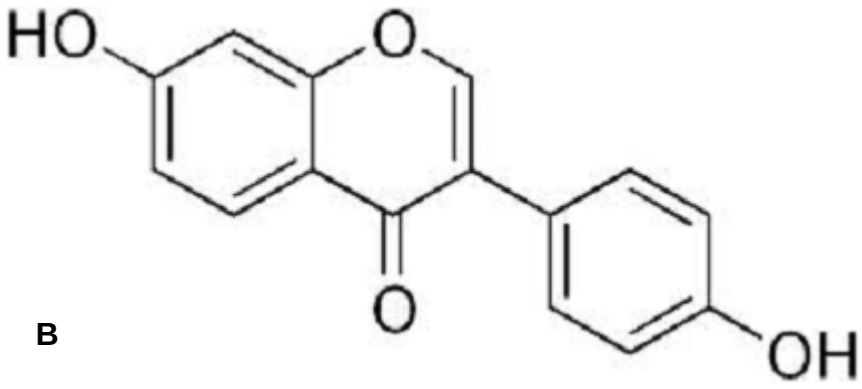
3.2.5.5 LC/MS/MS Assay of DES in Rat Plasma and Liver Samples

The LC/MS/MS assay was based on the assay previous validated and established (101). The assay was developed by using a 3200 QTRAP triple quadrupole LC/MS/MS mass spectrometer. The instrument was operated in the negative mode, and quantification was performed using multiple-reaction monitoring (MRM). The IonSpray heater was maintained at 500 °C, and the nebulizer and heater gases were set at 40 and 80 psi, respectively. The needle voltage was set to 4500 V, the curtain gas setting was 10 psi, and the collision activated dissociation (CAD) gas setting was medium. Optimal MRM was used to detect the transition ions from a specific precursor ion to the product ion; the ion transitions monitored were *m/z* 266.9 → 237.0 for DES and 253.0 → 132.0 for Daidzein (Figure 8). The collision energy was set at 38 eV for DES and 46 eV for Daidzein. Other compound parameters were determined for each drug using the QTRAP instrument and Analyst software version 1.5. The liquid chromatography part was an Agilent Technologies 1200 and the separation was achieved using an Agilent Eclipse XDB-C18 column (4.6 × 150 mm, 5 µm) maintained at 40 °C. The gradient mobile phases were 2.5 mM ammonium acetate, pH 7.4, (solvent A) and 100% acetonitrile (solvent B). A linear gradient profile was applied with the following proportions (v/v) of phase B [t (min), %B]: (0, 5), (1, 35), (2, 65), (5, 95), (6, 95), (7, 35), (8, 5). The flow rate was 1 mL/min, and the injection volume was

50 μL. The peak area ratios of DES/Diadzein were plotted against the corresponding DES concentrations. Linear regression was used to determine the slopes and y-intercepts of the curves.



A



B

Figure 8: Chemical Structures of Diethylstilbestrol (A) and Daidzein (B)

3.2.5.5.1 Preparation and Storage of Stock, Standard and Working Solutions

A stock solution of 1 mg/mL DES in methanol was diluted with 50% acetonitrile aqueous solution to 10 µg/mL and then with plasma to a working solution of 100 ng/mL. Serial dilutions were made from the working solution to prepare the calibration standards and quality control (QC) samples. A stock solution of 2.54 µg/mL Daidzein in dimethyl sulfoxide (DMSO) - methanol (25:75, v/v) was first diluted with the DMSO–methanol solvent to 25.4 ng/mL and then further diluted with 50% acetonitrile to a working solution of 12.7 ng/mL. The working solution of DES was stored at – 80 °C.

3.2.5.5.2 Preparation of DES Rat Plasma Samples

One hundred µL of sampled, spiked or blank rat plasma was transferred into a microcentrifuge tube. Ethyl acetate (500 µL) was added to the tube as the extraction solvent. Ten µL of internal standard (Diadzein) was added. The mixture was vortexed for 45 seconds and centrifuged at 14000 rpm for 30 minutes. The supernatant layer was transferred to another tube and evaporated to dryness using an evaporator for air drying. The dried extract was reconstituted in 100 µL of water/ACN (50/50 v/v diluent). The tube was centrifuged for 15 minutes at 13,000 rpm and 80 µL of aliquot was injected into the LC/MS/MS machine.

3.2.5.5.3 Preparation of DES and Blank Rat Liver Samples

The liver samples were allowed to thaw and 1 g of the liver was homogenized with a tissue tearer in 0.5 mL of normal saline. One hundred µL of the liver homogenate was mixed with 10 µL of internal standard. The rest of the process was exactly the same as mentioned in 3.2.5.5.2.

3.2.5.6 Pharmacokinetic Data Analysis

The concentrations of DES obtained from the plasma samples at various time points were fitted into compartmental models using Phoenix WinNonlin 6.3. The compartmental modeling was used to estimate the various pharmacokinetic parameters such as area under the curve (AUC), clearance (CL), volume of distribution (V_d), elimination half life ($t_{1/2,\beta}$) and distribution half life ($t_{1/2,\alpha}$) for the subcutaneous solution, NS-160, NS-500 and oral suspension groups.

3.2.6 *In Vitro In Vivo* Correlation (IVIVC)

The Level B correlation was used for the IVIVC. In this correlation, the mean *in vitro* dissolution rate (MDR $_{in\ vitro}$) was compared to the mean *in vivo* dissolution rate (MDR $_{in\ vivo}$) for the NS-160, NS-500 and solution groups. The mean in vitro dissolution time (MDT $_{in\ vitro}$) was calculated by Equation 6 and the mean in vivo dissolution time (MDT $_{in\ vivo}$) for the nanosuspension formulations NS-160 and NS-500 was given by Equation 7. The *in vivo* mean residence time (MRT$_{in\ vivo}$) required to calculate the MDT$_{in\ vivo}$ for nanosuspensions is obtained from the non compartmental analysis using Phoenix WinNonlin 6.3. Equations 8 and 9 give the calculations for the MDR $_{in\ vitro}$ and MDR $_{in\ vivo}$, respectively.

$$MDT_{in\ vitro} = \frac{\sum_{j=1}^{n} Tj\hat{}\ \Delta Mj}{\sum_{j=1}^{n} \Delta Mj} \quad \text{..... Equation (6)}$$

$$MDT_{in\ vivo} = MRT_{NS\ formulation} - MRT_{solution} \quad \text{..... Equation (7)}$$

$$MDR_{in\ vitro} = 1 / MDT_{in\ vitro} \quad \text{..... Equation (8)}$$

$$MDR_{in\ vivo} = 1 / MDT_{in\ vivo} \quad \text{..... Equation (9)}$$

Where:

j is the sample number

$T_{j\hat{}}$ is the time at mid-point between T_j and T_{j-1}

ΔM_j is the additional amount of drug dissolved between T_j and T_{j-1}

MRT is the mean residence time in vivo.

3.2.7 Toxicity (Pharmacodynamic) Evaluations for DES in Sprague-Dawley Rats.

3.2.7.1 Determination of Fibrinogen (FBG) and Antithrombin III (ATIII) Levels

The determinations of clotting factors (FBG and ATIII) levels were measured using respective ELISA kits from Genway Biotech, Inc. (San Diego, CA, USA). The test kits are a highly sensitive two-site enzyme linked immunoassay (ELISA) for measuring the FBG and ATIII levels in biological samples of rats. The assay for quantification of plasma requires that each test sample be diluted before use. A 1/10,000 dilution was used for most plasma samples. For absolute quantification, samples that yield results outside the range of the standard curve, a lesser or greater dilution was required. A 1/10,000 dilution of sample was prepared by transferring 5 µL of sample to 495 µL of 1X diluent. This gave a 1/100 dilution. Next, 5 µL of 1/100 diluted sample was added to 495 µL of diluents to give a 1/10,000 dilution. The samples were mixed thoroughly at each stage. All reagents were thawed to room temperature. One hundred µL of standards and samples (in duplicate) were pipetted into pre-designated wells. The microtiter plate was incubated at room temperature for sixty (60 ± 2) minutes. The plate was covered and kept level during incubation. The contents of the wells were aspirated following incubation. The wells were washed 4 times with the washing buffer. Following the wash, the residual buffer was removed by

striking the walls on absorbent paper. Later, 100 µL of diluted Enzyme Antibody Conjugate was added to each well and incubated at room temperature for 30 ± 2 minutes in dark, being protected from light. After washing the plate as previously stated, 100 µL of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution was added to each well and incubated in the dark for 10 minutes. Then 100 µL of stop solution was added to each well and absorbance was determined at 450 nm.

3.2.7.2 Determination of Rat Blood Clotting Time (CT)

Rat blood clotting was measured by sampling 25 µL of blood from the rat tail vein. The blood was collected in the microhematocrit glass capillary and a chronometer was started at a time when the blood first makes contact with the glass capillary. The flow of blood was evaluated between two marks on the capillary by tilting it at ± 60 º. The chronometer was stopped once the blood stops flowing. The time indicates the clotting time in rats (102).

3.2.7.3 Short Term Toxicity Studies in Sprague Dawley Rats

The SD rats were dosed and sampled in the same way and time as described in the section 3.2.5.3 for groups of NS-160, NS-500 and oral suspension. The levels of FBG and ATIII were evaluated in the plasma samples by the ELISA technique mentioned in 3.2.7.1. The CT levels were measured by using the technique described in section 3.2.7.2.

3.2.7.4 Long Term Toxicity Studies in Sprague Dawley Rats

The SD rats were dosed with 7 mg of DES with NS-160, NS-500 and oral suspension every 14 days for a period of 28 days (dosed twice). The blood sampling was collected at 0, 1, 7, 14, 21 and 28 days. The FBG, ATIII and CT levels were evaluated as described in the sections 3.2.7.1 and 3.2.7.2.

3.2.8 DES Nanosuspension Efficacy Study

From the pharmacokinetic and toxicity evaluations of the nanosuspensions, the NS-160 was selected as the candidate for the proof-of-concept efficacy study in a mouse prostate tumor model. The MDA PCA183 tumor cells and Xenograft model was used in this study. The study was performed at MD Anderson Cancer Center.

3.2.8.1 Passage of Tumor into SCID Mice

MDA PCa 183 xenograft is derived from androgen-dependent prostate carcinoma from drug naïve patient. The tumor tissue was rinsed 6 times by sterile PBS solution in cell culture condition. Dulbecco's Modified Eagle Medium (DMEM) and 10 % Fetal Bovine Serum (FBS) medium were added to the tumor and minced into approximately 1 mm pieces. Depending on the red cell numbers, the minced tumor was rinsed with the same medium twice or more. The tumor pieces were incubated with pre-warmed 1X Accumax (1,200-2,000 U/mL) and kept for 20 minutes at 37 ^{0}C under rotating conditions. The cells were filtered

through 70 µm cell strainer. The cells were observed under microscope for a definite cell count and subcutaneously delivered into the right and left flanks of SCID mice. The tumors were allowed to grow in the severe combined immunodeficiency (SCID) mice and the treatment was initiated when all the mice reached a minimum tumor volume of 50 mm^3. The tumors were then extracted, pooled, minced up and centrifuged. The same protocol as described above was performed for the passage of tumor into SCID mice for the efficacy study. About 50 µL of the suspension was injected into the left flank of the SCID mice to induce tumor growth. Four to five mice were used in each group for the efficacy study. The mice were dosed after the tumor volume reached a minimum volume of 50 mm^3. The dosing groups used for study were NS vehicle, Oral suspension (47 mg/kg), NS-160 groups such as NS-10 mg/kg, NS-23.5 mg/kg and NS-47 mg/kg.

The 47 mg/kg dose was selected for the mice since the dose given in rats for the pharmacokinetic study was around 23.3 mg/kg. It was calculated based on the dosing calculations given by Regan-Shaw et al., 2008.

Formula for dose translation based on Body Surface Area (BSA) (103):

Equivalent Dose (mg/kg) = Animal (rat) dose (mg/kg) * Rat K_m / Mouse K_m

K_m factor for Mouse = 3

K_m factor for Rat = 6

Equivalent Dose (mg/kg) in mouse = 23.33 (mg/kg) * 6/3 = 46.66 = 47 mg/kg

where:

K_m is the factor used to convert dose in mg/kg to dose in mg/m^2 (BSA normalized).

3.2.8.2 Randomization of Mice into Dosing Groups

When the estimated tumor volumes were about 50 mm^3, the mice were weighed and the tumor volumes were measured. Vernier calipers were used to measure tumor volume which is defined as the product of the tumor length (L), width (W) and height (H). The mice were randomized into different groups of NS vehicle, Oral suspension (47 mg/kg), NS-160 groups such as NS-10 mg/kg, NS-23.5 mg/kg and NS-47 mg/kg based on the tumor volumes.

3.2.8.3 Study Design

The mice were dosed and assessed weekly. The assessment included body weight for toxicity, as well as tumor volumes and prostate specific antigen (PSA) levels for efficacy. The end points of the study were tumor growth rate (defined as V/V_o, V is the tumor volume at the measurement days and V_o is the tumor volume on the first day of dosing), toxicity (body weight loss > 15%) and survival. The PSA levels on the first day of dosing was compared to the PSA levels at the end of the study.

3.2.8.4 Statistical Data Analysis

The data were represented as Means ± SD. Unpaired t-tests were conducted for comparing means between the different groups mentioned in section 3.2.8.2. Significance was set at $p < 0.05$.

Chapter 4 Results

The results have been summarized through the following topics: 1) Preparation of DES nanosuspension formulations, 2) Characterization of the nanosuspension formulations based on particle size, zeta potential and in vitro release, 3) Plasma pharmacokinetics and hepatic exposure of subcutaneous DES nanosuspension formulations, 4) Short-term and long-term toxicity evaluations with FBG, ATIII and RBCT, 5) Proof-of-concept efficacy study in a xenograft prostate tumor mouse model.

4.1 Preparation of DES Nanosuspension Formulations:

In order to evaluate the best conditions and factors for the formulation of the DES nanosuspension formulations, the different types of stabilizers, concentration of stabilizers, amount of drug in the formulation, milling time, varying ratios of glass beads were used. The stirring rate was kept constant at 1,600 rpm. The various factors were screened one factor at a time keeping the other factors constant.

4.1.1 Selection of Stabilizers

To select the stabilizer candidates for the DES nanosuspension formulations, different stabilizers such as Pluronic® 108 (F-108), Pluronic® 68 (F-68), Polysorbate 80 (Tween 80), Polyvinylpyrrolidone (PVP) 40 were used at a concentration of 10 % w/w. From the different stabilizers, Tween 80 was used in combination with F-108, F-68 and PVP 40. The F-68 and PVP 40 failed to stabilize the DES nanosuspension formulations. The combination of F-108 and Tween 80 stabilized the DES nanosuspension formulations effectively and led to lowering of the particle size of the formulation. Tween 80 (30 µL of 10 % w/w) was used at a lower amount than F-108 (130 µL of 10% w/w). This combination led to efficient stabilization and effective lowering of the particle size of DES nanosuspension formulation. F 108 used alone did not result in adequate stabilization and wetting of the nanosuspensions. Hence from the different stabilizers a combination of the F-108 and Tween 80 was selected. The different concentrations of F-108 and Tween 80 were evaluated at 5 %, 10 % and 15 % w/w (Table 1). The 10 % and 15 % w/w selections resulted in comparable or significant lowering of the particle size as compared to 5 % w/w of the stabilizers. The 10 % w/w group was selected as it has a lower concentration of the stabilizers in the formulation as compared to the 15 % w/w group.

4.1.2 Effect of Glass Bead Ratio and Amount of Drug

Different sizes of glass beads, S, M and L, were used in the ratio of 50:1, 40:1 and 30:1 (Beads : Drug amount) keeping the other factors constant at 20 mg of DES, 10 % w/w of F 108 and Tween 80 (Table 2). The 50:1 ratio had 0.5 g of L, 0.3 g of M and 0.2 g of S beads. The 40:1 ratio had 0.4 g of L, 0.2 g of M and 0.2 g of S beads. The 30:1 ratio had 0.3 g of L, 0.2 g of M and 0.1 g of S beads. The effect of above mentioned ratios were evaluated over duration of 32 hours. The particle size was not reduced effectively by using 30:1 ratio while 50:1 ratio causes too much packing in the vial and the milling is not efficient. The 40:1 ratio reduces the particle size efficiently and faster as compared to that of 50:1 ratio.

Three different amounts of DES at 10 mg, 20 mg and 30 mg were also used to test the effect of amount of drug in the formulation to the reduction in the particle size while keeping the other factors constant (Table 3). The particle size was effectively reduced for 10 and 20 mg of DES but 30 mg of DES did not reach lower particle sizes.

4.1.3 Effect of milling time

Milling time, 1 to 32 hours was evaluated for reduction in particle size by fixing all the other factors (Figure 9). The particle size decreased with the milling time. After 30 hours the mean particle size reached a plateau and hence 30 hours of milling was selected as total time of milling for the NS-160 group. The NS-500 group was achieved in 6 hours and NS-300 was achieved after 8 hours milling.

Table 1: Effect of Stabilizer Concentration on the Particle Size of the Nanosuspension with Increase in Milling Time by the Media Milling Method of Preparation.

Stabilizers (%w/w)	6 h Particle size (nm) ± SD	8 h Particle size (nm) ± SD	24 h Particle size (nm) ± SD	30 h Particle size (nm) ± SD	32 h Particle size (nm) ± SD
5	NA	NA	NA	NA	NA
10	503 ± 10.5	310 ± 5.4	220 ± 8.6	160.2 ± 3	160 ± 3.2
15	739.8 ± 18.4	654.7 ± 20.8	174.1 ± 15.2	160.3 ± 7.1	160.3 ± 5.6

NA: Drug is not wetted by the surfactant concentration. Data Reported as Mean ± SD (n=3). Formulation composition: 20 mg of DES + 200 µL of Distilled Water + 130 µL of F108 + 30 µL of Tween 80.

Table 2: Effect of Ratio of Glass Beads to Drug Amount on the Particle Size of the Nanosuspension with Increase in Milling Time by the Media Milling Method of Preparation.

Glass Beads : Drug	6 h Particle size (nm) ± SD	8 h Particle size (nm) ± SD	24 h Particle size (nm) ± SD	30 h Particle size (nm) ± SD	32 h Particle size (nm) ± SD
50:1	430.9 ± 42.7	389.1 ± 23.4	326 ± 26.8	176.5 ± 16.2	160.5 ± 16
40:1	503 ± 10.5	310 ± 5.4	220 ± 8.6	160.2 ± 3	160 ± 3.2
30:1	410 ± 30.2	391.3 ± 8.7	205 ± 18.3	200.8 ± 11.6	193.5 ± 12.8

Data Reported as Mean ± SD (n=3). Formulation composition: 20 mg of DES + 200 µL of Distilled Water + 130 µL of 10 % w/w F108 + 30 µL of 10% w/w Tween 80.

Table 3: Effect of Drug Amount on the Particle size of the Nanosuspension with Increase in Milling Time by the Media Milling Method of Preparation.

Drug Amount (mg)	6 h Particle size (nm) ± SD	8 h Particle size (nm) ± SD	24 h Particle size (nm) ± SD	30 h Particle size (nm) ± SD	32 h Particle size (nm) ± SD
10	452.1 ± 12.5	498.9 ± 56.1	215.3 ± 17.4	162.0 ± 8.3	160 ± 12.1
20	503 ± 10.5	310 ± 5.4	220 ± 8.6	160.2 ± 3	160 ± 3.2
30	880.9 ± 90.4	467.2 ± 76.7	350.1 ± 32.5	301.8 ± 43.2	257.4 ± 33.6

Data Reported as Mean ± SD. Formulation composition: DES + 200 µL of Distilled Water + 130 µL of 10 % w/w F108 + 30 µL of 10% w/w Tween 80.

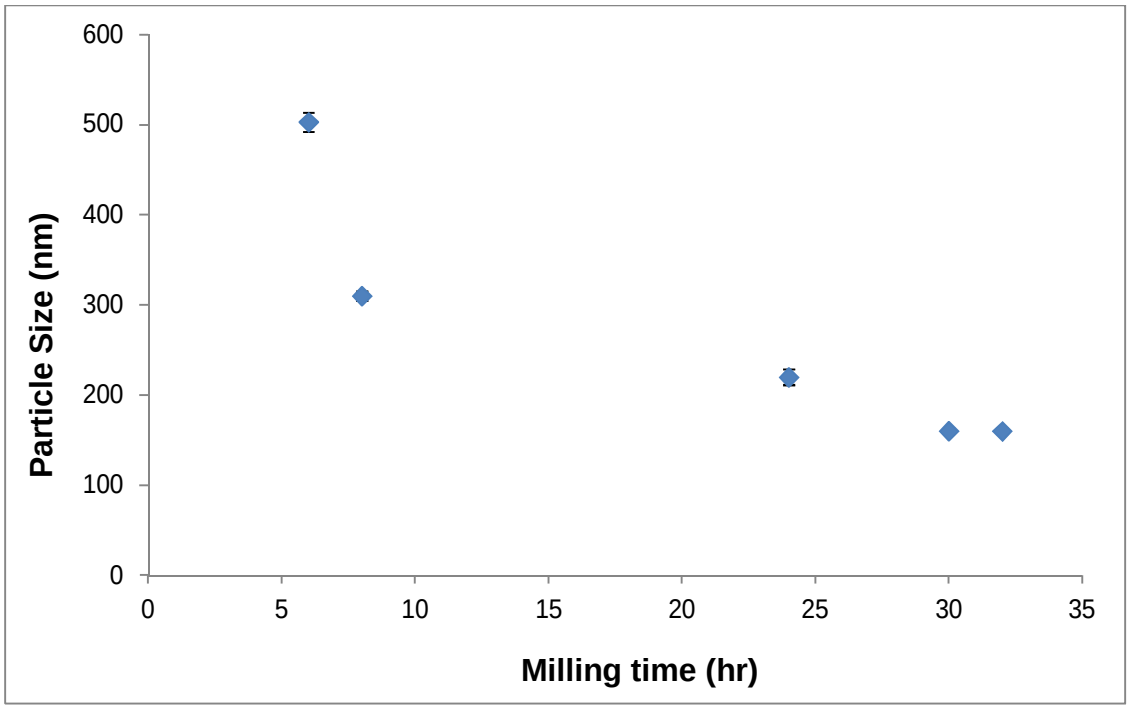


Figure 9: Dependence of Particle Size on Milling Time (n=3).

Data Reported as Mean ± SD.

Formulation composition: 20 mg of DES + 200 µL of Distilled Water + 130 µL of 10 % w/w F108 + 30 µL of 10% w/w Tween 80.

4.2 DES HPLC Assay

The HPLC assay was developed and validated for the quantification of DES in aqueous solutions. MT was used as the internal standard (IS). The assay was linear in the range 0.198 to 100 µg/mL. The retention times for the peaks of DES and IS were at 5.10 and 3.53 minutes, respectively (Figure 10). The assay was validated with the within-day variability (n=3) of 3.84% and the between day variability (n=6) of 9.63 %. The calibration curves were established in aqueous solution for DES. The correlation co-efficient (R^2) of the assay was 0.998 (Figure 11).

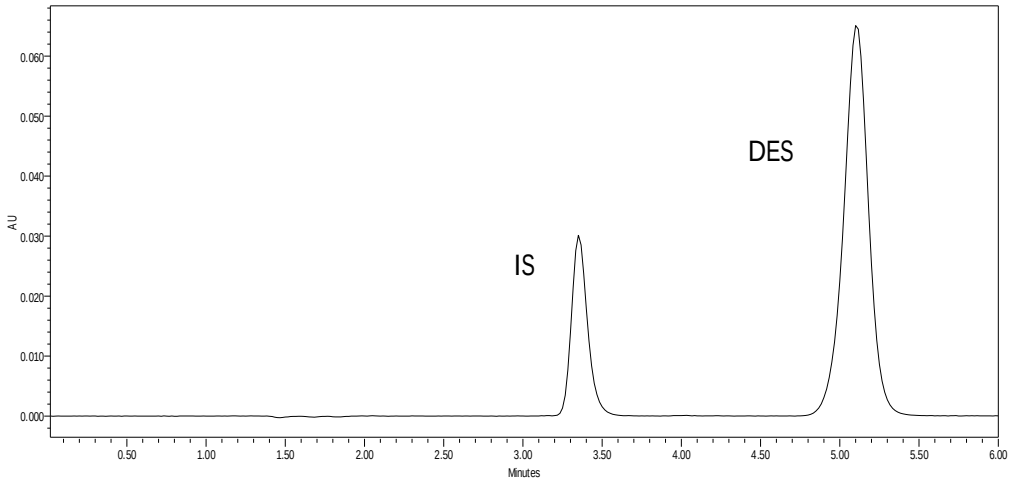


Figure 10: HPLC Chromatogram of DES (12.5 µg/mL) and IS in Aqueous Solution.

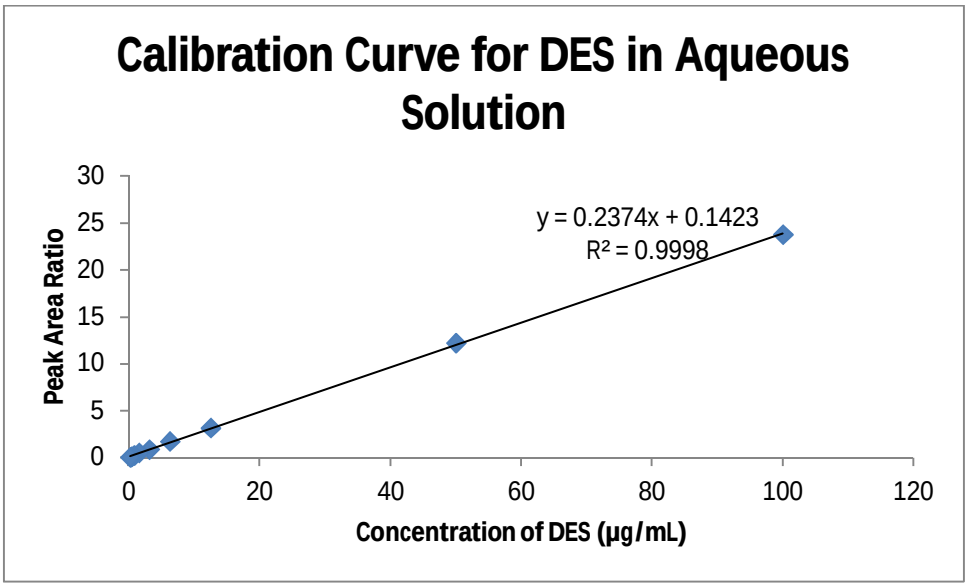


Figure 11: Representative Calibration Curve of DES in Aqueous Solution.

4.3 DES Nanosuspensions Characterization

4.3.1 Physical Characterization

Three nanosuspensions of particle sizes of about 160 nm (NS-160), 300 nm (NS-300) and 500 nm (NS-500) were formulated using the wet milling technique. All the three nanosuspensions were prepared by mixing with stabilizers and milled with glass beads. The particle sizes of the nanosuspensions depended on the milling time. The particle size distributions of the nanosuspensions showed a bi-modal distribution of the size based on the diameter (Figure 12). The polydispersity indices (PI) of the NS-160, NS-300 and NS-500 was 0.18 ± 0.01, 0.15 ± 0.01 and 0.20 ± 0.03 respectively. The values of PI are considered within the stable range for nanosuspensions. The zeta potentials for the three groups were in the negative range. The values of the zeta potentials for NS-160, NS-300 and NS-500 were - 25 ± 0.65, - 22.73 ± 0.58 and - 18.08 ± 0.45 mV, respectively (Table 4).

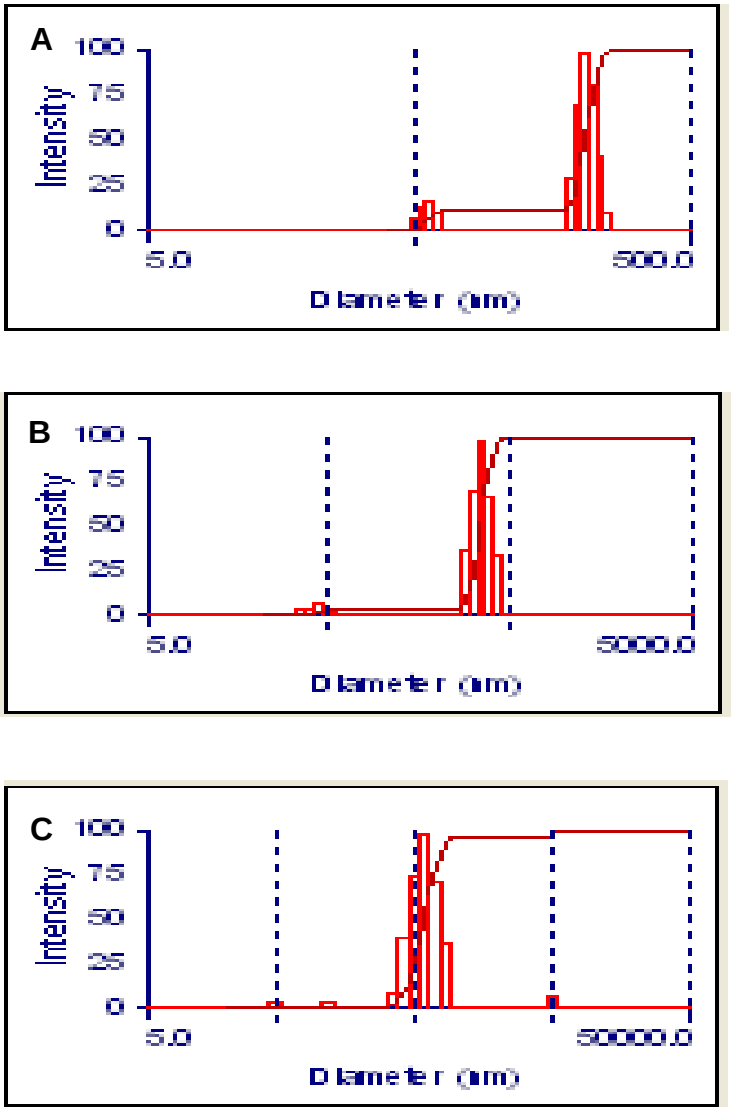


Figure 12: Particle Size Distribution of the NS Formulations of Particle Sizes NS-160 (A), NS-300 (B) and NS-500 (C).

In the figure, X-axis represents the diameter of the particles in nanometers (nm) and Y-axis represents the intensity.

Table 4: Physical Properties of DES Nanosuspensions and Milling time.

Formulation	Milling time (hr)	Particle Size (nm)	Zeta Potential (mV)	Polydispersity
NS-160	30	162.5 ± 1.5	-25 ± 0.65	0.18±0.01
NS-300	8	287.1 ± 2.7	-22.73 ± 0.58	0.15±0.01
NS-500	6	492.5 ± 8.3	-18.08 ± 0.45	0.20±0.03

Data Reported as Mean ± SD.

Formulation composition: 20 mg of DES + 200 µL of Distilled Water + 130 µL of 10 % w/w F108 + 30 µL of 10% w/w Tween 80.

4.3.2 *In vitro* Release of DES from Co-solvent and Nanosuspensions of Three Particle Sizes (NS-160, NS-300 and NS-500).

The *in vitro* release of DES formulations was evaluated in PBS with 0.2% Tween 80 at 37 ºC for a period of 14 days. Because of the low solubility of DES in water, 0.2% of Tween 80 was added to maintain the sink condition. The overall cumulative release of DES from the DES co-solvent was rapid. The entire amount of DES was released in 9 hours (Figure 13). As compared to the release from the DES co-solvent, the DES nanosuspension formulations exhibited a biphasic release pattern, with an initial burst rapid release followed by a slow sustained release.

Among the three nanosuspension formulations of NS-160, NS-300 and NS-500, the NS-160 had a significantly higher and faster mean initial release of DES (25.06 %/day) than the other two NS formulations. The mean initial releases of DES from the NS-300 and NS-500 formulations were comparable with 15.68 and 15.51%/day, respectively. There was no significantly difference between the initial release rates of the higher particle sizes of NS-300 and NS-500.

The faster release of DES from the nanosuspension formulations was observed till day 6 after which till the day 14 a slow sustained release was observed (Figure 13). By Day 6, the NS-160 and NS-300 had an extent of DES release of 65.76% and 67.38 %, respectively. This was significantly higher than that from

NS-500 formulation (55.42 %). By Day 14, 67.77% of DES was released from NS-160, 70.53% from NS-300 and 64.79% from NS-500 group (Table 5).

The release from the nanosuspension formulations was much slower than that of the DES solution. Also, the nanosuspension of the smallest particle size (NS-160) exhibited a significantly faster initial release than those of higher particle sizes (NS-300 and NS-500). The extent of drug release at 6 days for the largest particle size was the significantly slower than the smaller particle sizes of NS-160 and NS-300. It is interesting to note that the initial release profile of the NS-300 formulation is similar to that of the NS-500 formulation but later is similar to that of the NS-160 nm formulation.

The kinetics of DES release from the nanosuspension formulations were characterized by fitting the release profiles to the zero order (Figure 14), first order (Figure 15), Higuchi (Figure 16) and Weibull (Figure 17) models. The goodness of fit for the model was based on the values of co-efficient of determination (R^2) values (Table 6). There was no one model which perfectly fits the release of DES from the nanosuspensions. The release was best described by the Higuchi model which describes the dissolution and diffusion process based on the Fick's law and by the Weibull model which is adapted to the dissolution process and is applied to almost all kinds of dissolution curves. The

release kinetics of the nanosuspensions did not fit well with the zero order and first order release models.

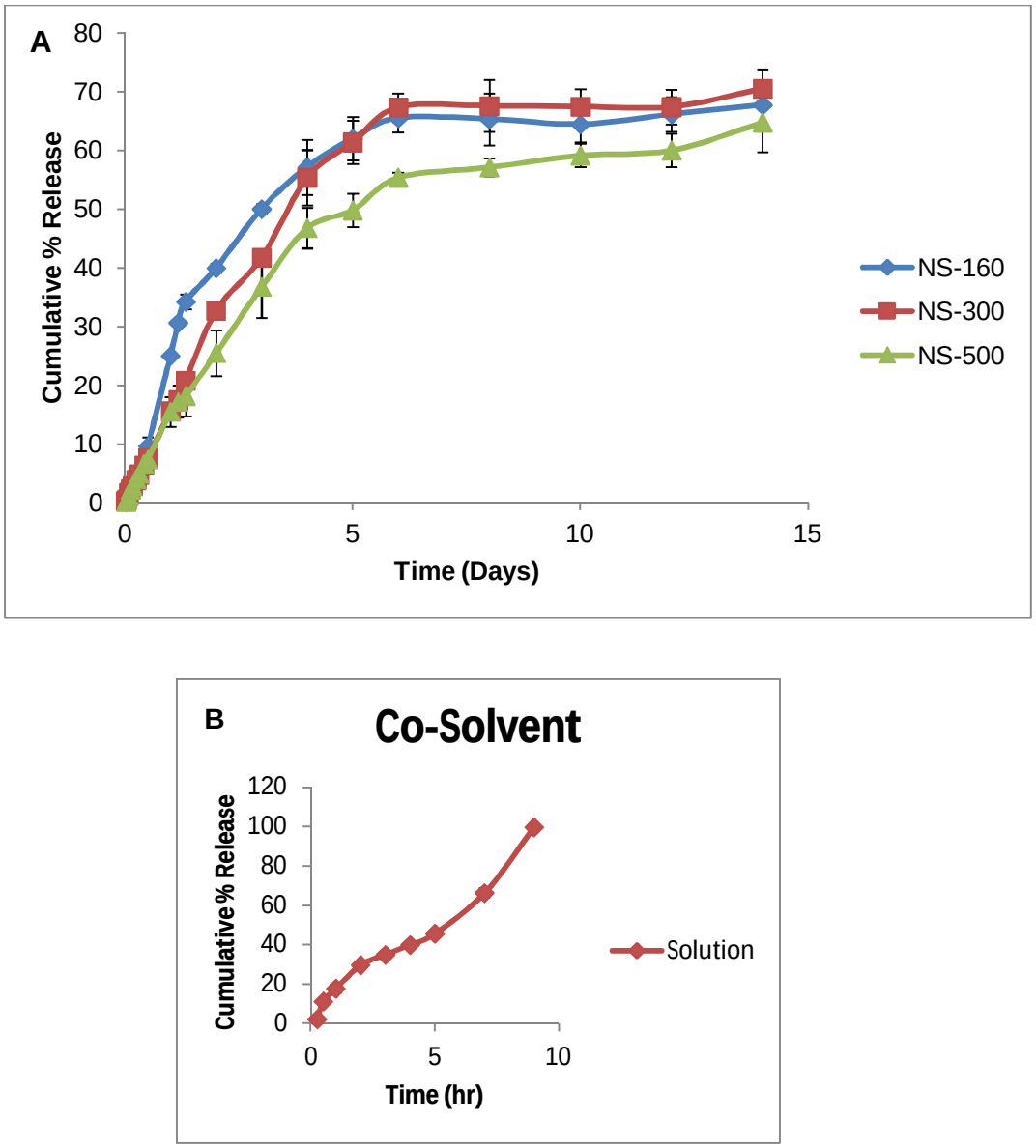


Figure 13: Release Profiles of DES Nanosuspensions of Different Particle Sizes (A) and Co-solvent (B) in PBS at 37 ºC.

Data Reported as Mean ± SD (n=3).

Table 5: Extents of Release of DES from Nanosuspensions.

Formulation	Initial % Release (1 day)	Extent of release (6 days)	Extent of release (14 days)
NS-160	25.06±0.15*	65.76±3.68	67.77±4.89
NS-300	15.68±2.69	67.38±6.03	70.53±4.80
NS-500	15.51±2.52	55.42±1.57*	64.79±7.78

* Significant difference in the extent of release among NS formulations. Unpaired T-test ($p < 0.05$)

Data Reported as Mean ± SD (n=3).

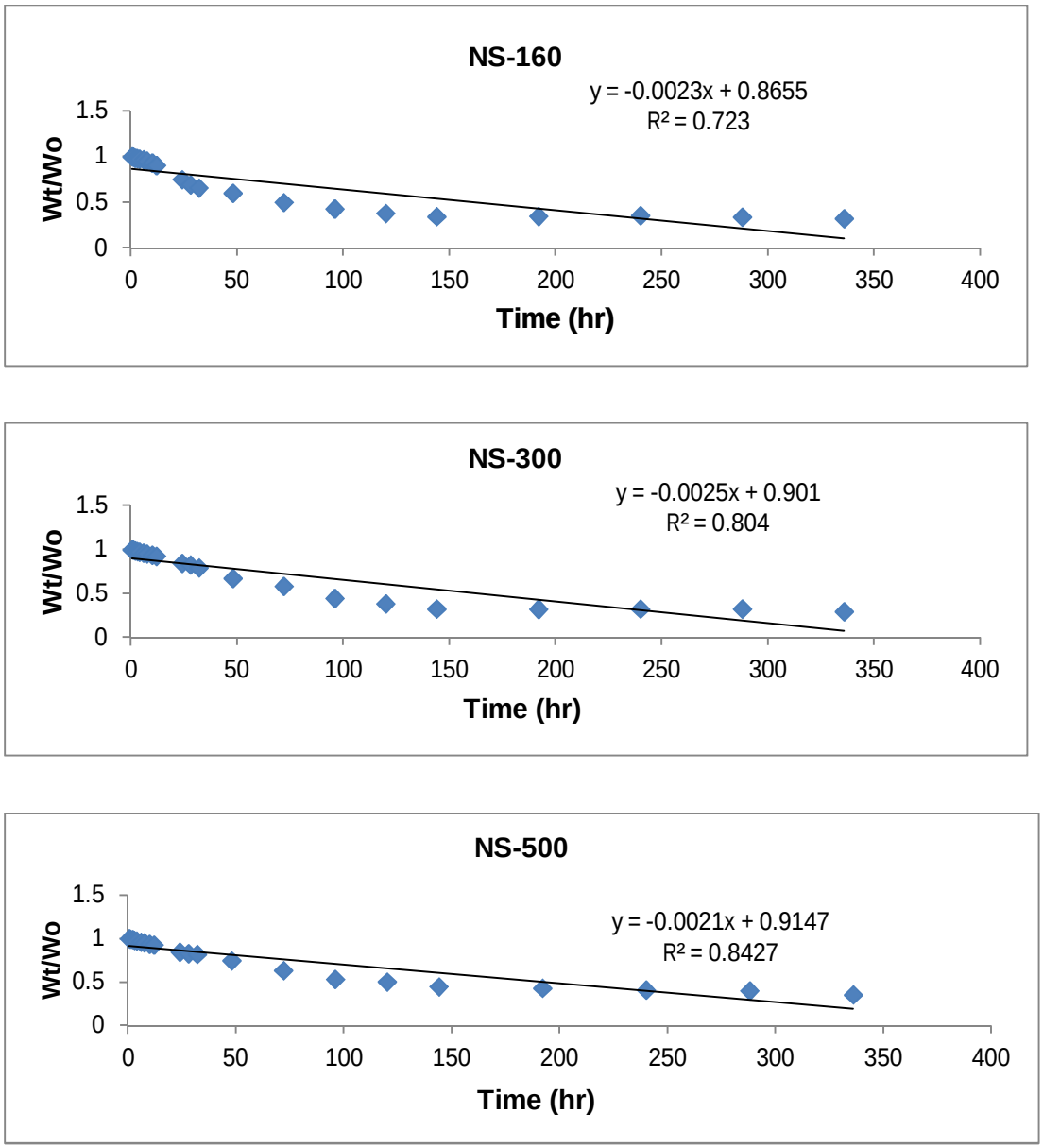


Figure 14: Zero Order Release Kinetics from NS-160, NS-300 and NS-500 Nanosuspensions.

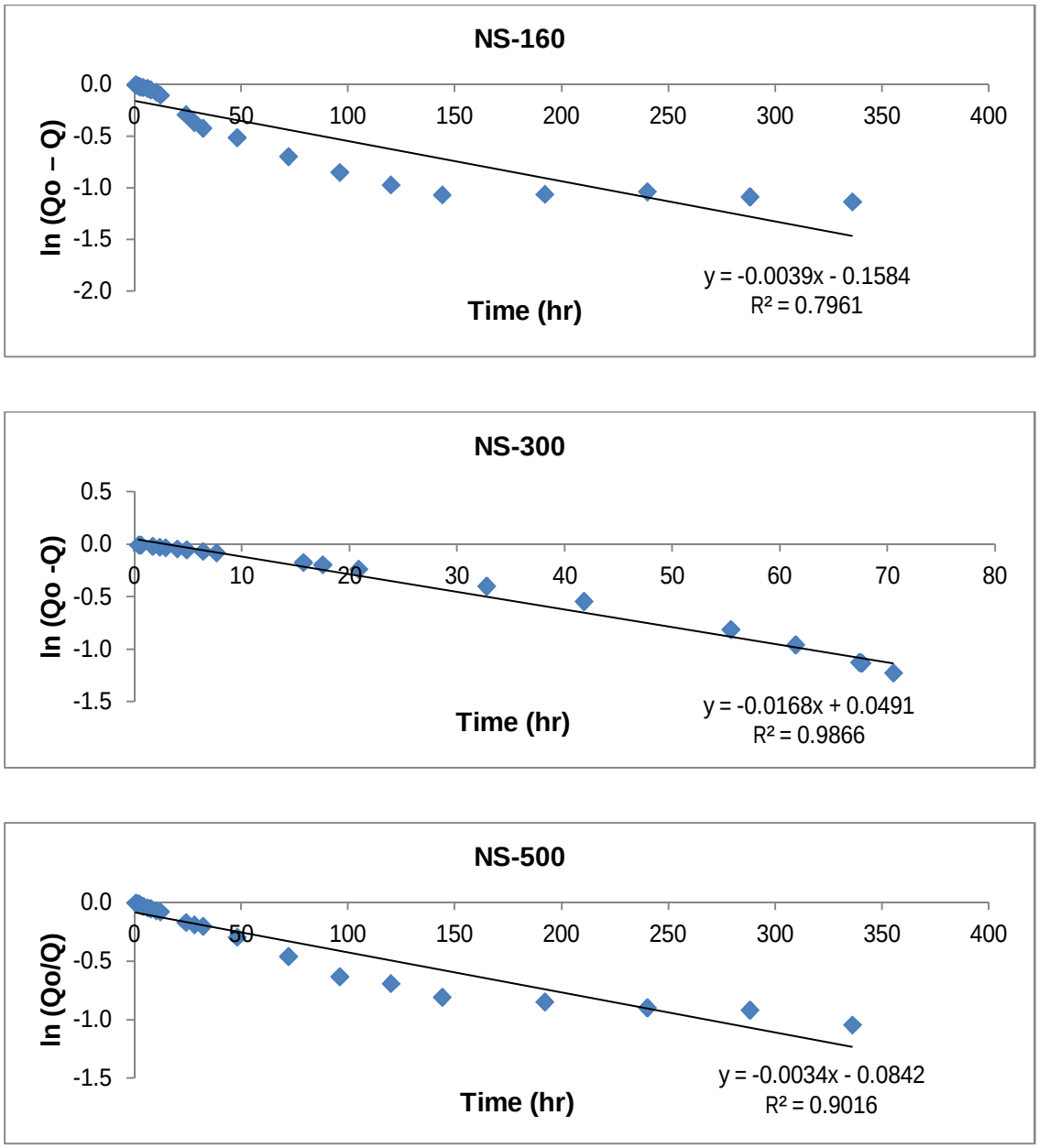


Figure 15: First Order Release Kinetics from NS-160, NS-300 and NS-500 Nanosuspensions.

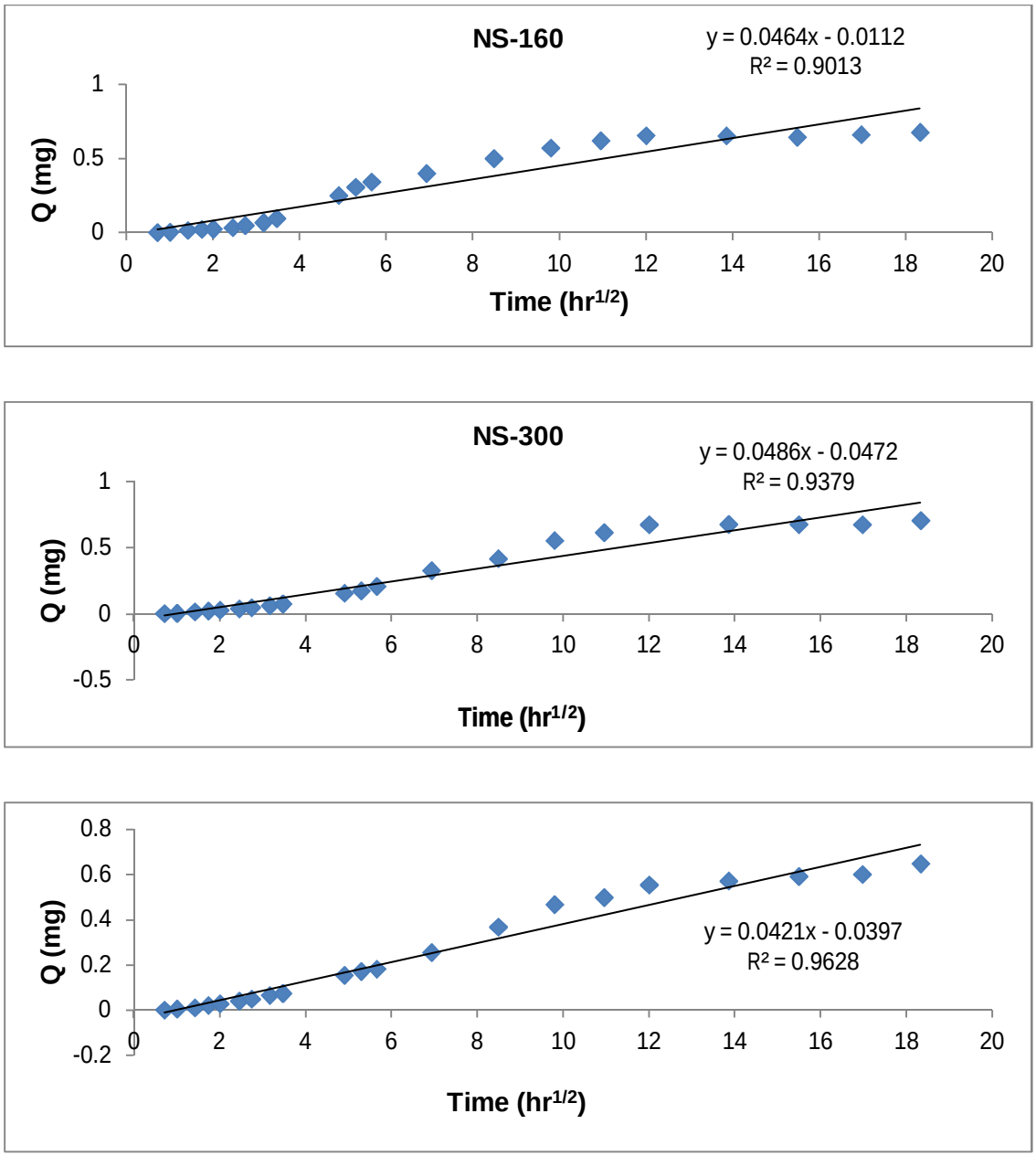


Figure 16: Higuchi Release Kinetics from NS-160, NS-300 and NS-500 Nanosuspensions.

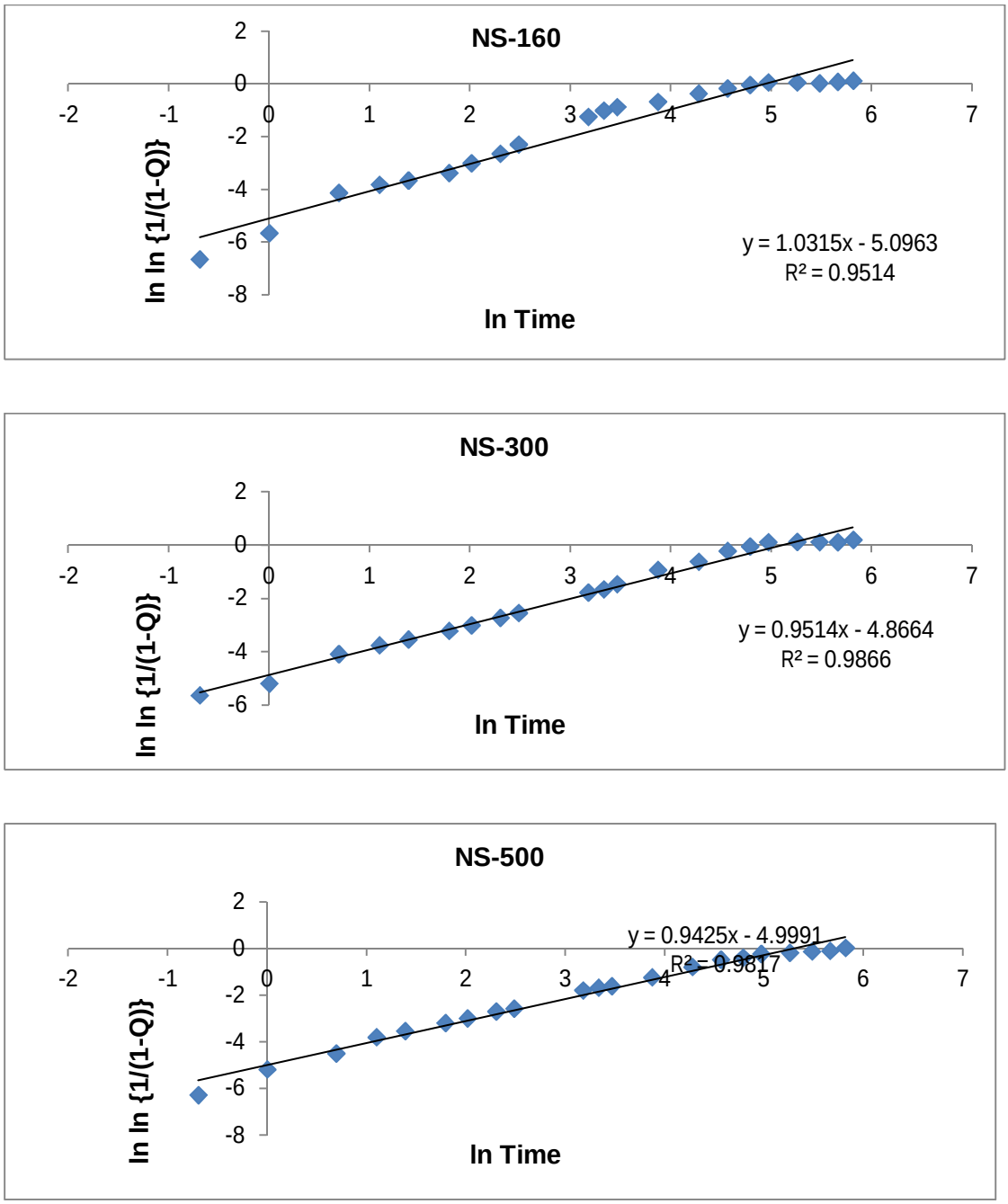


Figure 17: Weibull Release Kinetics from NS-160, NS-300 and NS-500 Nanosuspensions.

Table 6: Release Kinetics Criteria for NS-160, NS-300 and NS-500.

Particle Size	R^2			
	Zero Order	**First Order**	**Higuchi**	**Weibull**
NS-160	0.7230	0.7961	0.9013	0.9514
NS-300	0.8040	0.9866	0.9379	0.9866
NS-500	0.8427	0.9016	0.9628	0.9817

4.3.3 Stability of DES Nanosuspensions

The physical and chemical stability of the NS-160 and NS-500 formulations were evaluated for a period of 4 months. The formulations were measured for particle size and chemical stability at the time of formulation, Day 0 and stored at room temperature, RT (20 ± 2 ^{o}C) and 4 ^{o}C, respectively and then continued monitoring at 1 and 4 months. No apparent changes in the particle sizes were observed over a period of 4 months for NS-160 at both RT and 4 ^{o}C. The particles sizes for NS-500 were constant at 1 month, but significantly increased to 538.17 nm at 4 months at RT, but remained constant at 4 ^{o}C for 4 months (Figure 18). There was no chemical degradation in the formulations over a period of 4 months at both RT and 4 ^{o}C as evaluated by HPLC analysis (Figure 19).

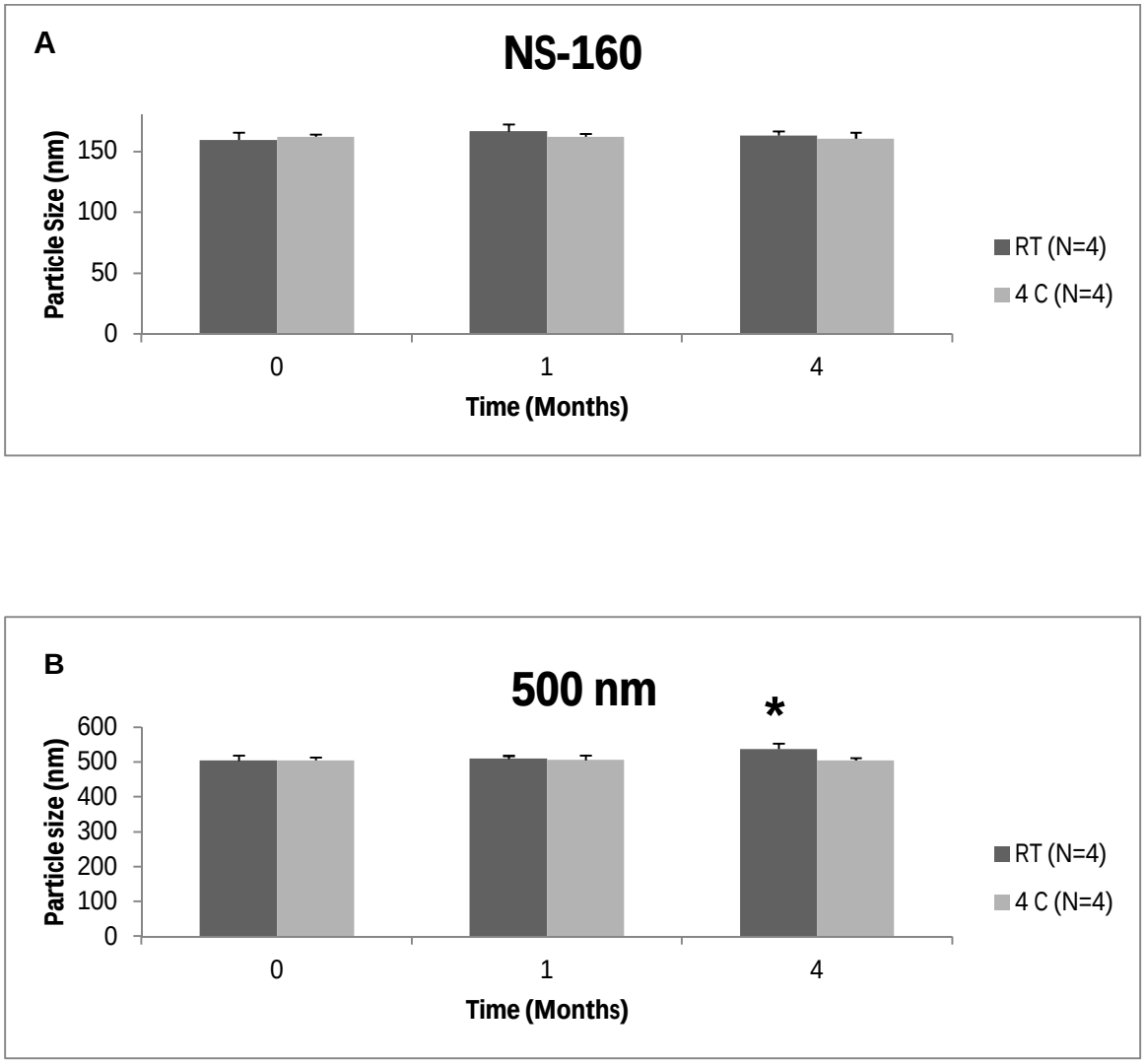


Figure 18: Physical Stability of NS-160 (A) and NS-500 (B) at RT and 4 ^{o}C.

*** Significant Increase in the Particle Size. Student's T-test ($p < 0.05$).**

Data Reported as Mean ± SD (n=4)

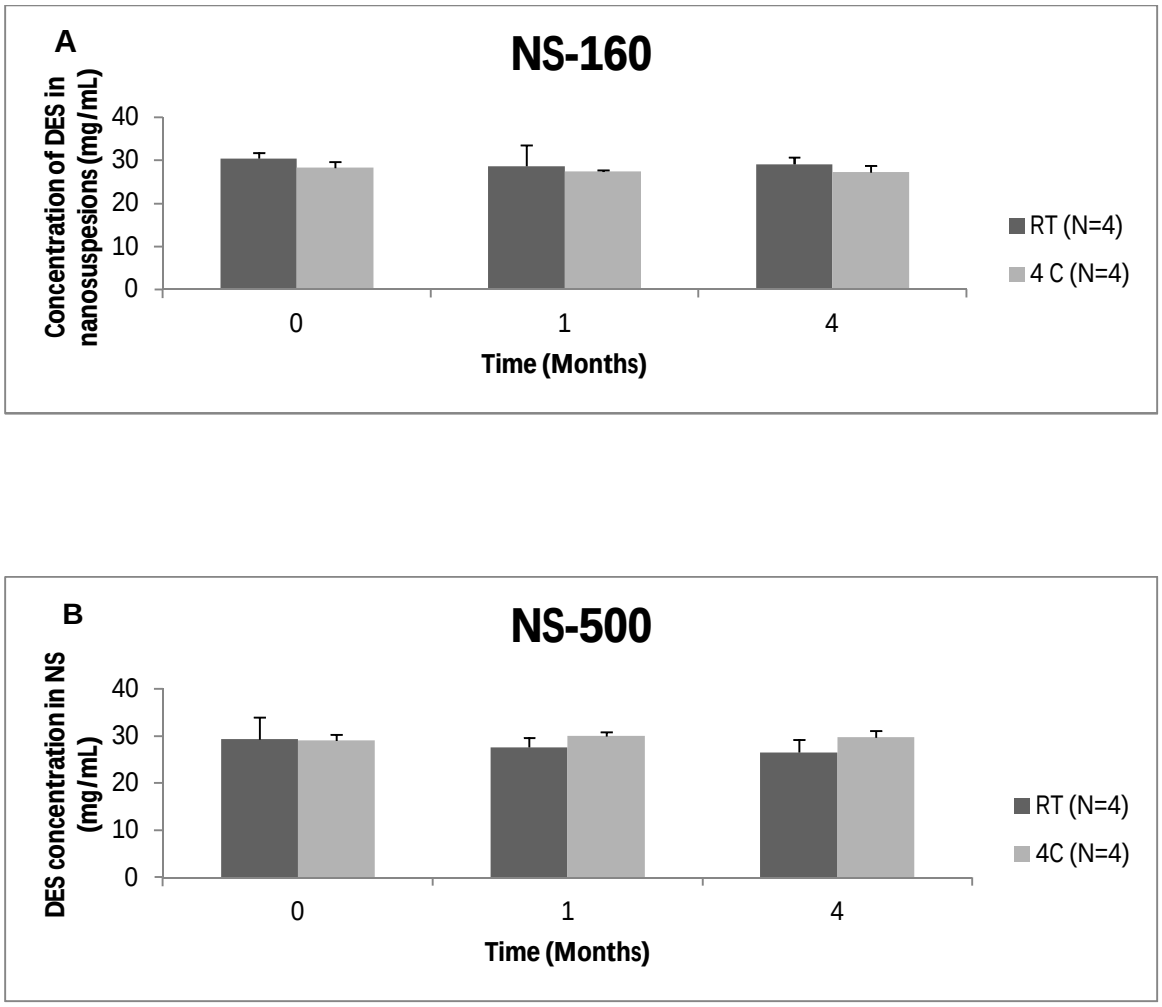


Figure 19: Chemical Stability of NS-160 (A) and NS-500 (B) at RT and 4 ^{o}C.

Data Reported as Mean ± SD (n=4).

4.4 DES LC-MS/MS Assay

A sensitive and precise LC-MS/MS assay which was successfully developed for the quantification of DES in the rat plasma and liver tissue (101) was used to quantify the DES in plasma and liver for the pharmacokinetic and liver bio-distribution studies. The internal standard used was diadzein at a concentration of 5 µM. The running time of the assay was 8 minutes with retention times of 4.61 for DES and 3.46 for IS with an isocratic elution (Figures 21 and 22). The mass spectra for DES and internal standard showed maximum intensities at ions 266.9/237 and 253.0/132.0, respectively. Good assay linearity was established within the range of 0.78 ng/mL – 200 ng/mL for plasma (Figure 23) and 0.78 ng/mL – 100 ng/mL (Figure 24) for liver samples. The method validation was performed using high (200 ng/mL), medium (50 ng/mL) and low (1.56 ng/mL) quality control (QC) samples for plasma and high (100 ng/mL), medium (50 ng/mL) and low (1.56 ng/mL) for the liver samples. The inter-day and intra-day accuracy and precision were below the FDA recommended guidelines (Guidance for Industry. Bioanalytical Method Validation, accessed June, 2010). The inter-day and intra-day precision for plasma was below 10% for the high and medium QC and below 15% for the low QC samples (Table 7). The inter-day and intra-day precision for liver tissue was below 12% for the high and medium QC and below 11% for the low QC samples (Table 8). The accuracy for all the groups

was between 90-100% (Table 7 and Table 8). The blank plasma and liver tissue chromatograms did not show any residue of drug or internal standard (Figure 20).

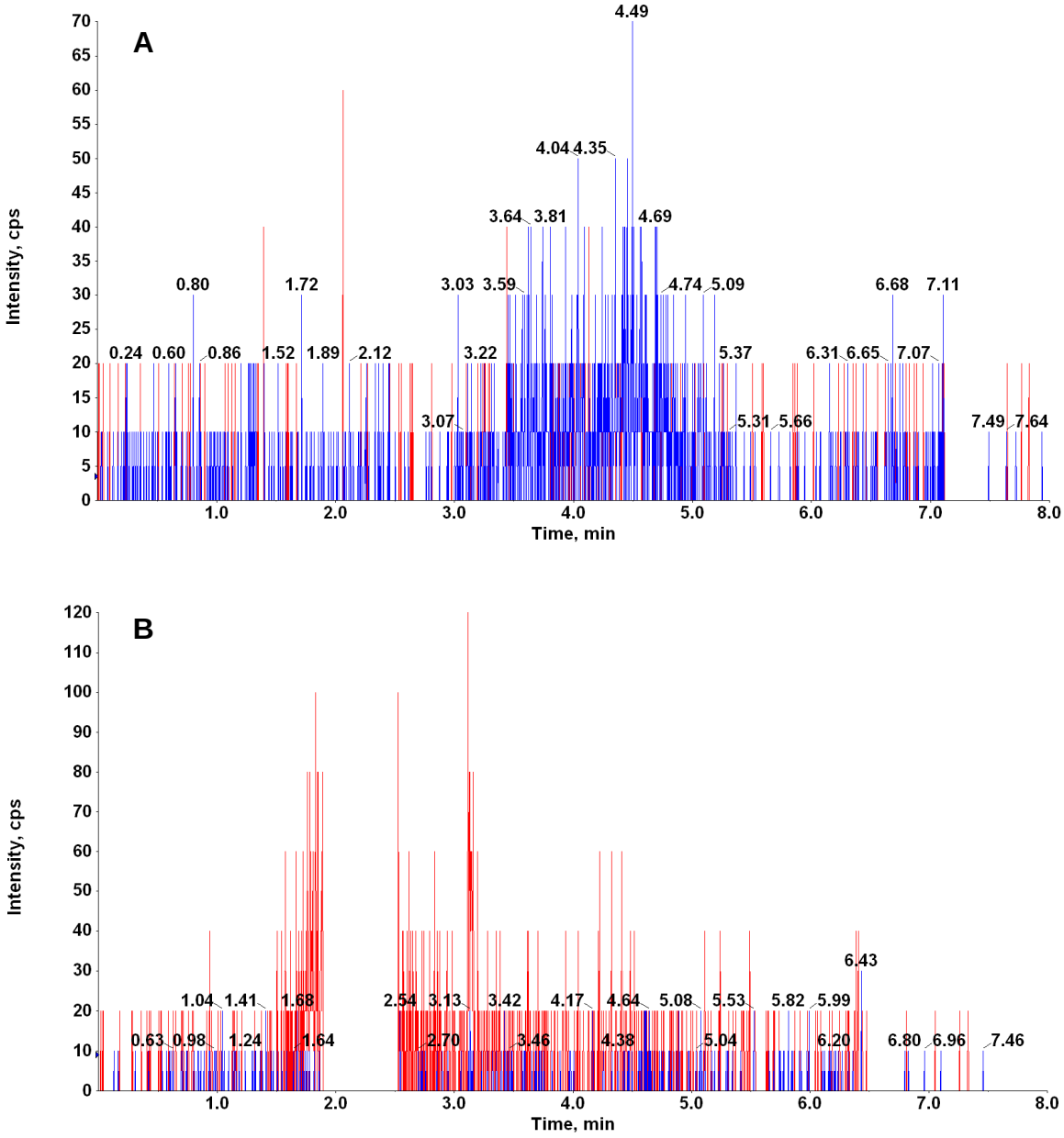


Figure 20: Chromatograms of Blank Plasma (A) and Blank Liver Tissue (B).

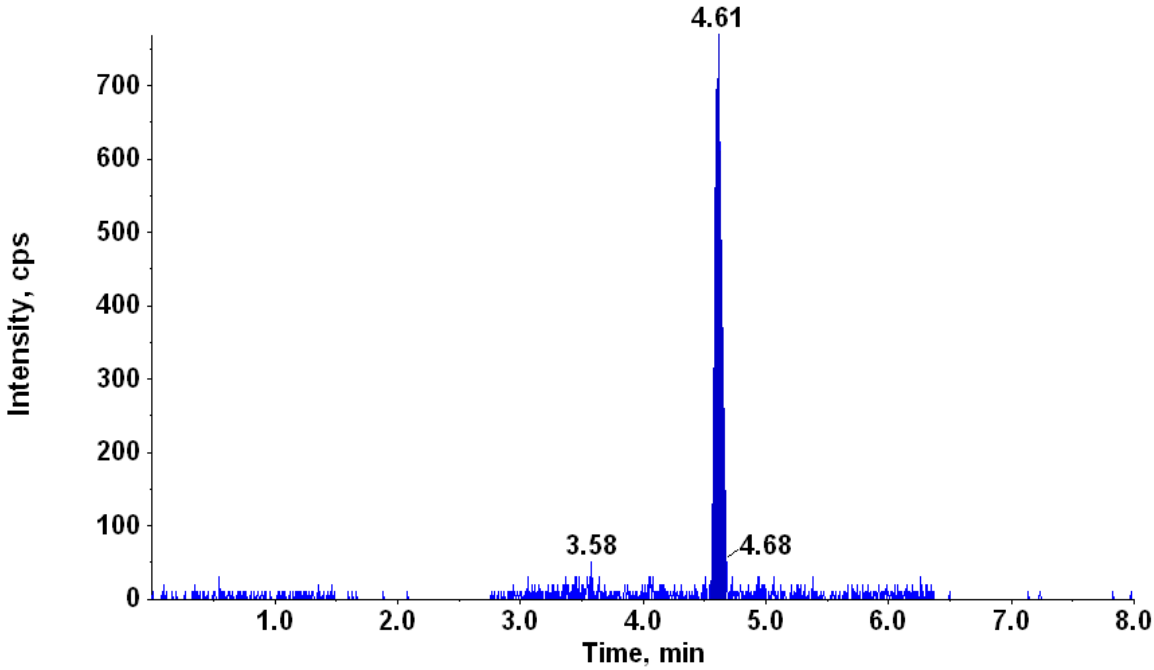


Figure 21: Chromatogram of DES at Lower Limit of Quantification (LLOQ = 0.78 ng/mL).

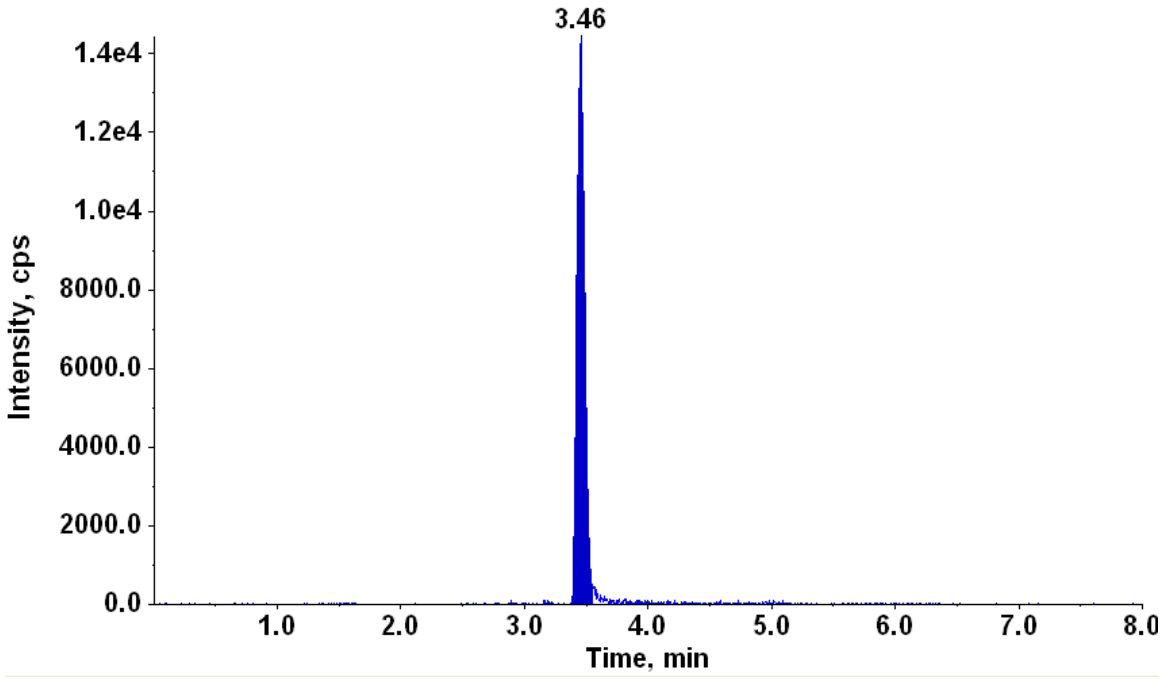


Figure 22: Chromatogram of Diadzein Internal Standard at 5 µM.

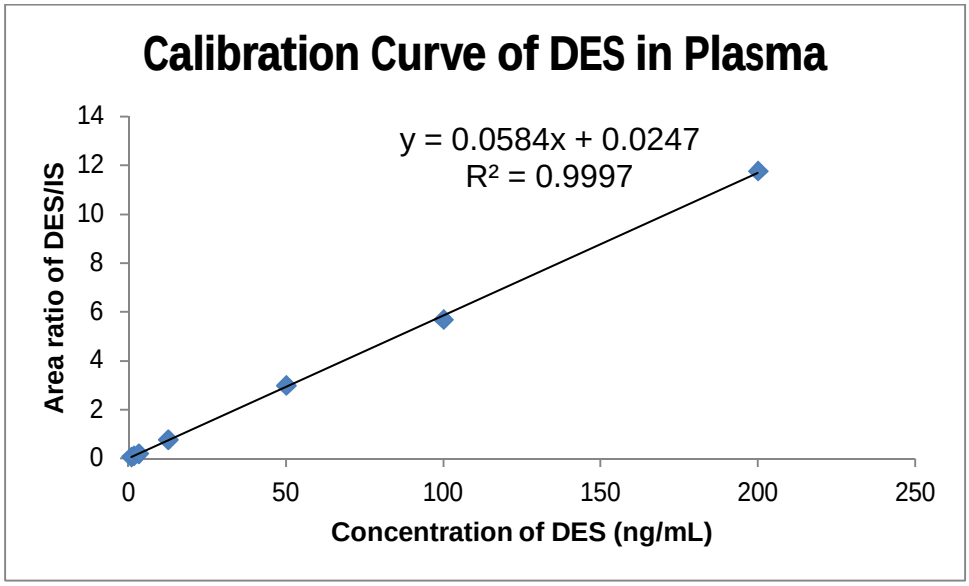


Figure 23: Representative LC/MS/MS Calibration Curve for DES in Plasma. Linear Range was 0.78 (LLOQ) - 200 ng/mL of DES in Plasma.

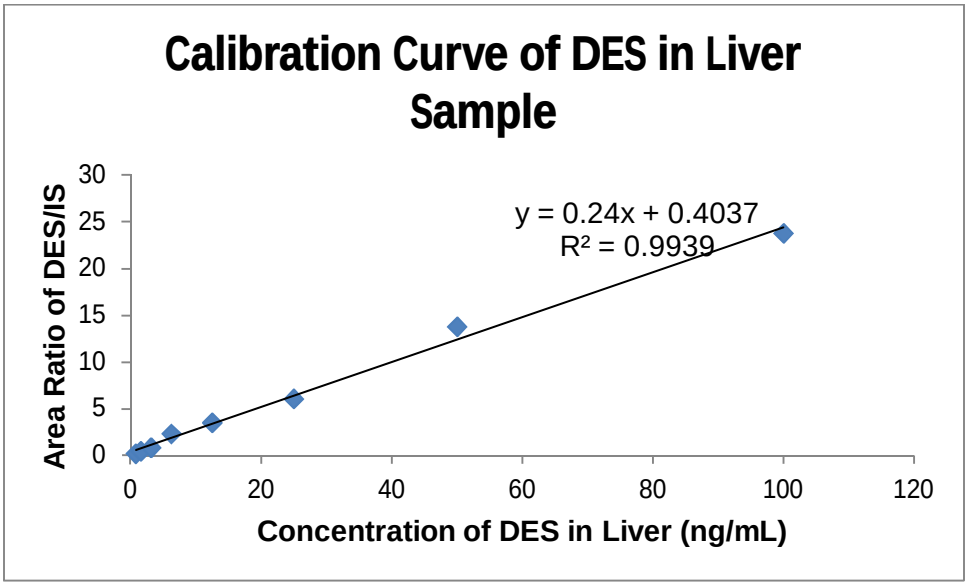


Figure 24: Representative LC/MS/MS Calibration Curve for DES in Liver Sample.

Linear Range was 0.78 (LLOQ) – 100 ng/mL of DES in Liver Sample.

Table 7: DES Assay Validation, Accuracy and Precision in Rat Plasma.

QC Sample (ng/mL)	Time	Calculated DES Concentration (ng/mL)	Accuracy[a] (%)	Precision[b] RSD (%)
Inter-day		N=3 each		
200	Day 1	186.67 ± 2.89	93.33	1.55
	Day 2	197.67 ± 14.01	97.86	7.09
	Day 3	198.73 ± 11.94	99.36	6.01
50	Day 1	50.70 ± 3.41	101.40	6.72
	Day 2	51.93 ± 0.81	103.87	1.56
	Day 3	51.92 ± 0.77	103.84	1.48
1.56	Day 1	1.48 ± 0.22	94.87	14.86
	Day 2	1.66 ± 0.15	106.62	8.76
	Day 3	1.59 ± 0.15	102.14	9.50
Intra-day		N=18		
200		197.73 ± 15.41	98.87	7.79
50		50.07 ± 4.61	100.14	9.20
1.56		1.52 ± 0.13	97.56	8.54

Assay was Validated Using Quality Control (QC) Samples at High (200 ng/mL), Medium (50 ng/mL) and Low (1.56 ng/mL). The Accuracy and Precision were within FDA Guidelines. Data Reported as Mean ± SD.

a is calculated DES concentration of QC samples divided by actual concentration × 100.

b is calculated by standard deviation of calculated DES concentration divided by the mean calculated DES concentration × 100 .

Table 8: DES Assay Validation, Accuracy and Precision in Rat Liver Sample.

QC Sample (ng/mL)	Time	Calculated DES Concentration (ng/mL)	Accuracy[a] (%)	Precision[b] RSD (%)
Inter-day		N=3 each		
100	Day 1	93.43 ± 10.88	93.43	11.56
	Day 2	101.84 ± 9.61	101.84	9.43
	Day 3	99.12 ± 2.96	99.12	2.99
50	Day 1	46.53 ± 3.01	93.07	6.46
	Day 2	49.74 ± 5.17	99.49	10.38
	Day 3	50.50 ± 0.71	101.00	1.40
1.56	Day 1	1.49 ± 0.16	95.51	10.80
	Day 2	1.55 ± 0.10	99.36	6.22
	Day 3	1.51 ± 0.06	97.01	3.64
Intra-day		N=18		
100		97.71 ± 6.97	97.71	7.13
50		49.60 ± 3.65	99.19	7.35
1.56		1.50 ± 0.09	96.23	6.18

Assay was Validated Using Quality Control (QC) Samples at High (100 ng/mL), Medium (50 ng/mL) and Low (1.56 ng/mL). The Accuracy and Precision were within FDA Guidelines. Data Reported as Mean ± SD.

a is calculated DES concentration of QC samples divided by actual concentration × 100.

b is calculated by standard deviation of calculated DES concentration divided by the mean calculated DES concentration × 100 .

4.5 Plasma Pharmacokinetics of DES Formulations in Rats:

The plasma pharmacokinetic profiles were evaluated for subcutaneously delivered NS-160, NS-500, DES co-solvent (solution group) and DES suspension (Figure 25). The nanosuspension pharmacokinetic profiles were also compared to the DES given PO in the form of DES oral suspension. The doses given for the NS-160, NS-500, DES suspension and DES oral suspension was 7 mg per rat and 0.125 mg per rat for the co-solvent group. The main objective of the pharmacokinetic evaluations was to evaluate whether the subcutaneous NS formulations were able to achieve sustained plasma levels of DES as compared to that of the other groups mainly that of the oral suspension group on the same dose basis.

After dosing the rats, blood samples were withdrawn at regular intervals and the plasma levels of DES in the blood samples were analyzed using the validated LC-MS/MS assay. Dose normalized plasma DES concentration-time profiles were constructed to evaluate the plasma exposure from the different formulations. The plasma DES concentrations from the NS, oral suspension and subcutaneous solution groups were used for compartmental modeling using the latest Phoenix WinNonlin 6.3 version. The compartmental modeling was used to determine and compare the various pharmacokinetic parameters of DES for each rat. Each parameter was given as the mean with standard deviation and statistical analysis

was performed using the t-test to determine the significance of the difference in the parameters.

From the different groups, the DES plasma levels were sustained for a period of 6 days from the NS-160 and NS-500 groups. The levels of DES from the DES solution group declined rapidly and were undetectable after 12 hours of dosing. The systemic exposure of DES with the subcutaneous suspension was undetectable after 48 hours. It is interesting to note that, on the same dose basis as compared to the nanosuspensions, the oral DES suspension had plasma DES exposure which declined rapidly and the DES levels were undetectable after 36 hours.

The nanosuspension formulations form a depot *in vivo* and provide a slow sustained release of DES over a period of 6 days.

The plasma pharmacokinetic profiles of DES were best described as 1 -compartmental model for subcutaneous solution and oral suspension groups whereas 2 -compartmental model for the NS-160 and NS-500.

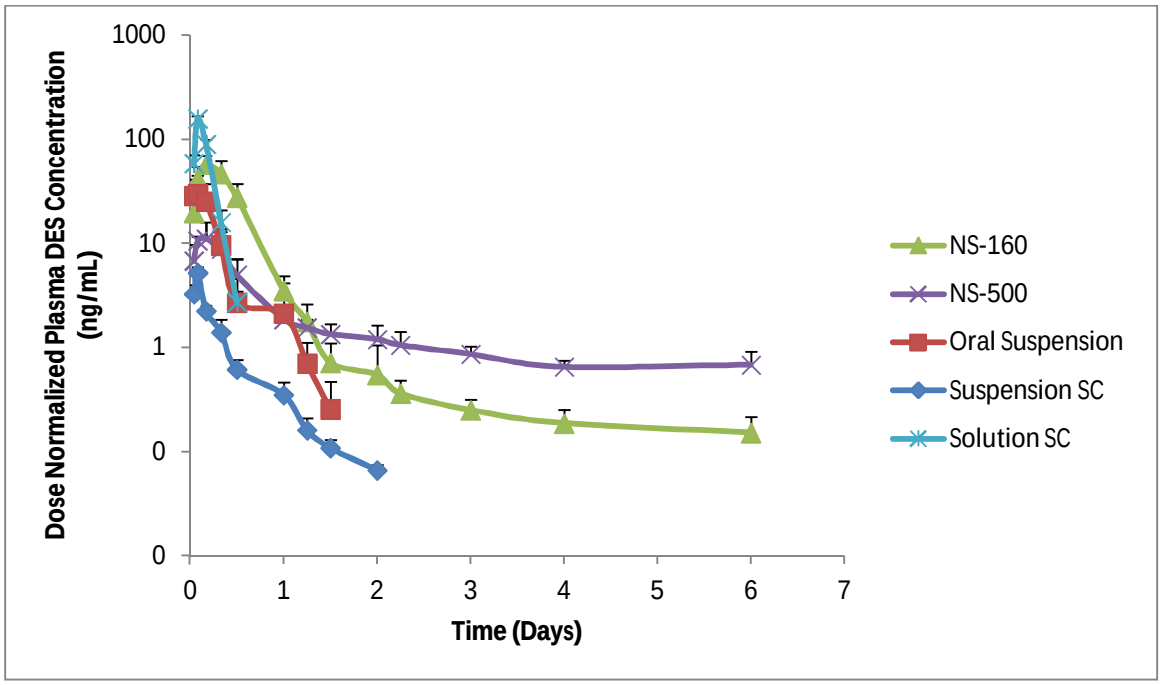


Figure 25: Plasma DES Concentration versus Time Profiles Following a Single Subcutaneous Dose of Diethylstilbestrol Nanosuspensions, Solution and Suspension as well as Oral Suspension *In Vivo*.

N=7 for NS-160 and NS-600, N=6 for Oral Suspension, N=3 for DES Solution SC and DES Suspension SC. All values are Mean ± SD.

4.5.1 Comparative Pharmacokinetics of DES in Rats Following Oral Suspension and Subcutaneous NS-160 and NS-500.

The pharmacokinetic parameters of NS-160, NS-500 and Oral suspension were compared in Table 9. The dose normalized $AUC_{0-\infty}$ of the subcutaneous nanosuspension formulations of NS-160 and NS-500 (736.70 ± 210.12 and 365.12 ± 99.90 ng*hr/mL respectively) were significantly higher than that of the oral DES suspension (207.06 ± 73.41 ng*hr/mL). The relative bioavailability was also higher with 3.56 for the NS-160 vs oral suspension and 1.76 for the NS-500 vs oral. The systemic exposure of NS-160 was significantly higher than that of NS-500. The nanosuspensions NS-160 and NS-500 not only yielded a longer systemic exposure but also prolonged the half life of the DES in the systemic circulation. The elimination half life was significantly prolonged for NS-160 and NS-500 (89.18 ± 31.88 and 146.22 ± 99.12 hr) as compared to that of the oral suspension (2.66 ± 0.84 hr). The dose normalized C_{max} was significantly higher for the NS-160 (58.35 ± 15.05 ng/mL) as compared to both the NS-500 and oral suspension (13.49 ± 2.29 ng/mL and 35.03 ± 12.39 ng/mL, respectively). The NS-160 and NS-500 had a significantly slower clearance (1.57 ± 0.54 and 2.98 ± 1.05 L/hr, respectively) from the systemic circulation as compared to that of the oral suspension (5.37 ± 1.86 L/hr). The NS-160 had a significantly slower clearance than that of the NS-500 group. The comparative time dependent systemic exposures of DES indicate a significantly higher AUC from 0 to 2 days

for NS-160 group as compared to NS-500. The NS-500 group shows a significantly higher AUC from 2-6 days (Table 11).

The subcutaneous NS-160 group had the highest systemic exposure, slowest clearance and highest C_{max} as compared to the subcutaneous NS-500 and oral suspension groups.

4.5.2 Comparative Pharmacokinetics of DES in Rats Following Subcutaneous Delivery of NS-160 and NS-500 and DES Solution.

The pharmacokinetic parameters of NS-160, NS-500 and subcutaneous solution were compared in Table 10. The dose normalized $AUC_{0-\infty}$ of solution group (600.34 ± 57.09) was similar to that of the NS-160 and significantly higher than that of the NS-500. The nanosuspensions NS-160 and NS-500 not only yielded a longer systemic exposure but also prolonged the half life of the DES in the systemic circulation. The elimination half life was significantly prolonged for NS-160 and NS-500 (89.18 ± 31.88 and 146.22 ± 99.12 hr) as compared to that of the solution group (1.55 ± 0.27 hr). The dose normalized C_{max} was significantly higher for the solution group (157.24 ± 10.47 ng/mL) as compared to both NS-160 and NS-500. The clearance of the solution group (1.67 ± 0.15 L/hr) was similar to that of NS-160 nm and significantly slower than that of NS-500 nm.

Table 9: Comparative Pharmacokinetic Parameters of DES from NS-160, NS-500 after Subcutaneous Administration and Oral DES Suspension.

Parameter	Unit	Mean ± SD		
		NS-160	NS-500	Oral
Cmax/dose	(ng/mL)/mg	58.35± 15.05*$	13.49 ± 2.29*	35.03 ± 12.39
Tmax	hr	4.28 ± 0.054*	3.06 ± 1.74	1.67 ± 0.97
AUC/Dose	(hr*ng/mL)/mg	736.70 ± 210.12*$	365.12 ± 99.90*	207.06 ± 73.41
Rel. F		3.56	1.76	
K01	1/hr	0.38 ± 0.16*	4.18 ± 4.99	3.10 ± 3.96
K10	1/hr	0.18 ± 0.03	0.08 ± 0.04$*	0.29 ± 0.12
K12	1/hr	0.015 ± 0.006$	0.17 ± 0.10	
K21	1/hr	0.010 ± 0.004	0.04 ± 0.05	
K01 HL	hr	2.11 ± 0.71*	1.63 ± 0.51	0.71 ± 0.07
K10 HL	hr	3.90 ± 0.59*$	11.54 ± 7.19*	2.66 ± 0.84
Alpha HL	hr	3.59 ± 0.56	3.24 ± 2.21	
Beta HL	hr	89.18 ± 31.88*	146.22 ± 99.12*	
V1_F	L	8.71 ± 2.90*$	48.02 ± 28.15*	20.81 ± 11.21
CL	L/hr	1.57 ± 0.54*$	2.98 ± 1.05*	5.37 ± 1.86
V2_F	L	15.07 ± 8.80$	322.58 ± 160.94	

* Significant difference in NS-160 and NS-500 with oral as reference. $Significant difference between NS-160 and NS-500. Unpaired t-test ($p < 0.05$)

Table 10: Comparative Pharmacokinetic Parameters of DES from NS-160, NS-500 and Solution after Subcutaneous Administration.

Parameter	Unit	Mean ± SD		
		NS-160	NS-500	Solution
Cmax/dose	(ng/mL)/mg	58.35 ± 15.05$^	13.49 ± 2.29^	157.24 ± 10.47
Tmax	hr	4.28 ± 0.054^	3.06 ± 1.74	2.00 ± 0.07
AUC/Dose	(hr*ng/mL)/mg	736.70± 210.12$	365.12±99.90^	600.34 ± 57.09
Rel. F		1.23	0.61	
K01	1/hr	0.38 ± 0.16	4.18 ± 4.99	1.44 ± 0.30
K10	1/hr	0.18 ± 0.03	0.08 ± 0.04$	0.46 ± 0.09
K12	1/hr	0.015 ± 0.006$	0.17 ± 0.10	
K21	1/hr	0.010 ± 0.004	0.04 ± 0.05	
K01 HL	hr	2.11 ± 0.71^	1.63 ± 0.51	0.50 ± 0.11
K10 HL	hr	3.90 ± 0.59$^	11.54 ± 7.19^	1.55 ± 0.27
Alpha HL	hr	3.59 ± 0.56	3.24 ± 2.21	
Beta HL	hr	89.18 ± 31.88^	146.22 ± 99.12^	
V1_F	L	8.71 ± 2.90$	48.02 ± 28.15	3.73 ± 0.60
CL	L/hr	1.57 ± 0.54$	2.98 ± 1.05^	1.67 ± 0.15
V2_F	L	15.07 ± 8.80$	322.58 ± 160.94	

^ significant difference in NS-160 and NS-500 with solution as reference.

$ significant difference between NS-160 and NS-500. Unpaired t-test ($p < 0.05$)

Table 11: Dose Normalized Comparative Time Dependent Systemic Exposures from NS-160 and NS-500.

Group	$AUC_{0\text{-}2\ days}$/Dose (ng*hr/mL/mg) Mean ± SD	$AUC_{2\text{-}6\ days}$/Dose (ng*hr/mL/mg) Mean ± SD	$AUC_{0\text{-}6\ days}$/Dose (ng*hr/mL/mg) Mean ± SD
NS-160	714.16 ± 207.32*	21.50 ± 6.82	735.85 ± 209.82*
NS-500	176.68 ± 36.41	74.50 ± 14.06*	251.18 ± 36.07

* Significantly greater AUC. Student's T-Test ($p < 0.05$).

Data Reported as Mean ± SD (n=7).

4.6 *In Vitro In Vivo* Correlation (IVIVC)

The $MDT_{in\ vitro}$ was calculated with the release of DES from the nanosuspensions upto 6 days since it showed increases in the cumulative release of the drug from the nanosuspension formulations. From the *in vitro* release studies after the rapid release of DES after 6 days, the levels of DES release are sustained but at very low concentrations. Also, the levels of DES *in vivo* were determined till 6 days. The *in vitro* and *in vivo* dissolution rates were the fastest for the subcutaneous solution group, with NS-160 and NS-500 in a descending order (Table 12). The lower particle size of the NS-160 group yielded a faster dissolution rate, both *in vivo* and *in vitro*, as compared to the NS-500 group. Thus particle size of the DES NS formulations plays a crucial role in the dissolution kinetics *in vivo.*

Linear relationship was established, R^2 = 0.8773 for the developed NS-160 and NS-500 formulations, between the *in vivo and in vitro* dissolution times (Figure 26). This linear relationship would enable us to estimate the *in vivo* dissolution rates from the *in vitro* dissolution evaluation for NS formulations with different particle sizes.

Table 12: IVIVC of Subcutaneous DES Formulations.

Formulation	MDT $_{in\ vivo}$	MDT $_{in\ vitro}$	*In vivo* dissolution rate (1/hr)	*In vitro* dissolution rate (1/hr)
Solution	3.44	4.74	0.29	0.211
NS-160	7.90	46.04	0.12	0.021
NS-500	33.55	56.53	0.03	0.017

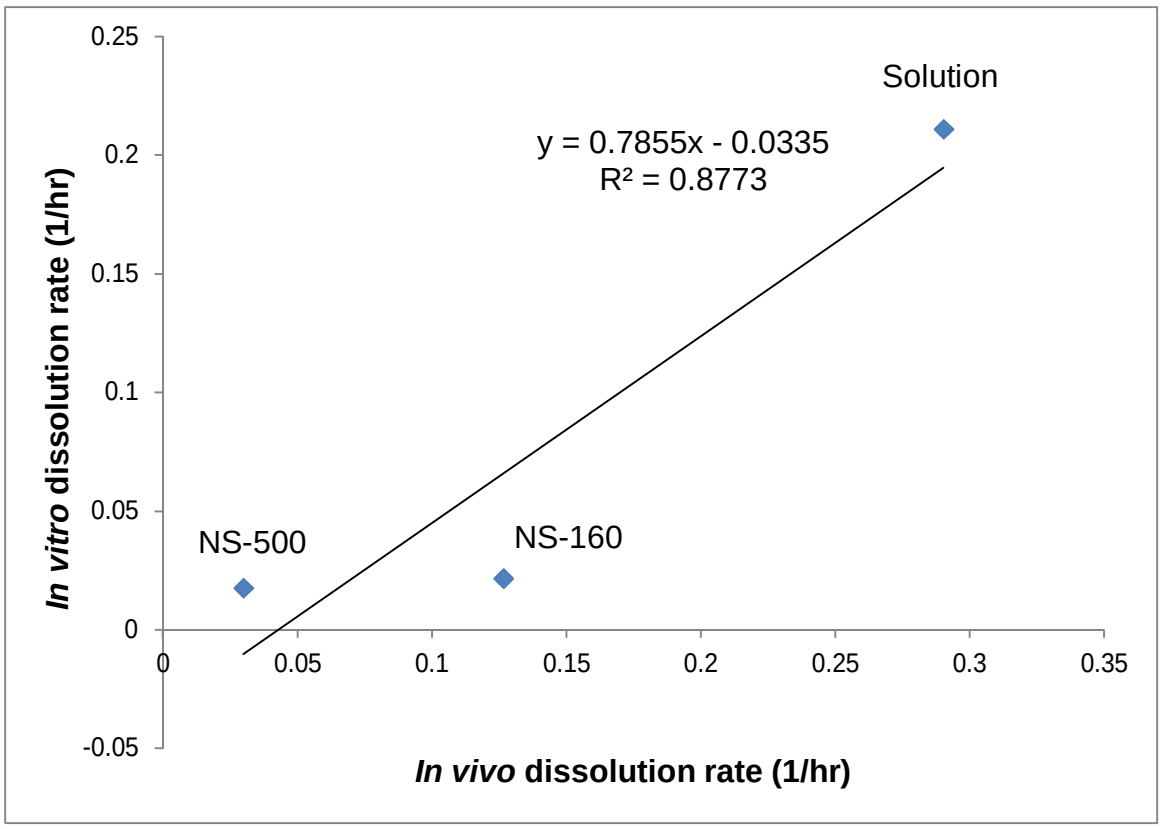


Figure 26: Level B Correlation: *In Vitro* Dissolution Rate Versus In Vivo Dissolution Rate.

4.7 Hepatic Exposure (Biodistribution) of DES in Rat Liver Following Subcutaneous NS-160, NS-500 and Oral Suspension Groups.

The concentrations of DES in rat livers were evaluated (n=4 at each time point) (Figure 27). The animals were administered a single dose of 7 mg of NS-160, NS-500 and oral suspension, respectively. At each sampling point the animals were sacrificed and liver was perfused with saline and harvested. The DES from the liver was extracted and evaluated by the validated LC-MS/MS assay.

The liver concentrations of DES from the oral suspension were significantly higher for the oral suspension than those of the two nanosuspension formulations (NS-160 and NS-500) delivered subcutaneously on the same dose basis. The hepatic exposure of DES was about 5 times higher for the oral route as compared to the subcutaneous nanosuspensions (Figure 28). Also, the L/P exposure ratio was more than 12 times higher in the oral group as compared to those of NS-160 and NS-500 (Table 13). This observation demonstrated the potential merit of NS formulations that on the same dose basis, even with significantly higher systemic exposures of DES, the subcutaneous NS-160 and NS-500 nanosuspension formulations yielded a significantly lower hepatic exposure of DES. This is an extremely intriguing and promising finding, since the hepatic exposure of estrogens (DES) has been directly linked to the changes in the blood clotting proteins which are synthesized by the liver leading to the

thromboembolic toxicity and cardiovascular complications. Decreasing the hepatic exposure may potentially result in a reduced DES associated thromboembolic toxicity and cardiovascular complications.

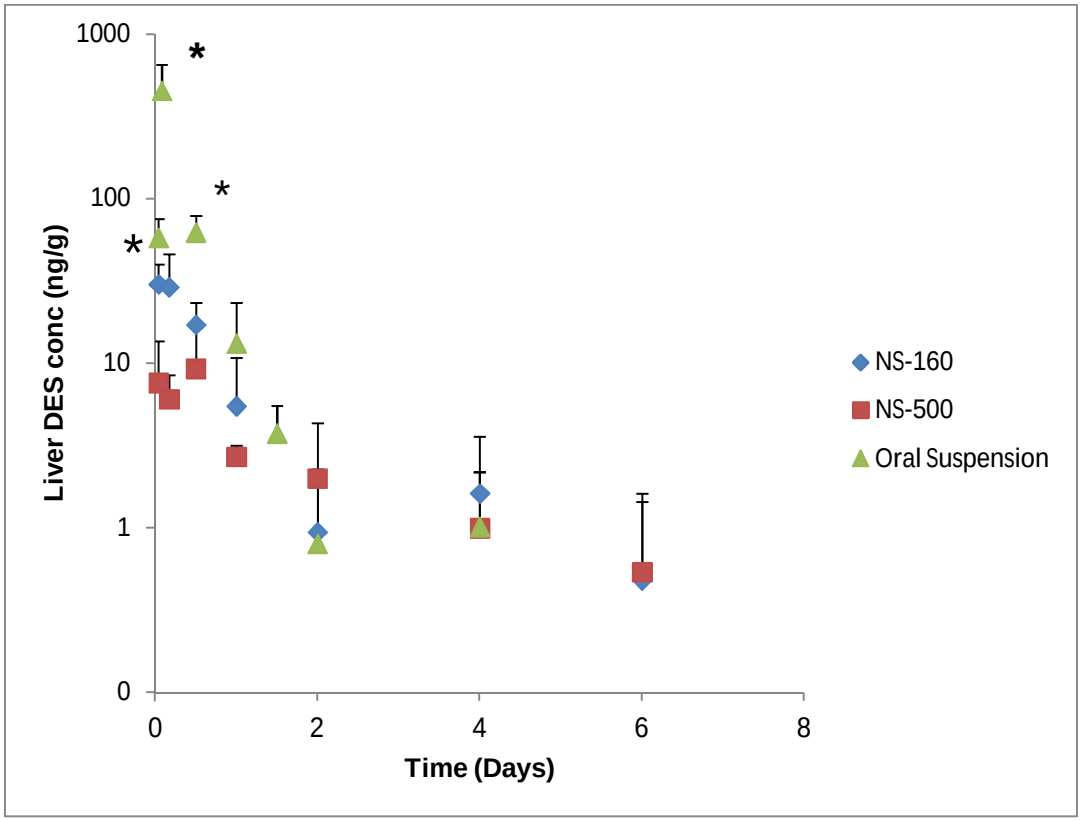


Figure 27: Liver DES Concentration versus Time Profiles Following a Single Subcutaneous Dose of Diethylstilbestrol Nanosuspensions and Oral Suspension *In Vivo*.

n=4 at Each Time Point. * Significantly Higher DES Concentrations. Student's T-Test ($p < 0.05$). All values are Mean ± SD

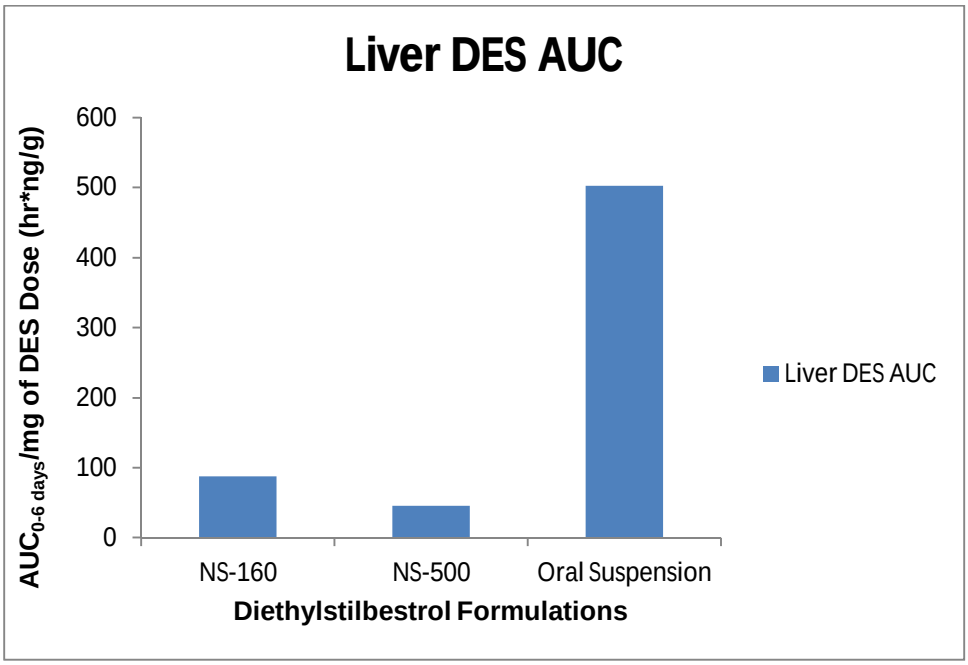


Figure 28: Dose Normalized $AUC_{0\text{-}6\ days}$ of NS-160, NS-500 and Oral Suspension.

N = 4 at each time point.

Table 13: Liver/Plasma (L/P) Ratio of NS-160, NS-500 and Oral Suspension after a Single Dose of 7 mg.

Formulation	AUC_{0-6}/mg of DES dose (hr*ng/mL)		
	Liver	Plasma	L/P ratio
NS-160	87.66	735.84	**0.12**
NS-500	45.97	290	**0.16**
Oral Suspension	502.60	243.18	**2.07**

4.8 Toxicity (Pharmacodynamic) Evaluations for Subcutaneous DES NS-160, NS-500 and Oral Suspension in Sprague-Dawley Rats.

4.8.1 Short-term Toxicity Study

The short term toxicity of the three groups of NS-160, NS-500 and oral suspension was evaluated based on the changes in the plasma FBG, ATIII levels and the actual rat blood clotting time (RBCT). A single 7 mg dose of DES was delivered in the three groups. Similarly two sham control groups of nanosuspension vehicle and oral suspending vehicle, respectively were evaluated for fibrinogen and ATIII levels.

As evaluated earlier, the NS-160 and NS-500 groups not only showed a significantly higher systemic exposure of DES but also lower hepatic exposures of DES than the oral DES suspension. Thus it is important to evaluate the resulting effects of the different groups on the coagulation cascade, particularly with effects on fibrinogen and ATIII. Significant changes in the levels of fibrinogen and ATIII in the coagulation cascade may indicate a potential increase in the toxicity complications.

The fibrinogen levels were detected till 6 days after a single dose of DES (Figure 29). The fibrinogen levels ranged from 4.13 to 0.16 mg/mL for NS-160, 3.71 to 0.17 mg/mL for NS-500 and 3.19 to 0.14 mg/mL for oral suspension. The levels of sham control remained constant for both the NS and oral sham control groups (Figure 30). However, when compared to the sham control, there was a

significantly greater decrease in the mean percent change of FBG for the oral group versus NS-160 at 0.33, 1.5 and 2 days; for the oral group versus NS-500 there was a significantly greater decrease in the mean percent change at 0.33 and 2 days (Figure 31). This indicates that there is decreasing trend in the change of fibrinogen levels in the oral suspension as compared to the NS-160 and NS-500 groups.

The antithrombin III levels were also detected till 6 days after the single dosing of DES for the three groups of NS-160, NS-500 and oral suspension group (Figure 32). The ATIII levels ranged from 325.68 to 445.84 µg/mL for NS-160, 317.81 to 393.21 µg/mL for NS-500 and 276.60 to 322.64 µg/mL for oral suspension. The levels of sham controls also remained constant for both the NS and oral sham control groups (Figure 33). When compared to the sham control, there was a significantly greater decrease in the mean percent change of FBG for the oral group versus NS-160 from 0.04 to 6 days except for 0.17 and 0.33 days and for the oral group versus NS-500 there was significantly greater decrease in the mean percent change from 0.04 to 6 days except 0.33 day (Figure 34). This demonstrated a significantly greater decrease in the levels of ATIII in presence of DES oral suspension as compared to the NS-160 and NS-500 groups.

The comparison with the sham control is important since it gives us the range of levels of FBG and ATIII level profiles over the same duration of time (6 days) without the effect of DES.

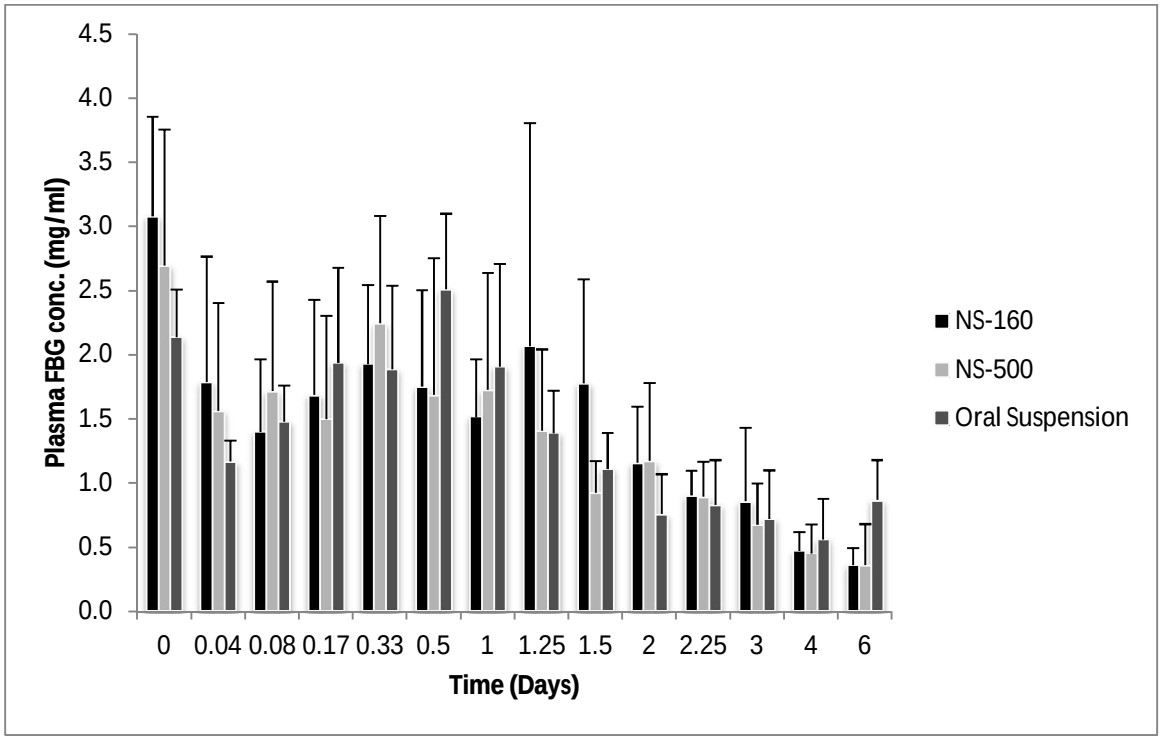


Figure 29: Plasma Fibrinogen Profiles after Single Dose (7 mg) of Subcutaneous NS-160, NS-500 and Oral Suspension of DES in Rats for 6 days.

Data Reported as Mean ± SD (n=5).

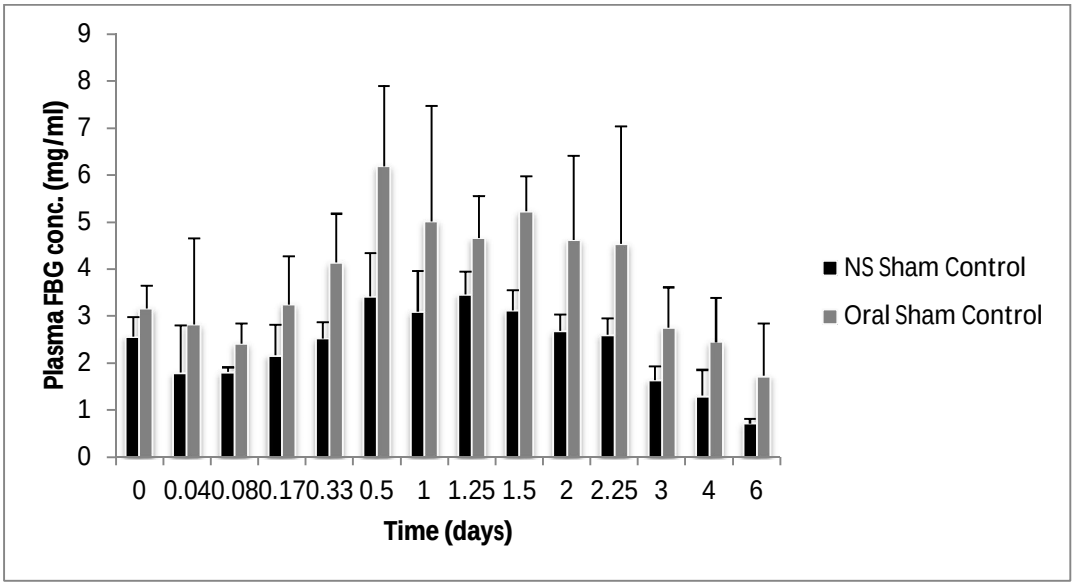


Figure 30: Plasma Fibrinogen Profiles from Sham Controls of Nanosuspension and Oral Suspension after a Single Dose of Nanosuspension Vehicle and Oral Suspending Vehicle in Rats.

Data Reported as Mean ± SD (n=3).

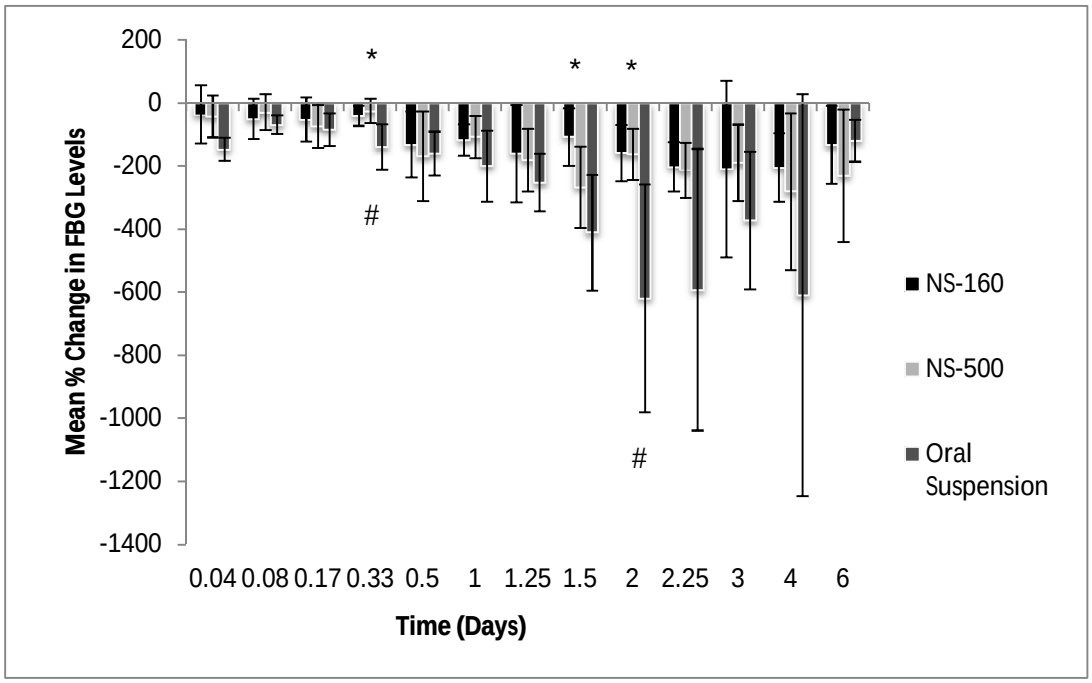


Figure 31: Mean Percentage Change Profiles in Fibrinogen after Single Dose (7 mg) of NS-160, NS-500 and Oral Suspension.

Data reported as Mean ± SD (n=5).

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

* indicates significant difference between NS-160 and Oral Suspension ($p<0.05$).

# indicates significant difference between NS-500 and Oral Suspension ($p<0.05$).

ANOVA with post-hoc Multiple Tukey's.

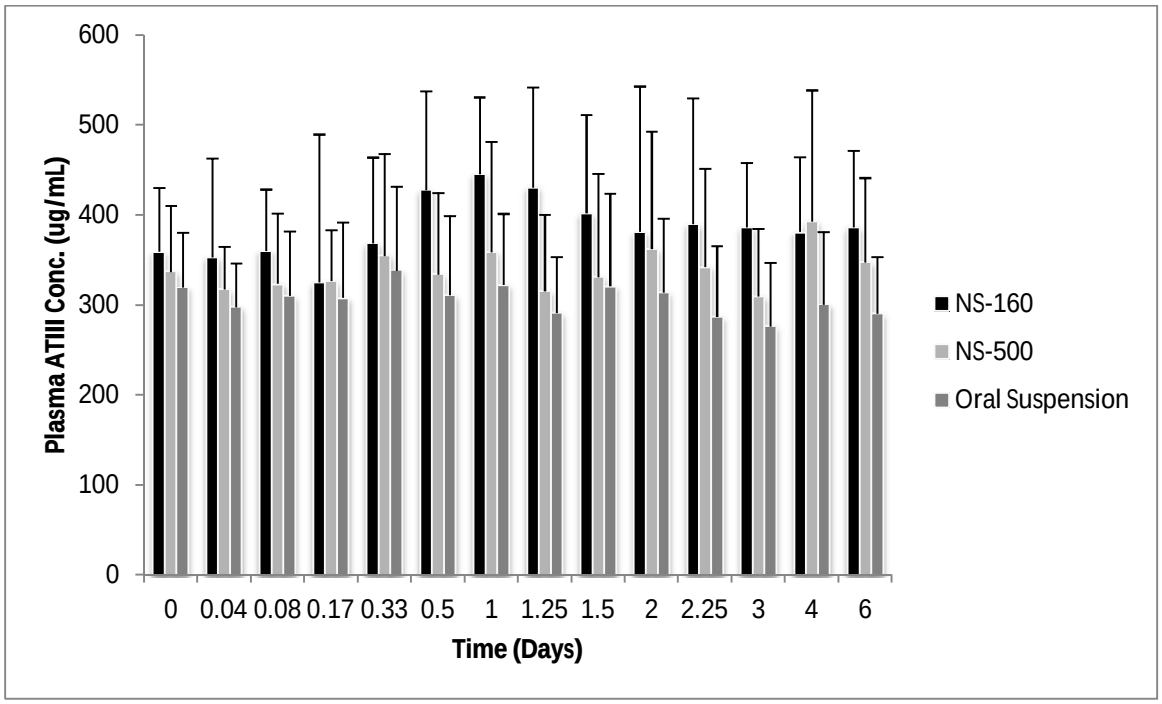


Figure 32: Plasma Anti-thrombin III Profiles after Single Dose (7 mg) of Subcutaneous NS-160, NS-500 and Oral Suspension of DES in Rats for 6 days.

Data Reported as Mean ± SD (n=5).

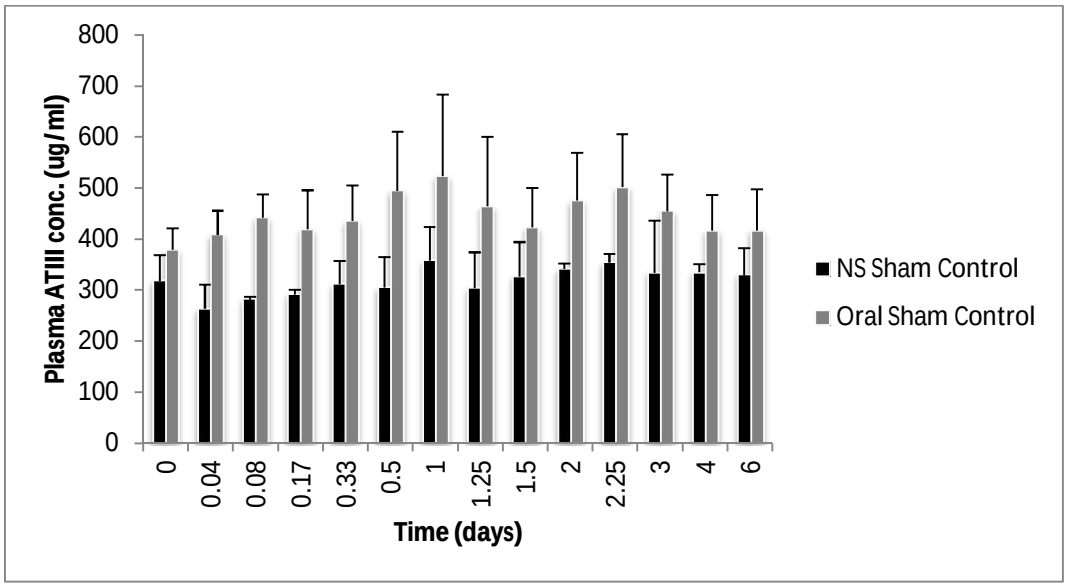


Figure 33: Plasma Anti-thrombin III Profiles from Sham Controls of Nanosuspension and Oral Suspension after a Single Dose of Nanosuspension Vehicle and Oral Suspending Vehicle in Rats.

Data Reported as Mean ± SD (n=3).

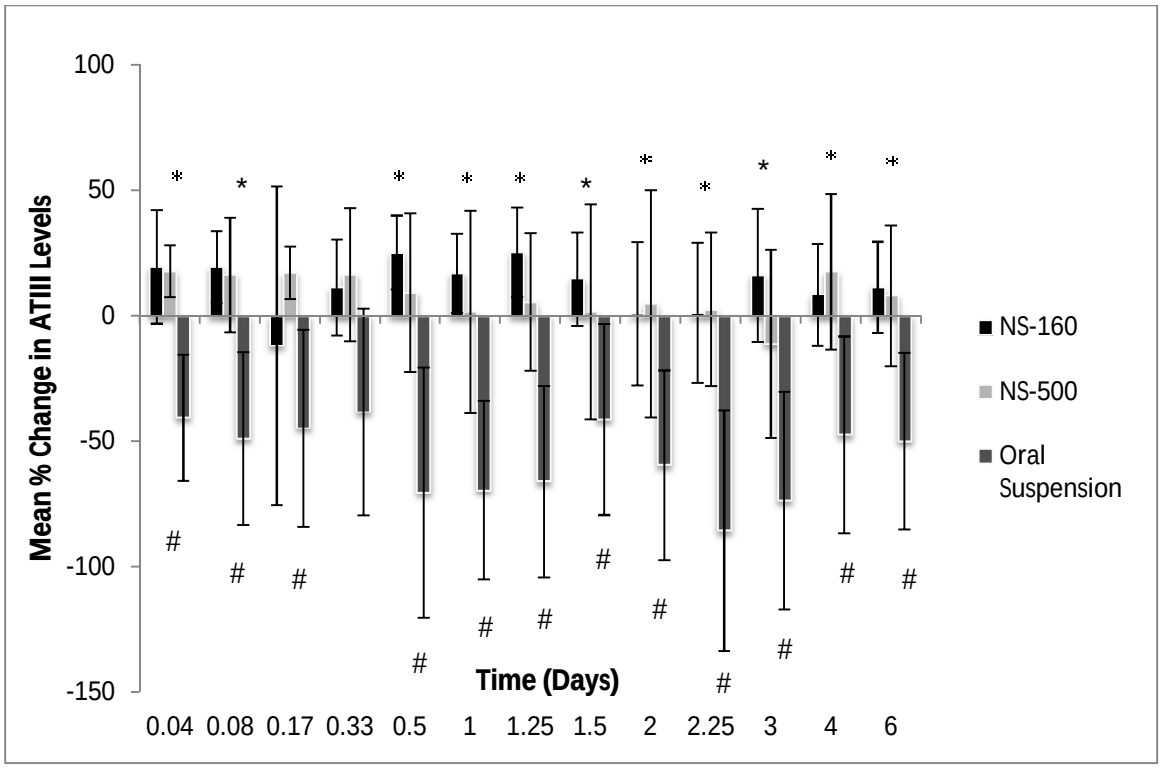


Figure 34: Mean Percentage Change Profiles in Anti-thrombin III after Single Dose (7 mg) of NS-160, NS-500 and Oral Suspension.

Data reported as Mean ± SD (n=5).

The Anti-Thrombin III levels for the study animals were compared to the sham controls.

* significant difference between NS-160 and Oral Suspension ($p<0.05$).

significant difference between NS-500 and Oral Suspension ($p<0.05$). ANOVA with post-hoc Multiple Tukey's.

4.8.2 Correlation between Levels of Fibrinogen and Antithrombin III

The percent changes of FBG as compared to the respective sham control were correlated with the percent changes in ATIII over a period of 6 days (Figure 35). A visible trend of higher percent changes in the levels of FBG and ATIII for the oral suspension was observed as compared to the two nanosuspension formulations (NS-160 and NS-500) given subcutaneously. There was a greater decrease in the fibrinogen and ATIII levels for the oral group as compared to NS-160 and NS-500. The levels of percent change in ATIII showed a positive change for NS-160 and NS-500 but a negative change for the oral suspension group. This indicated that on the same dose basis, the oral suspension of DES showed larger changes in the clotting factors which was less prominent in the subcutaneous NS-160 and NS-500 formulations.

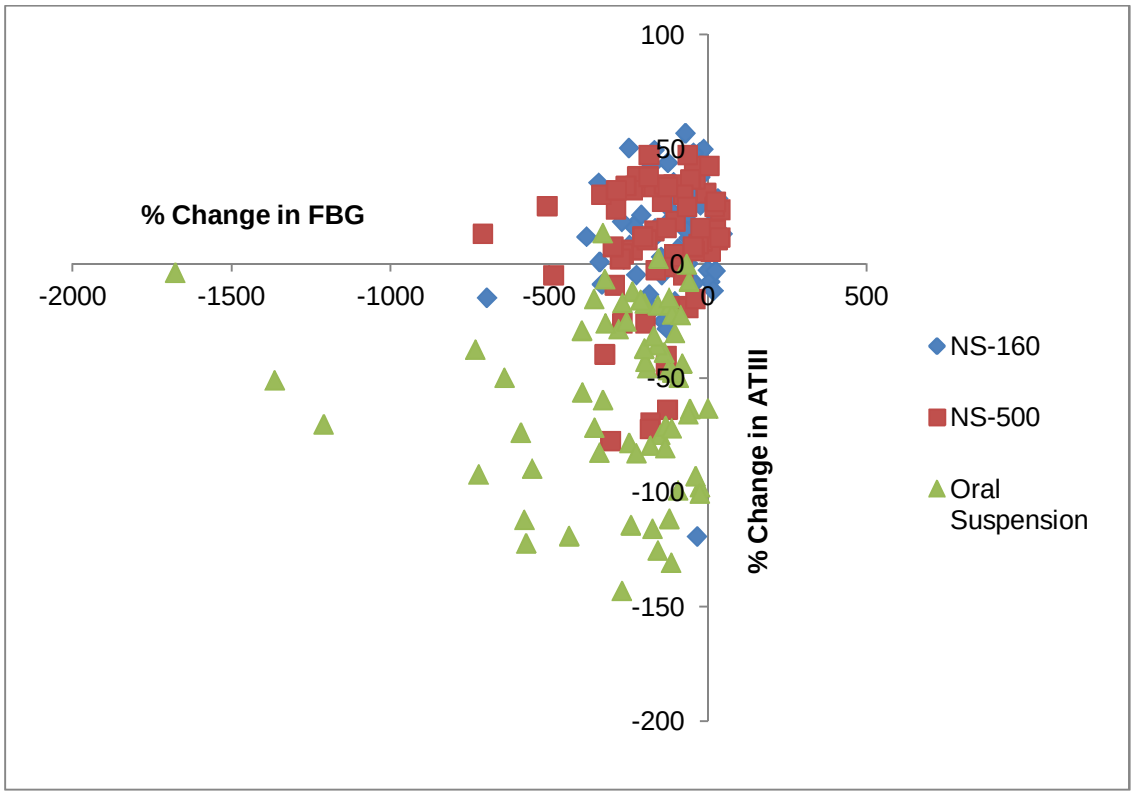


Figure 35: Correlation between Mean Percent Changes in Fibrinogen versus Mean Percent Changes in Anti-thrombin III for NS-160, NS-500 and Oral Suspension.

4.8.3 Changes in the Rat Blood Clotting Time with NS-160, NS-500 and Oral Suspension (Short-Term Evaluations).

The changes in the rat blood clotting time were evaluated after a single dose of 7 mg of DES NS-160, NS-500 and oral suspension groups for a period of 6 days (Figure 36). The clotting time remained constant over a period of 6 days for the two subcutaneous nanosuspension formulations NS-160 and NS-500. The clotting time decreased significantly with the oral suspension over the same period of 6 days. There is no significant change in the clotting time from NS-160, NS-500 and oral suspension till 1.5 days but from day 2 onwards there was a significant decrease in the levels of clotting time. This indicates that the blood clots significantly faster in the oral group as compared to the two nanosuspensions. This could mean that the tilt and changes in the clotting factors could have a direct effect on the rapid clotting of blood internally. This could lead to thromboembolism and cardiovascular toxicities.

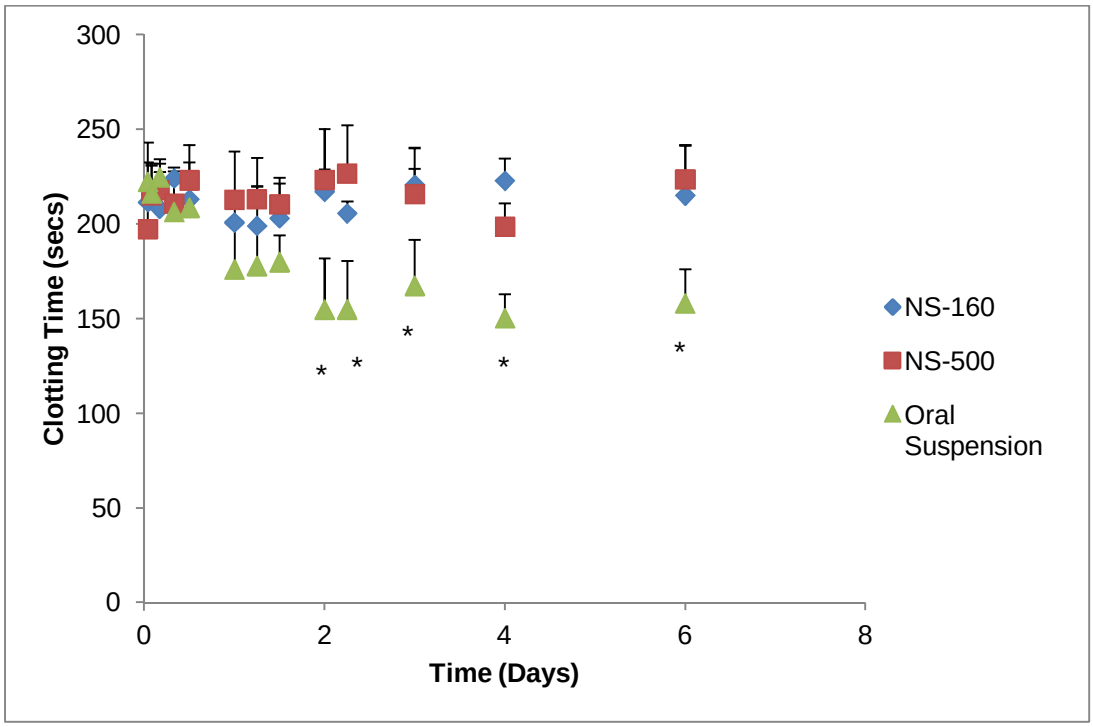


Figure 36: Rat Blood Clotting Time Measured Over 6 days for NS-160, NS-500 and Oral Suspension.

* Significant difference between Oral Suspension as compared to NS-160 and NS-500. n= 4 for NS-160, NS-500 and Oral Suspension.

4.8.4 Long-Term Toxicity Study

The long term toxicity of the three groups NS-160, NS-500 and oral suspension was evaluated based on the changes in the plasma FBG, ATIII levels. Two 7 mg doses of DES of NS-160 and NS-500 groups were delivered every 14 days for a period of 28 days (2 doses), whereas four 7 mg doses of the DES oral suspension were delivered weekly for a period of 28 days. Similarly, two sham control groups of nanosuspension vehicle and oral suspending vehicle, respectively, were evaluated for FBG, ATIII levels.

Similar to that evaluated earlier, in the short-term toxicity evaluations, it was observed that the oral DES suspensions caused significantly greater decreases in the levels of FBG and ATIII over a period of 28 days as compared to the two nanosuspension formulations given subcutaneously. Since, DES therapy is a long-term treatment option for prostate cancer, it is important to study the effect of the different groups on the changes in the coagulation cascade on a long-term basis (28 days). The greater decrease in FBG and ATIII as compared to the sham control could lead to a potential increase in the thromboembolic toxicity and cardiovascular complications.

The fibrinogen levels were detected till 28 days after multiple dosing of DES (Figure 37). The FBG levels ranged from 1.6 to 0.13 mg/mL for NS-160, 0.95 to 0.2 mg/mL for NS-500 and 1.38 to 0.063 mg/mL for oral suspension. There was

an overall trend of decrease in levels of fibrinogen over 28 days. The decrease was more pronounced for the oral group than the NS-160 and NS-500 groups. The levels of sham controls remained constant for both the NS and oral sham control groups (Figure 38). But when compared to the sham control, there was a significantly greater decrease in the mean percent change of FBG for the oral group versus NS-160 and NS-500 at 1, 7, 14 and 28 days (Figure 39). This indicates that there is a greater decreasing trend in the change of fibrinogen levels in the oral suspension as compared to the NS-160 and NS-500 groups. This significant decrease for the oral suspension was observed at different time points as compared to the NS-160 and NS-500 group but there was no difference between the NS groups.

The antithrombin III levels were also detected for 28 days after multiple dosing of DES for the three groups of NS-160, NS-500 and oral suspension group (Figure 40). The ATIII levels ranged from 334.78 to 338.58 µg/mL for NS-160, 302.07 to 256.2 µg/mL for NS-500 and 270.91 to 250.33 µg/mL for oral suspension. The levels of sham controls also remained constant for both the NS and oral sham control groups (Figure 41). But when compared to the sham control, there is a significantly greater decrease in the mean percent change of FBG for the oral group versus NS-160 at 1, 7, 14, 21 and 28 days, and for the oral group versus NS-500 there was significantly greater decrease in the mean percent change at 1 and 7 days (Figure 42). This indicated that there is a significantly greater

decrease in the levels of ATIII in presence of DES oral suspension as compared to the NS-160 and NS-500 groups. Also, the decrease in the levels of ATIII was less pronounced in the NS-160 group as compared to the NS-500 group.

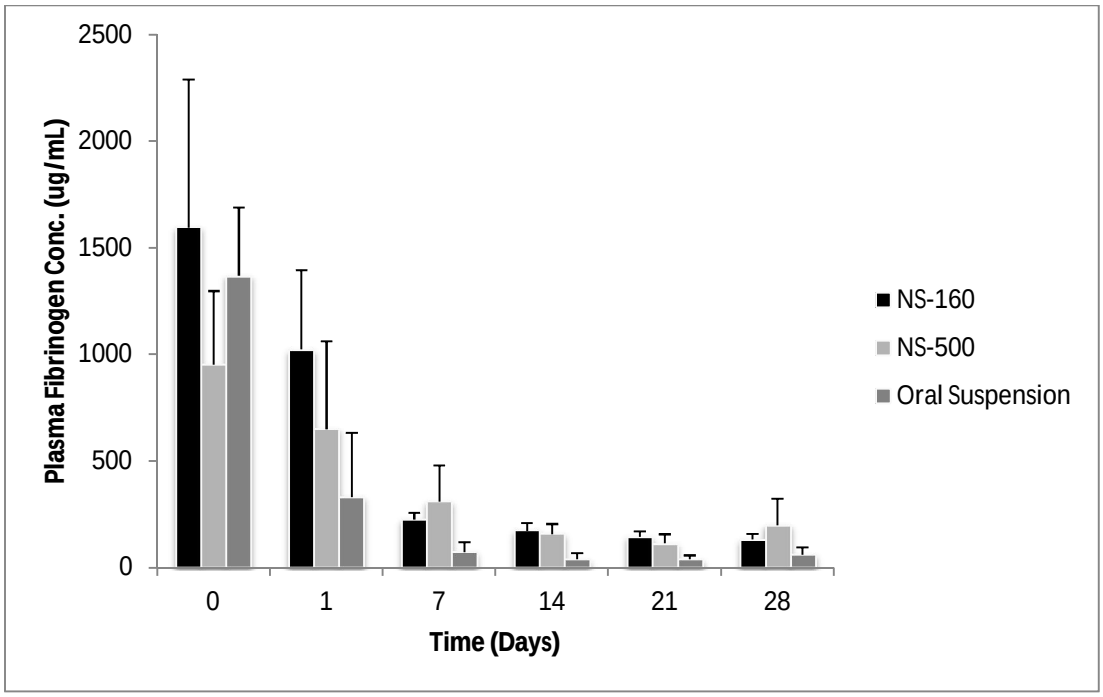


Figure 37: Plasma Fibrinogen Profiles after Multiple Doses of Subcutaneous NS-160, NS-500 and Oral Suspension of DES in Rats for 28 days.

Data Reported as Mean ± SD (n=6).

The NS-160 and NS-500 groups were dosed with 7 mg DES every two weeks for a total of two doses over 28 days.

The Oral Suspension was dosed with 7 mg / week DES for a total of four doses over 28 days.

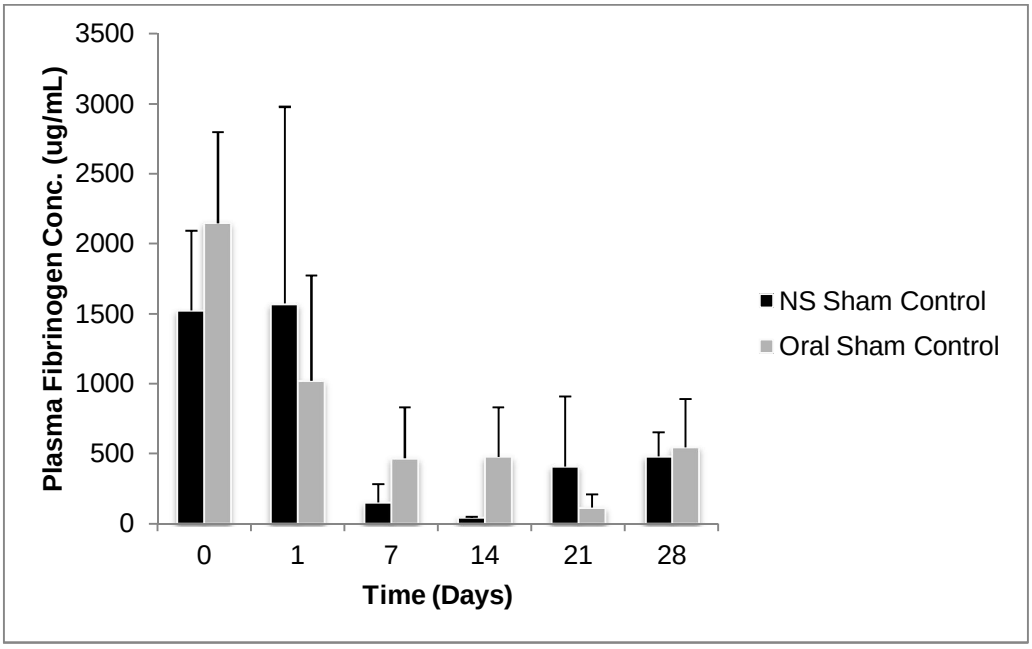


Figure 38: Plasma Fibrinogen Profiles from Sham Controls of Nanosuspension and Oral Suspension after Multiple Doses of Nanosuspension Vehicle and Oral Suspending Vehicle in Rats.

Data Reported as Mean ± SD (n=4).

The NS Vehicle group was dosed every two weeks for a total of two doses over 28 days.

The Oral Suspension Vehicle was dosed every week for a total of four doses over 28 days.

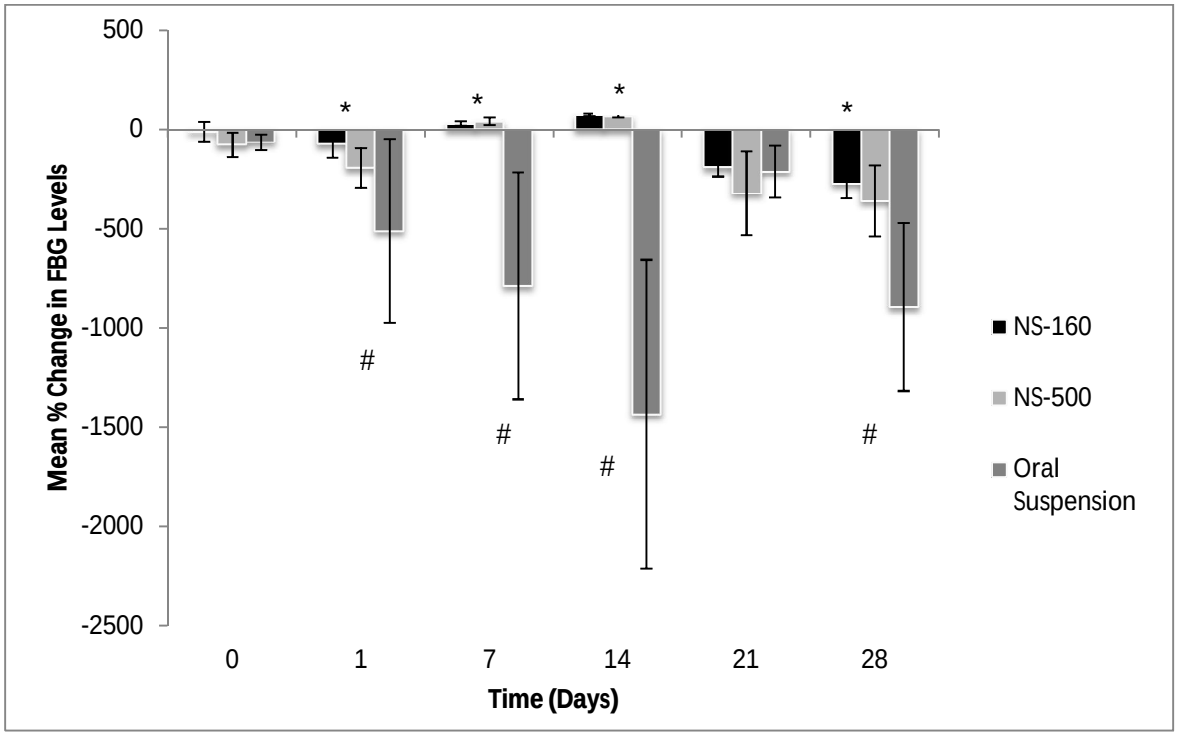


Figure 39: Mean Percentage Change Profiles in Fibrinogen after Multiple Doses of NS-160, NS-500 and Oral Suspension.

Data reported as Mean ± SD (n=6).

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

* indicates significant difference between NS-160 and Oral Suspension ($p<0.05$).

# indicates significant difference between NS-500 and Oral Suspension ($p<0.05$).

Unpaired T-Test ($p < 0.05$)

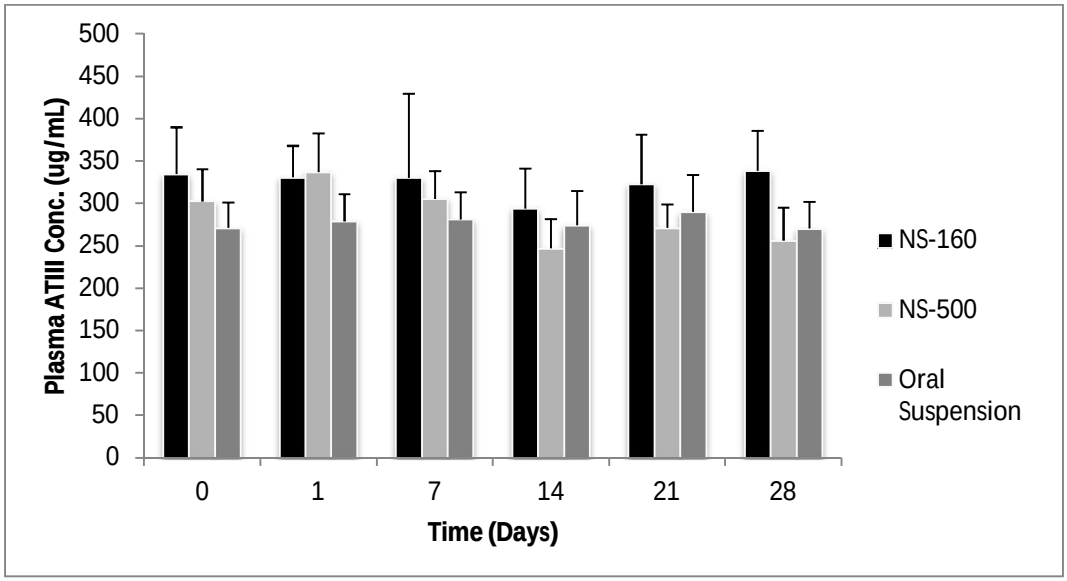


Figure 40: Plasma Anti-thrombin III Profiles after Multiple Doses of Subcutaneous NS-160, NS-500 and Oral Suspension of DES in Rats for 28 days.

Data Reported as Mean ± SD (n=6).

The NS-160 and NS-500 groups were dosed with 7 mg DES every two weeks for a total of two doses over 28 days.

The Oral Suspension was dosed with 7 mg / week DES for a total of four doses over 28 days.

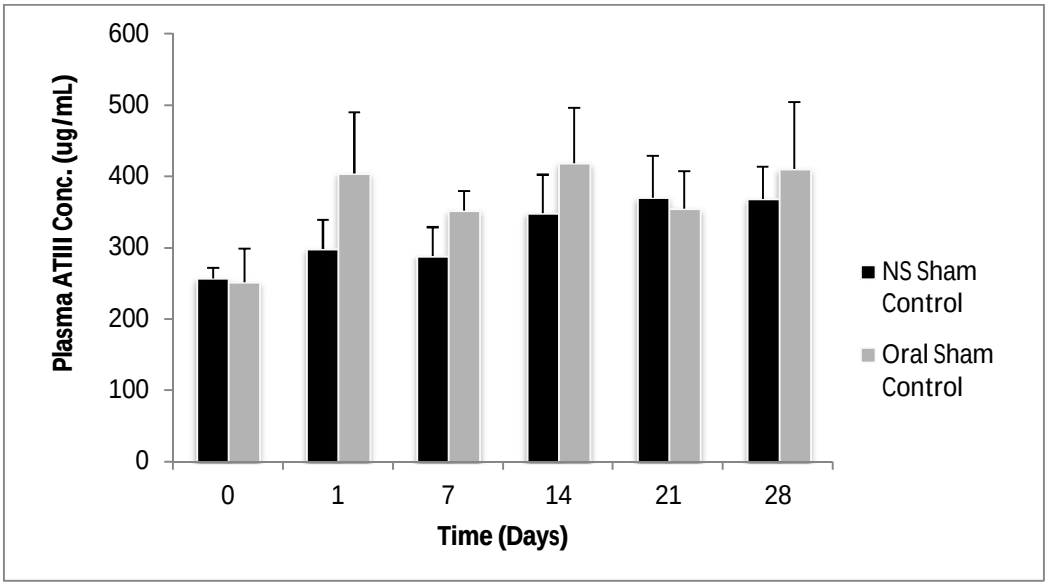


Figure 41: Plasma Anti-thrombin III Profiles from Sham Controls of Nanosuspension and Oral Suspension after Multiple Doses of Nanosuspension Vehicle and Oral Suspending Vehicle in Rats.

Data Reported as Mean ± SD (n=4).

The NS Vehicle group was dosed every two weeks for a total of two doses over 28 days.

The Oral Suspension Vehicle was dosed every week for a total of four doses over 28 days.

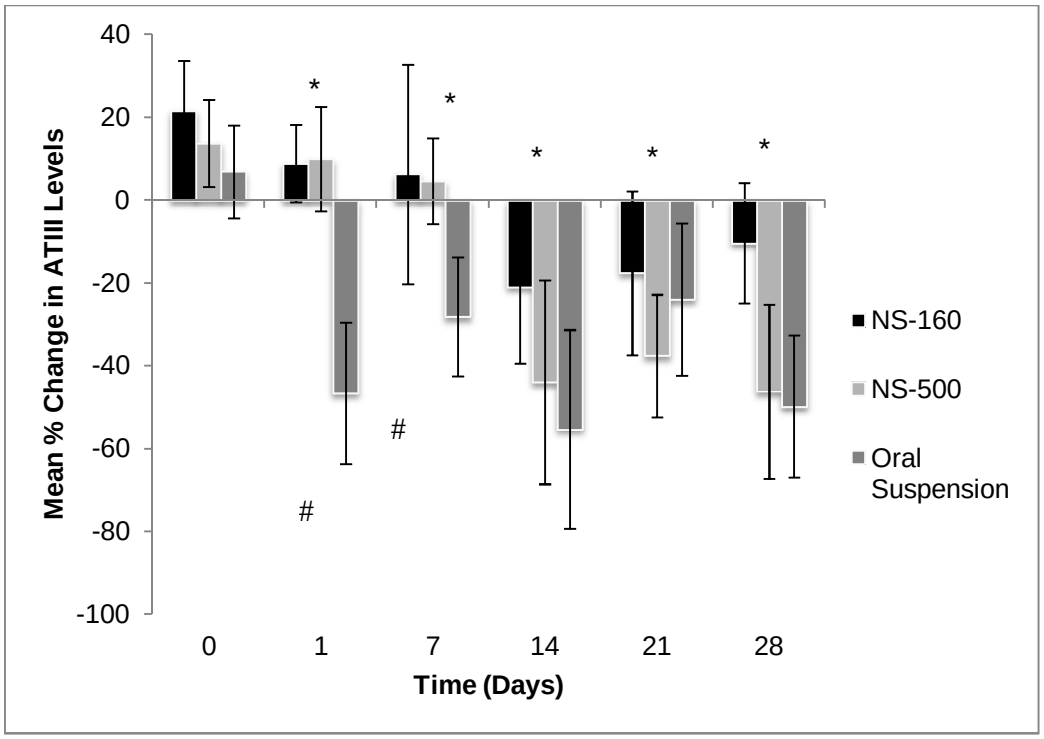


Figure 42: Mean Percentage Change Profiles in Anti-thrombin III after Multiple Doses of NS-160, NS-500 and Oral Suspension.

Data reported as Mean ± SD (n=6).

The Anti-thrombin III levels for the study animals were compared to the sham controls.

* indicates significant difference between NS-160 and Oral Suspension ($p<0.05$).

# indicates significant difference between NS-500 and Oral Suspension ($p<0.05$).

Unpaired T-Test.

4.8.5 Changes in the Rat Blood Clotting Time with NS-160, NS-500 and Oral (Long term).

The changes in the rat blood clotting time were evaluated after multiple dosing of DES NS-160, NS-500 and oral suspension groups for a period of 28 days (Figure 43). The dosing was the same as mentioned in the section 4.8.4. The clotting time remained constant over a period of 28 days for the two subcutaneous nanosuspension formulations NS-160 and NS-500. The clotting time decreased significantly with the oral suspension over the period of 28 days. There was no significant change in the clotting time of NS-160, NS-500 and oral suspension at 0 days but from day 1 on, there was a significant decrease in the levels of clotting time with the oral suspension. This indicated that even though the clotting times of the animals in the three groups before dosing the animals with DES was the same, the blood clots significantly faster in the oral group as compared to the two nanosuspensions over a period of 28 days. The mean change in clotting time as compared to its own pre-dosing values also, indicated a significant decrease in the oral suspension group as compared to the NS-160 and NS-500 (Figure 44). The sham control groups of NS vehicle and oral suspending vehicle without DES showed constant CT over a period of 28 days (Figure 45). This could mean that the tilt and changes in the clotting factors could have a direct effect on the rapid clotting of blood internally. Thus, long-term dosing of subcutaneous DES

nanosuspension formulations could be potentially safer than the long term oral DES on the same dose basis.

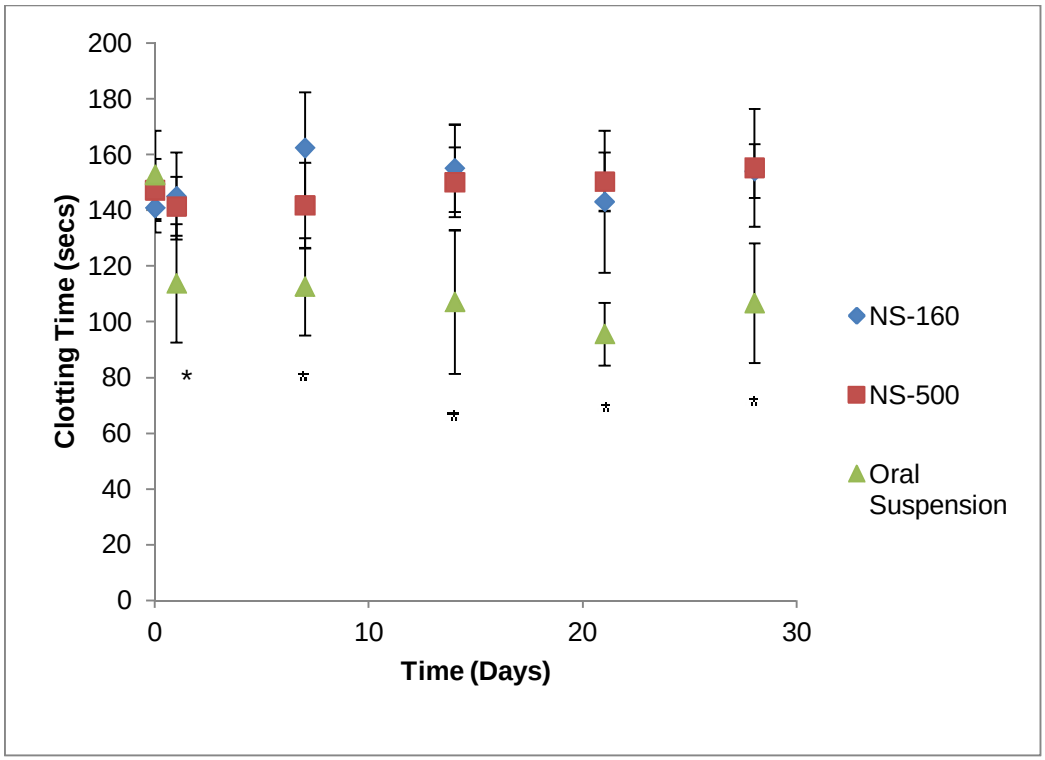


Figure 43: Rat Blood Clotting Time Measured over 28 Days for NS-160, NS-500 and Oral Suspension.

* indicates a significant difference between oral group as compared to NS-160 and NS-500 groups using one way ANOVA with post hoc analysis. ($p < 0.05$). All values are Mean ± SD (n=6).

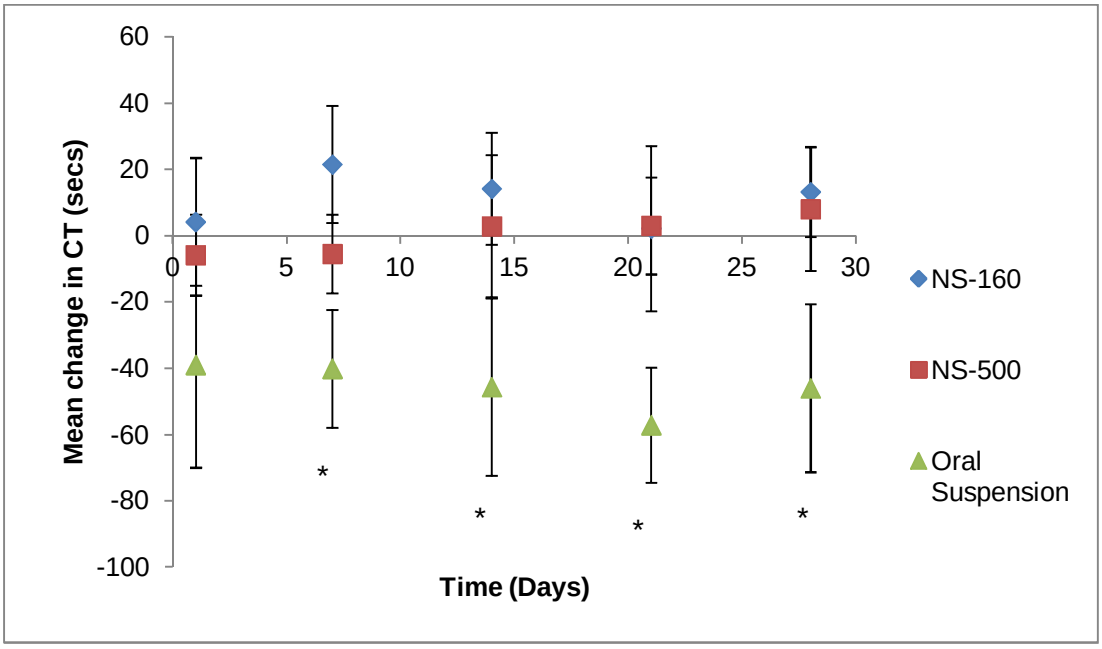


Figure 44: Mean Change in Clotting Time as Compared to Clotting Time at 0 Day.

* indicates a significant difference between oral group as compared to NS-160 and NS-500 groups using one way ANOVA with Multiple Tukey's post hoc analysis ($p < 0.05$). All values are Mean ± SD (n=6).

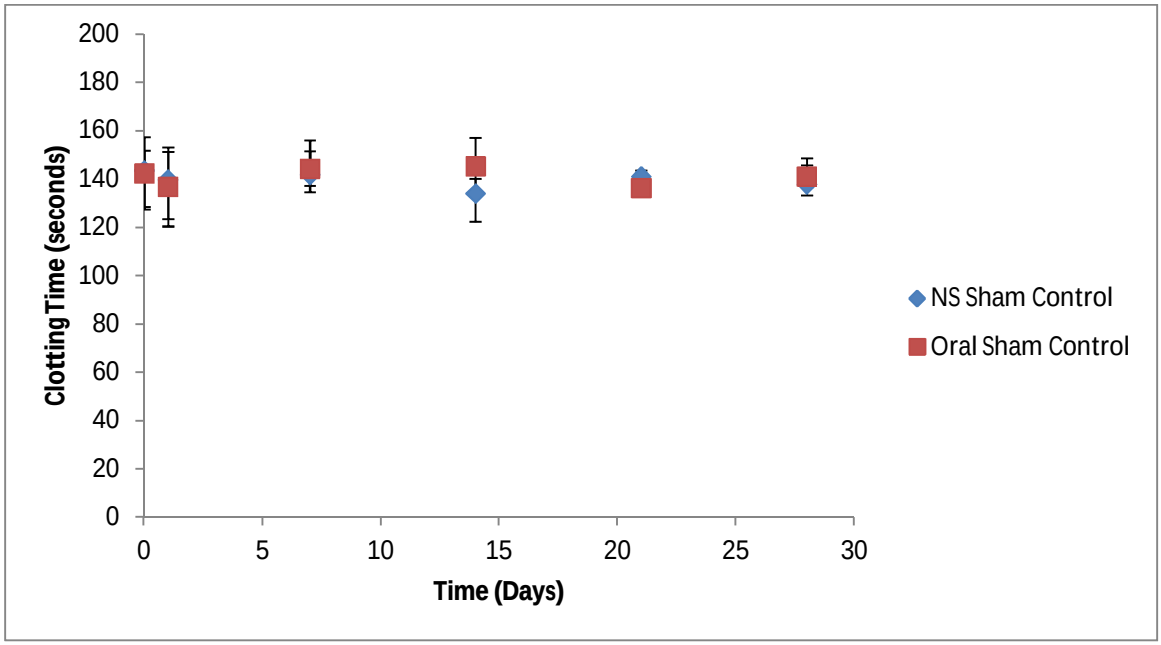


Figure 45: Rat Blood Clotting Time Measured over 28 Days from Sham Controls of Nanosuspension and Oral Suspension after Multiple Doses of Nanosuspension Vehicle and Oral Suspending Vehicle in Rats.

Data Reported as Mean ± SD (n=4).

The NS Vehicle group was dosed every two weeks for a total of two doses over 28 days.

The Oral Suspension Vehicle was dosed every week for a total of four doses over 28 days.

4.9 Proof-of-Concept Efficacy Study of Developed DES Nanosuspension

Based on the pharmacokinetic and toxicity profiles of the nanosuspension formulations, the NS-160 group was selected to evaluate the efficacy of DES. The five groups of tumor bearing SCID mice evaluated were NS vehicle (Control), Oral Suspension (47 mg/kg), NS-160 groups of NS-10 mg/kg, NS-23.5 mg/kg and NS-47 mg/kg. The dose of 47 mg/kg was selected as the highest dose based on the pharmacokinetic and toxicity studies in rats. This particular dose was selected as its equivalent dose given orally in rats was found to have potential toxicity as compared to the nanosuspension formulations. Two lower doses of 10 mg/kg and 23.5 mg/kg were selected to evaluate the effect of various doses of NS on the efficacy. The NS vehicle group was selected as the sham control group, and the oral suspension group as reference for comparison. The concentration of DES nanosuspensions were diluted to the required concentrations with distilled water to the intended doses. The volume of subcutaneous injections and oral suspensions was limited to 0.2 mL or less. The control group also received equal quantities of the nanosuspension vehicle. All the groups were dosed once every 14 days for a total of 2 doses.

The tumor size was measured before the first dose and then continuously monitored at 7, 14, 21 and 28 days (Figure 46). The size was recorded as

volume (Volume = Length × Width × Height). The tumor growth is expressed as V/V_0, the ratio of tumor volume at the respective day of measurement (V) to the initial volume on day 0. The plasma PSA levels at 0, 7, 14, 21 and 28 days were evaluated using ELISA kit. The efficacy of DES formulations was defined as the suppression of tumor growth, tumor volume and elevating PSA levels over a period of 28 days.

4.9.1 Tumor Growth when Dosed with Different Doses of DES NS-160, Oral Suspension and NS Control.

The comparative tumor growths, V/V_o ratio, versus the time from the first day of dosing to 28 days of the different groups were plotted (Figure 47). The tumor growth for the control group was similar to that of the oral suspension, NS-10 mg/kg and NS-23.5 mg/kg but the tumor growth was significantly suppressed for the NS-47 mg/kg (V/V_o = 0.95 ± 0.22) group as compared to the control (2.08 ± 1.070.95 ± 0.22) at day 7. The two groups of oral suspension and NS-47 mg/kg was compared on the same dose basis. The tumor suppression for the NS-47 mg/kg group was significantly greater at 7, 14 and 21 days as compared to the oral suspension (47 mg/kg) group. At 28 days, the tumor suppression values for the NS-47 mg/kg was greater (3.02 ± 2.17) than and that for oral suspension (5.79 ± 1.33) ($p = 0.056$). At the end of 28 days, the V/V_0 of NS-47 mg/kg was 3 which was 2.5 and 1.9 times smaller than those of control and oral suspension, 8 and 5.8 respectively (Table 14).

The actual tumor volumes of the two nanosuspension doses at 10 mg/kg and 47 mg/kg were compared to evaluate the reduction in tumor volumes at the highest and the lowest doses (Figure 48). The tumor volumes did not show a significant difference at 0 day. But the tumor volumes were significantly smaller for NS-47 mg/kg group as compared to those of the NS-10 mg/kg at 21 and 28 days. The tumor volumes were 454.23 ± 318.88 mm^3 and 1577.18 ± 621.14 mm^3 for NS-47 mg/kg and NS-10 mg/kg, respectively at 21 days and 623.54 ± 370.08 mm^3 and 1557.08 ± 329.06 mm^3 for NS-47 mg/kg and NS-10 mg/kg, respectively at 28 days.

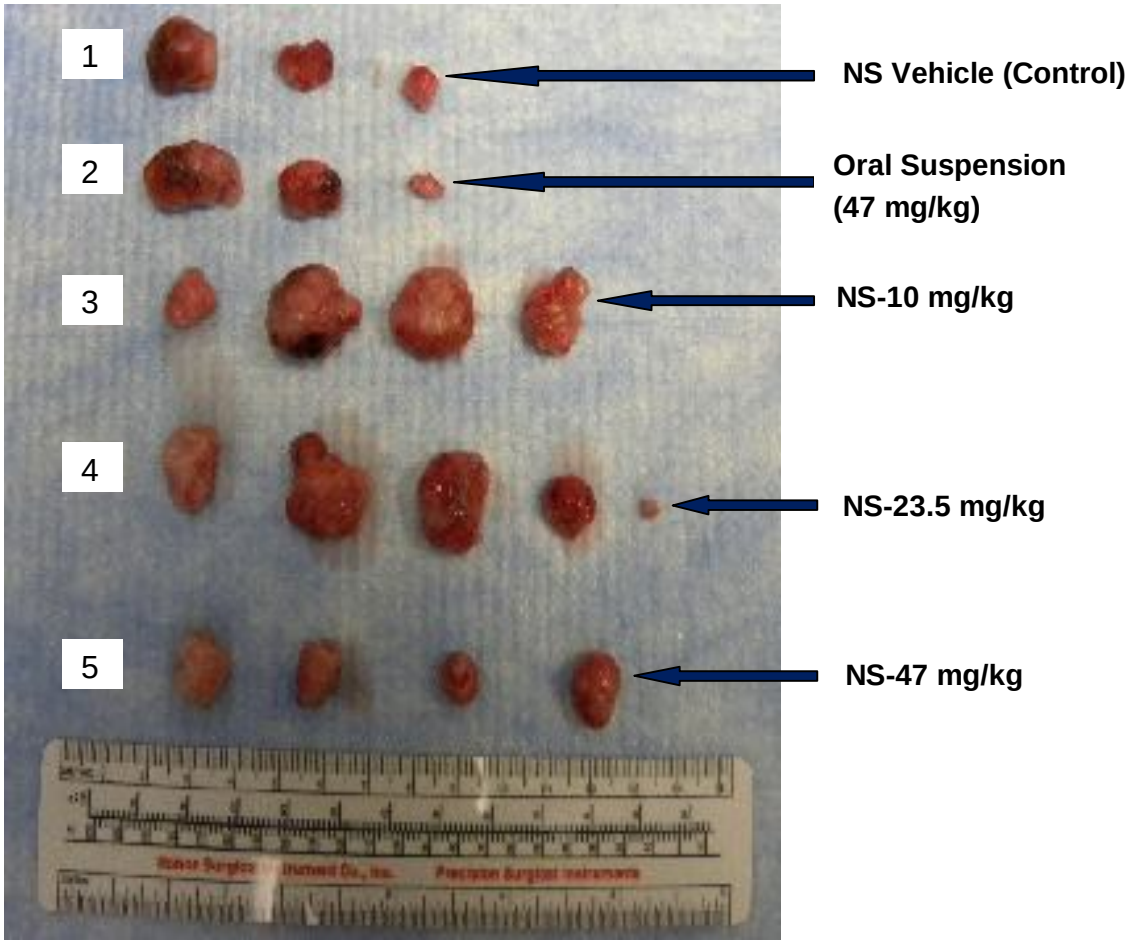


Figure 46: Extract Tumors from the SCID Tumor Bearing Mice at 28 Days for NS Vehicle, Oral Suspension, NS-10 mg/kg, NS-23.5 mg/kg and NS-47 mg/kg groups.

1 indicates n = 3 for NS Vehicle; 2 indicates n = 3 for oral suspension; 3 indicates n = 4 for NS-10 mg/kg; 4 indicates n = 5 for NS-23.5 mg/kg; 5 indicates n = 4 for NS-47 mg/kg. Each tumor was harvested from an individual mouse at day 28.

Table 14: Comparative Tumor Growth at 28 Days.

Group	Day 28 Mean $V/V_0 \pm SD$
Control	**8.03** ± 6.03
Oral suspension 47 mg/kg	**5.79** ± 1.33
NS-10 mg/kg	**7.16** ± 5.05
NS-23.5 mg/kg	**3.83** ± 3.09
NS-47 mg/kg	**3.02** ± 2.17

Mean $V/V_0 \pm$ SD for the tumor volume at day 28 (V) as compared to the tumor volumes at day 0 (V_0). All data reported as Mean ± SD. Control group (n=3), Oral Suspension group (n=3), NS-10 mg/kg (n=4), NS-23.5 mg/kg (n=5) and NS-47 mg/kg (n=4).

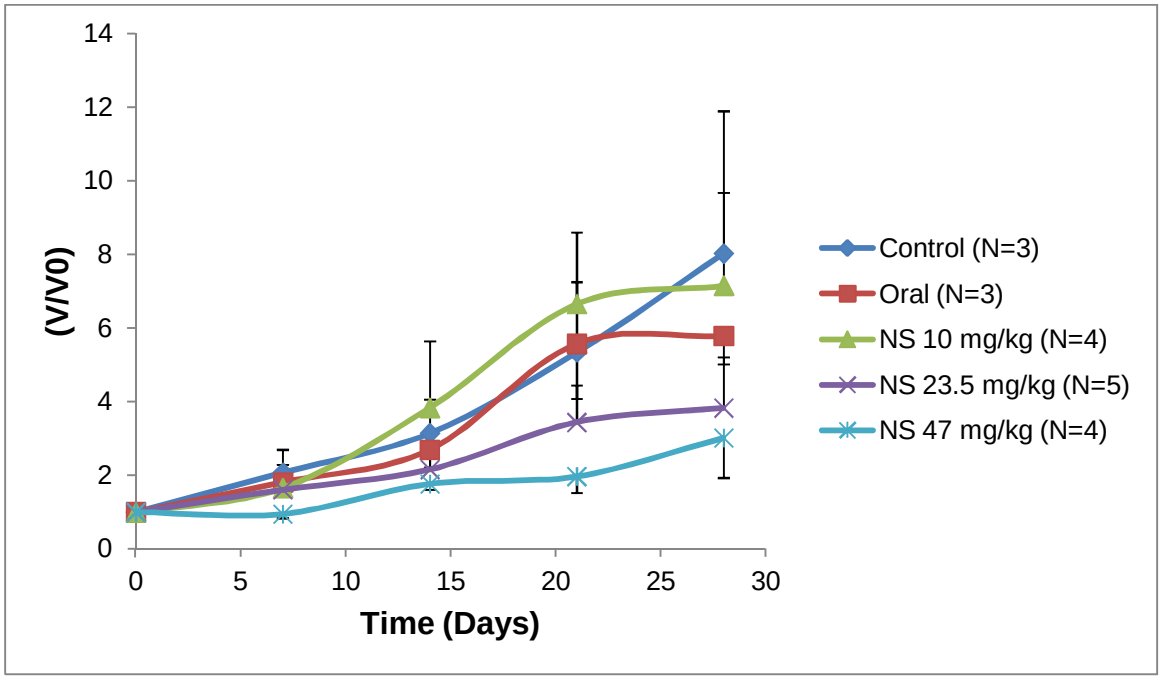


Figure 47: Tumor Growth (V/V_o) Profiles over 28 days.

V = Volume at time t, V_o = Initial volume before the start of treatment. Dose at Time = 0, 14 days. Data reported in Mean ± SE. n = 3 in control and oral suspension groups. n = 4 in NS-10 mg/kg and NS-47 mg/kg groups. n = 5 in NS-23.5 mg/kg group.

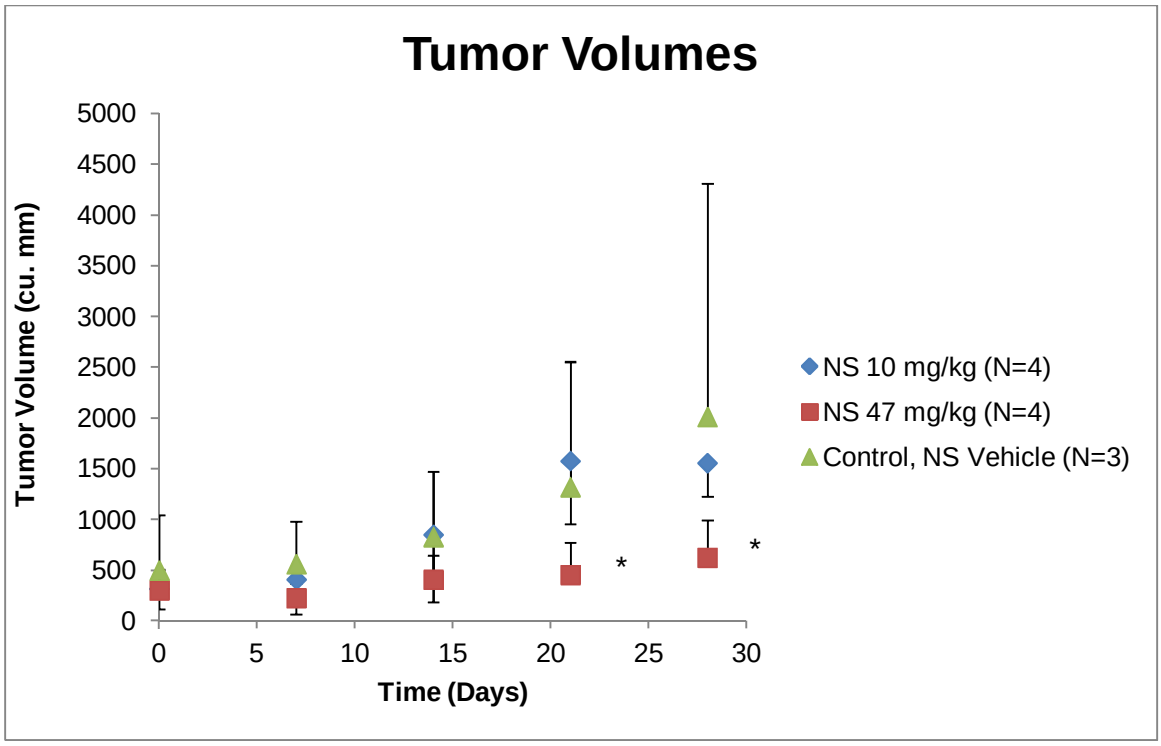


Figure 48: Tumor Volume versus Time over 28 days for NS-10 mg/kg as Compared to NS-47 mg/kg.

* indicates significant suppression in tumor volume of NS 47 mg/kg as compared to NS 10 mg/kg. Unpaired t-test ($p < 0.05$).

Data reported as Mean ± SD.

4.9.2 Effect of DES Formulations on the Prostate Specific Antigen (PSA) Levels

The PSA levels in the tumor-bearing mice were measured at 0 (pre-treatment) and 28 days for Control, Oral suspension, NS-10 mg/kg, NS-23.5 mg/kg and NS-47 mg/kg groups by ELISA (Table 15, Figure 49). The levels of PSA in the control, oral suspension, NS-10 mg/kg and NS-23.5 mg/kg increased significantly from 0 day to 28 days. The PSA levels were maintained for NS-47 mg/kg between Day 0 and Day 28. Thus, even on the same dose basis between the oral suspension (47 mg/kg) and NS-47 mg/kg, the nanosuspension formulation delivered subcutaneously showed a better profile for controlling the PSA levels over a period of 28 days. These results were consistent with the tumor suppression results evaluated earlier for a period of 28 days.

Table 15: PSA Values of Different Groups Measured at Day 0 and Day 28.

Groups	PSA levels (ng/mL) ± SD	
	Day 0	Day 28
Control	87.28 ± 59.98	284.00 ± 115.49*
Oral Suspension (47 mg/kg)	129.17 ± 48.22	317.31 ± 27.98*
NS-10 mg/kg	107.93 ± 45.29	345.06 ± 225.58*
NS-23.5 mg/kg	150.37 ± 88.08	380.92 ± 116.65*
NS-47 mg/kg	121.07 ± 39.55	196.50 ± 61.68

* significant increase in the PSA levels from 0 day to 28 days. Unpaired t-test ($p < 0.05$). Data reported in Mean ± SD. n = 3 in control and oral suspension groups. n = 4 in NS-10 mg/kg and NS-47 mg/kg groups. n = 5 in NS-23.5 mg/kg group.

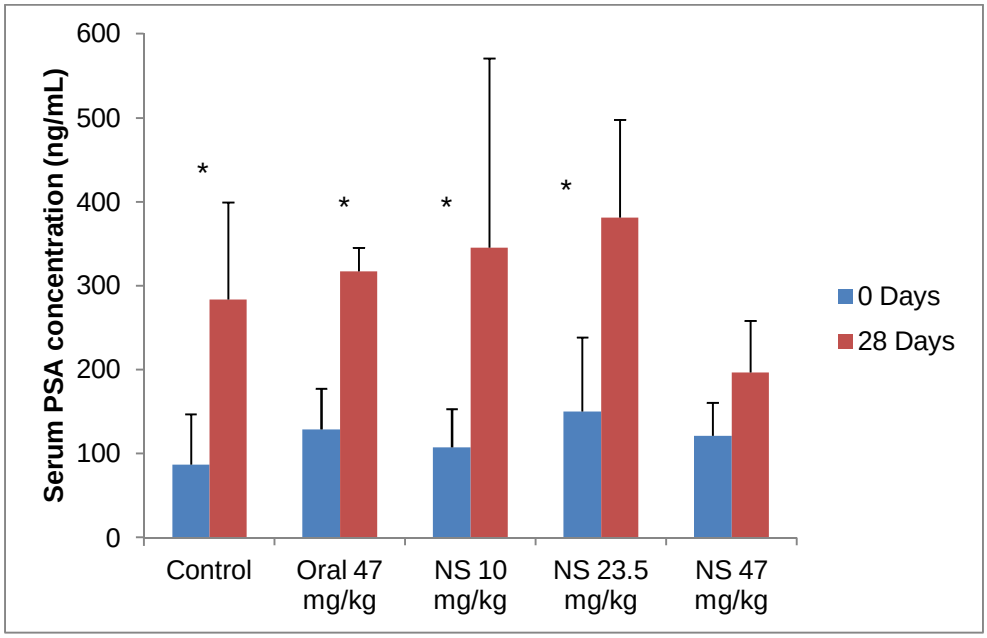


Figure 49: Changes in Plasma PSA Levels at Day 0 and Day 28.

* indicates significant increase in PSA levels ($p < 0.05$). Unpaired t-test ($p < 0.05$).

All values reported in Mean ± SD. n = 3 in control and oral suspension groups. n = 4 in NS-10 mg/kg and NS-47 mg/kg groups. n = 5 in NS-23.5 mg/kg group.

Chapter 5 Discussion

After the initial fall out and withdrawal from the market, the case for Diethylstilbestrol as a major player for use against both androgen dependent prostate cancer (ADPCa) and castrate resistant prostate cancer (CRPC) is regaining clinical interest. Orally administered diethylstilbestrol which leads to the cardiovascular toxicities and thromboembolic complications may become the past history. The focus has shifted to developing new formulations such as transdermal DES and parental estrogens which can by-pass the first pass effect leading to a lower hepatic exposure and potential lower toxicity. The proposed research of developing a novel subcutaneously delivered nanosuspension formulation of DES is innovative, in our opinion because it focuses on an entirely different approach of DES drug delivery by using a novel nanosuspension formulation and a different subcutaneous route of administration to potentially reduce the cardiovascular toxicity. Nanosuspensions inherently exhibit significantly different pharmacokinetic profiles as compared to the conventional oral formulations. The subcutaneous route of administration provides a novel route for delivery of nanosuspensions leading to a potential increase in the systemic exposure with a significant decrease in the hepatic exposure. The knowledge of the effect of particle size of the nanosuspensions delivered subcutaneously is limited. Also, there are no studies performed on the comparative pharmacokinetics and toxicity profiles of the subcutaneous

nanosuspension formulations of DES with oral administration of DES in a pre-clinical setting.

Our project is of prime importance towards an effort to bring DES back as the mainstay treatment for prostate cancer on account of the following factors:

Completely novel nanosuspensions with different particle sizes are formulated for subcutaneous delivery of DES. The DES nanosuspensions effectively modify the dissolution profile of DES when delivered subcutaneously and form a depot to give a slow sustained release.

The effects of particle size on the *in vitro* and *in vivo* release and pharmacokinetics of the nanosuspension formulation are characterized. Also, the systemic exposure and hepatic exposure of subcutaneous nanosuspensions are compared to those of the conventional oral DES. The nanosuspension formulations significantly improve the systemic exposures of DES and significantly reduce the hepatic exposures.

The pharmacodynamic and toxicity evaluations of the DES nanosuspension indicate that it could potentially reduce the cardiovascular toxicity and thromboembolic complications as compared to orally administered DES. The subcutaneous DES formulation provides an edge over the orally delivered DES

in its antitumor activity as demonstrated in a MDA PCa 183 xenograft mouse model.

5.1 Subcutaneous DES Nanosuspension Preparation

Diethylstilbestrol, in its salt free form is practically insoluble in water with a solubility of 12 µg/mL as given in Report on Carcinogens, 12th Edition. The poor solubility is because of the hydrophobicity which is the lack of the functional groups to form hydrogen bonds and/or with high lattice energy. The insolubility of a compound is defined as more than 10,000 parts of solvent (water) for one part of solute or < 0.1 mg/mL (DES has an even lower solubility, 12 µg/mL) (104). Also, if a high dose is required, formulation of nanosuspension is a prime candidate. Thus, considering these factors a nanosuspension of DES is a promising option. For formulating the nanosuspension formulations, there are two main methods that are used, the first being the top-down process and the second being the bottom-up process approach (86) (91). The top down approach mainly involves media milling in which the larger particles are broken down into smaller sized particles (nano-crystals) because of the attrition forces of the moving glass beads. This impaction produces a large amount of energy which in turn leads to smaller particles. The bottom-up process involves the use of organic solvents which when evaporated lead to the production of nanoparticles. The main

advantage of using the wet milling technique is that the drugs that are insoluble in water can be easily formulated and scale-up is extremely easy (89).

The use of stabilizers is an integral part of a stable pharmaceutical nanosuspension formulation. When the particle size is reduced a surface energy is generated which leads to positive gain in free energy. If this free energy is not negated by the use of a stabilizer, it leads to agglomeration/aggregation of the nanoparticles (105). Hence, the proper use of stabilizers is of utmost importance. From the different stabilizers available, non-ionic surfactants offer better adsorption and wetting potential (106). Also, there are safety concerns with the use of anionic surfactants. Combination of stabilizers is critical for the long term stability of the nanosuspension formulations (107) (91). Out of the different surfactants used, a combination of Pluronics F108 and Tween 80 were selected for formulating the nanosuspension formulations. Both surfactants have been widely used for formulating nanosuspensions (108) (109) (110). Pluronics F108 alone has also been successfully and safely used for formulating stable nanosuspensions (111).

To achieve lower, well distributed particle sizes, multi-sized beads were used instead of single sized beads. The smaller sized beads efficiently enter into the void spaces of the larger beads leading to an efficient, consistent shear of the larger particles to form nano-sized particles. The single sized glass beads

produces particles with larger polydispersity indices (bi-modal distributions) but the mixture of multi-sized glass beads gives a lower polydispersity index (mono-modal particle size distributions) (112).

It is commonly observed that, suspensions with a zeta potential of an absolute value of 30 mV are physically stable. Suspensions with a zeta potential above 60 mV (absolute value) shows excellent stability but with a value less than 20 mV are of limited stability but below 5 mV show pronounced aggregation. But these values are not strictly applied with systems containing stearic stabilizers such as Pluronics F108 and Tween 80. The stabilizers tend to adsorb on the surface of the particles and cause a drastic decrease in the zeta potential due to the shift in the shear plane of the particle (113). The minimum zeta potential required for electrostatic and stearic stabilization, is sufficient around ± 20 mV (114). The zeta potentials for NS-160 and NS-300 are less than – 20 mV but for the NS-500 the zeta potential was around – 15 mV which could lead to lower long term stability for the NS-500 group.

5.2 *In vitro* Release of DES Nanosuspensions and Solution

The *in vitro* studies of the three nanosuspension formulations of NS-160, NS-300, NS-500 and DES solution were evaluated. The release profiles of the three NS formulations was compared to each other and then with that of the solution. The release medium used in the study was 0.2 % Tween 80 dissolved in PBS. Tween

80 was used to maintain the sink condition and facilitate the dissolution of DES in the medium. A dialysis bag method was used for characterizing the release kinetics of DES from the formulations. It is a practical and convenient method since the media replacement and sampling can be easily performed (115). This method is efficient to be used for subcutaneous or intramuscular administrations as it mimics the *in vivo* conditions where the formulation particles are immobilized upon delivery (116).

From the release study, the release of the DES from solution was rapid and almost complete by 9 hours. In contrast, the release of DES from the nanosuspension formulations was significantly slower as only 15-25 % of DES was released by the end of 24 hours. The physical properties of the nanosuspensions and the solution govern the release profiles characterized by immediate release for the solution group and a slow sustained release for the nanosuspension formulations. In the solution group, the DES is already solubilized and completely dissolved in the co-solvent. Hence it gets rapidly released from the dialysis bag. On the contrary, the nanosuspensions contain solid drug particles which need to dissolve in the diffusion layer and the bulk medium in the dialysis bag before being released out of the dialysis bag. Hence the NS formulations resulted in the slow sustained release of DES. The initial extent of release for the NS-160 (smallest size) was significantly faster than the NS of higher particle sizes, NS-300 and NS-500. This is consistent with the

earlier results published in our lab (117) which shows that the mean initial release rates for the nanosuspensions are increased as the particle size decreases. According to the Noyes-Whitney equation, the dissolution rate increases because of the increase in surface area of the particles as the particle sizes decreases. The NS-300 and NS-500 had similar initial extent of release. But, at 6 days, the extent of release of the NS-160 and NS-300 groups was significantly higher than that of the larger particle size for the NS-500 group. Thus, the NS-300 group behaved initially as the NS-500 group and subsequently as the NS-160 group. The NS-160 and NS-500 have an inherently different release profiles *in vitro*. Thus, the particle size of the DES nanosuspension has an effect on the release kinetics of the nanosuspensions which could be anticipated in the subcutaneous environment *in vivo*. Hence, the NS-160 and NS-500 which exhibited significant difference in the release profiles were selected as the candidates for the pharmacokinetic and pharmacodynamic evaluations *in vivo.*

5.3 Stability of Nanosuspensions

The NS-160 and NS-500 were evaluated for the physical and chemical stability. These two groups were selected for the stability evaluations based on the *in vitro* release profiles. The physical and chemical stability of the nanosuspensions was evaluated 4 months at RT and 4 ^{o}C. The NS-160 and NS-500 are stable at 4 ^{o}C. But on account of its larger particle size and lower zeta potential of - 15 mV, the

NS-500 showed increase in particle size at 4 months when stored at RT. This increase in particle is mainly because of agglomeration and aggregation. As indicated by a zeta potential of – 25 mV for the NS-160 it remained stable with constant particle size at RT and 4 ^{o}C. DES remained chemically stable at both RT and 4 ^{o}C.

5.4 DES Pharmacokinetics of Subcutaneous NS-160, NS-500, Solution as well as Oral Suspension.

The rat plasma pharmacokinetics for the NS-160, NS-500, oral suspension and subcutaneous DES solution showed varied pharmacokinetic profiles. The two-compartment pharmacokinetic model was used to best describe the plasma DES concentration profiles for the subcutaneous NS-160 and NS-500 formulations, while a one-compartment pharmacokinetic model best described the subcutaneous solution and oral suspension groups. The systemic exposures characterized by AUC of the NS-160 and NS-500 were significantly higher than that of the oral suspension. This higher systemic exposure can be directly related to the significantly slower clearance of the two nanosuspension formulations as compared to that of the oral suspension. The NS-160 had a significantly slower clearance and a significantly higher systemic exposure than those of NS-500 nanosuspension. The absorption rate constant for the NS-160 was significantly slower than that of the oral suspension. Because of the smaller particle size, NS-

160 shows a faster dissolution in the subcutaneous site leading to a significantly higher C_{max} than that of NS-500. The elimination half life (T_{β}) was significantly prolonged for the NS-160 and NS-500 as compared to that of oral suspension. The slow, sustained release of DES from the nanosuspension formulations in the systemic circulation could be attributed to the depot effect at the subcutaneous site of administration as compared to the GI tract where the absorption of the oral suspension takes place. The bioavailability of the NS-160 and NS-500 was 3.5 times and 1.8 times higher than that of the oral suspension. This enhanced bioavailability can be attributed to the decreased clearance of the DES from NS-160 and NS-500 as compared to the oral DES. Because of the first pass effect most of the DES delivered orally entered the liver through the portal vein circulation. Oral DES has a high hepatic clearance and is extensively metabolized by the liver (118).

The increase in the elimination half life for the subcutaneous NS formulations can be attributed to the enterohepatic circulation and recycling of DES. The evaluation of DES disposition after intravenous and duodenal administration lead to the following conclusions: a) the DES is conjugated with glucuronic acid in the liver and follows enterohepatic recycling, b) a two compartment model best describes the disposition of DES and c) the bioavailability after duodenal administration is very low (119). Since DES is a lipophilic compound, it is hypothesized that the drug will distribute into fatty tissues and remain there for a

long period of time and re-enter the systemic circulation leading to prolonged elimination half life and a two compartment pharmacokinetic profile (119). Another set of experiments with intravenous administration performed in humans, dogs and rats indicate that the distribution half life of DES is around 1 hr and the elimination half life is around 24 hours. The terminal half life of DES decreases when the dogs are fed with cholestyramine to interrupt enterohepatic recycling. It is concluded that DES undergoes enterohepatic recycling and the elimination half life is dependent on the amount of drug that is recycled (120).

The systemic exposure of NS-160 was comparable to that of the DES solution delivered subcutaneously. The NS-500 group had a lower systemic exposure. The DES in solution form does not show a slow sustained release as observed in the NS groups. The elimination half life of the NS formulations was significantly prolonged as compared to the DES solution. It is interesting to note that the NS-160 group has an equivalent bioavailability as compared to the DES solution.

It is claimed that the higher the systemic exposure of DES (higher dose) the better is the efficacy in a prostate tumor animal model (121). Heston and Lazan found that in an androgen independent prostate tumor animal model, a dose of 20 mg/kg had a significantly better efficacy profile than the lower dose of 0.1 mg/kg. Thus, the NS-160 with its significantly highest systemic exposure as

compared to the NS-500 and oral suspension could potentially offer to be a treatment of choice for prostatic carcinoma.

This pharmacokinetic study represents a rare case of comparative pharmacokinetic evaluation of DES pharmacokinetic disposition in a pre-clinical animal model. The novel NS formulations showed tremendous merits over the conventional oral DES with increase bioavailability and systemic exposure but lower hepatic exposure suggesting potentially less concerns on DES adverse effects.

5.5 IVIVC

A level B correlation was attempted in comparing the subcutaneous NS formulations with DES in solution form. The MDR $_{in\ vitro}$ was compared to the MDR $_{in\ vivo}$ for the different formulations. A good correlation with an R^2 value 0.88 was established. The mean *in vivo* dissolution rate for the NS-160 was 4 times faster than that of the NS-500 group. Thus, the smaller particle size nanosuspension dissolves much faster than the larger particle size nanosuspension in the *in vivo* subcutaneous site. This leads to a significantly higher C_{max} for the NS-160 group as compared to that of NS-500. Because of the slow sustained release of DES from the nanosuspension formulations at the subcutaneous site in rats we detected the levels of DES for 6 days. This is consistent with the *in vitro* release profiles of DES nanosuspension formulations of NS-160 and NS-500, in which

DES was continuously released in a biphasic manner with the plateau being achieved after about 6 days. Hence, we took into consideration the first 6 days release profiles of the *in vitro* release data to correlate with the *in vivo* pharmacokinetic parameters. The good IVIVC correlation can enable us to estimate the *in vivo* dissolution rates of nanosuspension formulations with various particle sizes based on their *in vitro* release kinetics.

5.6 Hepatic Exposure of DES

The cardiovascular toxicities and thromboembolic complications of DES have been mainly linked to the route of administration. The direct exposure of liver to the high doses of estrogens through the first pass circulation up-regulates the metabolism of hormones, lipids and coagulation proteins. These changes are thought to be responsible for the short-term and long-term toxicity of DES (47, 122). In contrast, the parenteral estrogens reduce the first pass exposure of DES and have been shown to minimize the toxicity of DES (84). Our present study compared the subcutaneously delivered NS-160 and NS-500 to the oral suspension of DES over a period of 6 days. The levels of hepatic exposures of DES from oral suspension were more than 5 times higher than that of the subcutaneous NS formulations on the same dose basis. This is consistent with the literature which states that at equilibrium, after oral administration, the estrogen concentrations in the liver are about five times higher than in other

organs while the parenteral estrogens that reach the liver are those that do not reach the peripheral target organs and the concentrations are quite lower in the liver (47). This indicates that on the same dose basis, the levels of hepatic exposure of DES after oral administration would be higher and with parenteral administration the exposure of DES would be higher in the peripheral tissues.

The systemic exposure of the DES from the NS formulations was found to be significantly higher than that of the DES oral suspension. The liver to plasma (L/P) ratio of the DES exposures was evaluated for NS-160, NS-500 and oral suspension. The L/P ratio of oral suspension was more than 12 times higher than that of the NS formulations. This indicated that even with significantly higher systemic exposures of DES with the subcutaneous NS formulations, the hepatic exposures were significantly lower. This could directly lead to a better or similar efficacy of DES as compared to the conventionally given oral DES but with a potential reduction in cardiovascular toxicities and thromboembolic complications.

5.7 Short-term Toxicity Evaluations of the NS-160 and NS-500 Groups as Compared to the Oral Suspension of DES.

The changes in the levels of plasma coagulation factors such fibrinogen and ATIII and the direct effect of DES on the actual rat blood clotting times after a single dose of NS-160, NS-500 and oral suspension containing DES were evaluated. The systemic exposure (AUC) of DES from the NS formulations was

significantly higher and the hepatic exposures significantly lower as compared to the oral suspension. The impact of these parameters on the changes in the coagulation factors and clotting time were evaluated. DES has been shown to have a profound effect on the clotting factors in humans. The levels of fibrinogen and ATIII have been shown to decrease with estrogen administration orally (46). But with parenteral estrogens the levels of fibrinogen remain constant but still there is a significant decrease in the levels of ATIII which could still lead to thromboembolic complications (83). Also, Owens and Cimino delivered a 5 mg pellet of DES subcutaneously in rats but with external perfusion of liver with the addition of DES. They reported a significant decrease in the plasma levels of ATIII but no change in the levels of fibrinogen (123). But in this study, the effect of changes was evaluated at a single end point and with external evaluations of the perfused rat liver. There was no comparison with the conventionally delivered oral DES. In our study the effect of formulations with subcutaneous NS-160 and NS-500 were compared to that of the oral suspension. We also compared the effects of the DES in the formulation with a sham control. The sham control groups include the NS vehicle and the oral suspending vehicle, respectively. The sham control studies were conducted in order to evaluate the changes in the levels of the clotting factors and clotting time over the duration of 6 days when DES is not present in the formulations. Thus the changes in factors would be dependent on the internal injury caused by the route of administration, the

delivery of formulations, sampling of blood over the entire duration of 6 days causing additional injury and changes in the levels which cannot be attributed to DES. Hence, whatever changes that are obtained to compare among the groups are solely because of DES and its systemic and hepatic exposure. The levels of fibrinogen and ATIII obtained from the formulations were compared to the sham control to get a mean percent change which was used to compare the changes among formulations in the clotting factors over time.

There is a decreasing trend in the levels of fibrinogen over a period of 6 days for the NS-160, NS-500 and oral suspension. When compared to the sham control fibrinogen levels, the mean percent change of fibrinogen has a significantly greater decrease for the oral suspension as compared to the two NS-160 and NS-500 groups. This decrease is significant at 0.33, 1.5 and 2 days for NS-160 and at 0.33 and 2 days for NS-500 as compared to that of the oral suspension.

The levels of ATIII remained constant over a period of 6 days for the oral suspension as compared to the NS-160 and NS-500. When compared to the sham control ATIII levels, the mean percent change of ATIII has a significantly greater decrease for the oral suspension as compared to the two NS-160 and NS-500 groups. This decrease is significant at all time points except at 0.17 and 0.33 days for NS-160 and at 0.33 days for NS-500 as compared to that of the oral suspension.

Prostate cancer has been directly linked to the coagulation disorders and complications with metastatic prostate cancer leading frequently to disseminated intravascular coagulation (DIC) (124). The DIC has been shown to lead to thrombosis. DIC is often characterized by decrease in levels of fibrinogen and ATIII (51). Thus an additional decrease in the levels of fibrinogen and ATIII by orally administered DES could lead to aggravated changes in the coagulopathy and activation of the coagulation cascade leading to thrombosis.

These results are significant considering the fact that there is no significant change in the levels of both fibrinogen and ATIII in the NS-160 and NS-500 groups which could lead to lower changes in the coagulation cascade and a potential reduction in the thromboembolic complications and the consequent cardiovascular toxicity.

The rat blood clotting time (CT) was also evaluated to observe the direct changes in the clotting mechanism. The levels of CT remained constant for the NS-160 and NS-500 groups but decreased significantly for the oral suspension on the same dose basis. This indicates that the oral suspension with its pro-coagulation and anti-coagulant effects because of the increased hepatic exposure of DES leads to a faster clotting of blood as compared to the two nanosuspension formulations NS-160 and NS-500.

5.8 Correlation between the Mean Percent Change in Fibrinogen versus Mean Percent Change in ATIII.

This correlation gives a good representation of all mean percent changes in the fibrinogen and ATIII levels over a period of 6 days. The changes are more pronounced for the oral suspension group than the NS-160 and NS-500 groups. Most of the changes are even in the positive range for the ATIII levels for the NS-160 and NS-500 groups as compared to oral suspension which shows a decreasing trend. This indicates that the changes in the fibrinogen and ATIII levels are more pronounced in the oral suspension than the subcutaneously delivered NS formulations on the same dose basis.

5.9 Long-term Toxicity Evaluations of the NS-160 and NS-500 Groups as Compared to the Oral Suspension of DES.

The short-term toxicity evaluation showed a better safety profile for the NS-160 and NS-500 formulations as compared to the oral suspension. Hence, we wanted to evaluate the long-term toxicity of the DES formulations with multiple dosing. The NS-160 and NS-500 were dosed once every two weeks for 28 days for a total of two doses and the oral suspension was doses every week for a total of 4 doses. This dosing schedule was selected since the systemic exposure of DES from the NS formulations is about two times of that with the oral suspension. Hence, we wanted to evaluate the changes in the clotting factors and the clotting

times with comparable systemic exposures of DES. Owens and Cimino, also evaluated the long-term changes for 28 days in the coagulation proteins with a single subcutaneous pellet of 5 mg DES (57). They found that the fibrinogen levels remain constant and the ATIII levels decreased slightly but there was no significant decrease in the levels of ATIII as compared to that of the control. As in the short-term study, a long term sham control study was also evaluated for a period of 28 days with two doses of the NS vehicle and 4 doses of the oral suspending vehicle in our long-term toxicity evaluations.

The levels of fibrinogen over 28 days showed a decreasing trend for all the three formulations. But when compared to the sham control, the mean percent decrease for the oral suspension group was significantly greater than those from the two NS-160 and NS-500 groups.

Also, the ATIII levels remained constant for 28 days for oral suspension, NS-160 and NS-500. But when compared to the sham control the mean percent change of the oral suspension was significantly lower at 1, 7, 14, 21 and 28 days than for the NS-160 group. The change in ATIII was significantly lower for the oral suspension group as compared to NS-500 at 1 and 7 days but did not exhibit a significant change at 14, 21 and 28 days. This better toxicity profile for the NS-160 as compared to the NS-500 might be due to its significantly lower systemic exposure from 2 to 6 days as given by the AUC of the two groups.

The rat blood clotting time also decreased significantly over 28 days for the oral suspension group as compared to the NS-160 and NS-500 groups. The sham control CT for all the groups remained constant indicating that the changes in the clotting times are DES associated.

5.10 Proof-of-Concept Efficacy of DES NS-160 in a PCa 183 Prostate Tumor Mouse Model.

The pharmacokinetic and pharmacodynamic studies were performed using NS-160 and NS-500 groups. But NS-160 was found to have a significantly higher systemic exposure with slower clearance than the NS-500 groups. Also, the NS-160 has a better long-term safety profile with respect to the decrease in the ATIII levels as compared to the NS-500 group. The NS-160 has a better stability as compared to NS-500. Hence, NS-160 was selected as the formulation of choice for the proof-of-concept evaluation of the efficacy of a subcutaneously delivered nanosuspension of DES to compare with oral DES. The main objective behind this evaluation is to prove that the novel NS formulations have a better or similar efficacy as compared to the conventionally delivered oral DES. The efficacy of oral DES in prostate cancer has been well established (42) (125) (43) (126). The MDA PCa 183 model was derived from a drug naïve patient with androgen-dependent prostate carcinoma (127). The 47 mg/kg of dose for the NS-160 and the oral suspension in the tumor mice was based on the dose equivalent to that

dosed in the pharmacokinetic and pharmacokinetic study in rats (103). The dosing for all the groups in the study was administered once in two weeks for a total of two doses over a period of 28 days. Equivalent doses of NS and oral suspension were used since equivalent doses of DES delivered orally shows greater potential toxicity as compared to the NS formulations. The NS-47 mg/kg showed better tumor suppression over a period of 28 days as compared to the oral suspension. The decrease in the tumor volumes was also significantly higher in the NS-47 mg/kg group than that of the NS-10 mg/kg.

The MDA PCa 183 tumor model is an important model as it allows us to evaluate the efficacy based on Prostate Specific Antigen (PSA) levels as well. PSA levels have been successfully used to evaluate prostate tumor progression in humans and the decrease in PSA levels indicates increased efficacy (128). The PSA levels of all the groups including control, oral suspension, NS-10 mg/kg and NS-23.5 mg/kg increased significantly from Day 0 to Day 28. But level of PSA was maintained and did not increase for the NS-47 mg/kg group over a period of 28 days. The weight of the mice remained constant or even increased in all the dosing groups, indicating that the doses are tolerable and non-toxic.

Thus, the NS-47 mg/kg delivered subcutaneous had a better efficacy profile as compared to the oral suspension on the same dose basis. These results are

promising enough to consider the subcutaneous nanosuspension delivery of DES for further development and evaluations.

Chapter 6 Summary

6.1 Formulation of DES Nanosuspensions

The media milling technique was successfully used in the preparation of DES nanosuspensions. The nanosuspensions were effectively milled and sheared between attrition forces provided by the sliding surfaces of the glass beads. The stabilizers such as Pluronics F108 and Tween 80 were effectively used to stabilize the nanosuspension and to overcome the attractive forces between the nanoparticles. A narrow polydispersity and particle size distribution were achieved by using a mixture of glass beads rather than single size glass beads.

6.2 *In vitro* Release of DES from Three Nanosuspensions (NS-160, NS-300 and NS-500) and DES Solution.

The *in vitro* release study was carried out in PBS medium with 0.2% Tween 80 at 37 ^{o}C. The nanosuspension formulations showed a slow, sustained release as compared to the DES solution. A biphasic release was observed from the nanosuspensions with a rapid burst release followed by slow sustained release over a period of 14 days. The extent of drug release inversely depends on the particle size. The smallest particle size NS-160, showed a significantly higher initial extent of release than NS-300 and NS-500. Even at day 6 the extent of drug release from the NS-160 and NS-300 was significantly higher than that of NS-500. At 14 days, there was no significant difference in the DES release

among the nanosuspension groups. The differences in the release profiles between NS-160 and NS-500 might contribute to the differences in the pharmacokinetic characteristics of DES. The release profiles were best described by equations of the Higuchi and Weibull release kinetic models.

6.3 Plasma Pharmacokinetics of Subcutaneous DES Nanosuspensions, Solution and Oral Suspension.

The rat plasma DES pharmacokinetics profiles for the NS-160 and NS-500 groups were compared to that of the oral suspension. The plasma profiles for the NS-160 and NS-500 followed two-compartment kinetics, whereas the oral suspension and subcutaneous solution followed one-compartment pharmacokinetics. The AUCs of the NS-160 and NS-500 were significantly higher than that of oral suspension on the same dose basis. The elimination half lives of DES from the NS-160 and NS-500 were significantly prolonged resulting from a slower clearance as compared to that of oral suspension. The relative bioavailability was about 3.6 and 1.8 times higher for the NS-160 and NS-500 groups respectively, as compared to that of the oral suspension group. As compared to the subcutaneous solution group, the relative bioavailability was 1.2 and 0.61 for NS-160 and NS-500, respectively. The NS-160 group has a significantly higher AUC, slower clearance and comparable elimination half life as compared to NS-500 group. Both NS-160 and NS-500 groups showed a

sustained release for a period of 6 days as compared to the solution and oral groups. The novel nanosuspension formulations of DES are the first of its kind for subcutaneous delivery.

6.4 IVIVC

This correlation is based on the statistical moment analysis. The IVIVC correlates the mean *in vivo* summary parameter, in this case the *in vivo* dissolution rate with the *in vitro* summary parameter, such as the *in vitro* dissolution rate. Good correlation was established with an R^2 value of 0.877 between the two parameters.

6.5 Hepatic Exposure of DES Nanosuspensions and Oral Suspension.

The thromboembolic toxicity and cardiovascular complications of orally delivered DES have been directly linked to the hepatic exposure of the drug. The hepatic exposures of DES from NS-160 and NS-500 were significantly lower as compared to the orally delivered DES on the same dose basis. The first pass effect and portal circulation after oral administration led to the drug being exposed to the liver on a higher basis. The subcutaneously delivered DES by-passed the first pass effect and majorly distributed into the peripheral tissues and the residual drug has hepatic exposure. The liver to plasma (L/P) ratio for the oral DES suspension was more than 12 times higher than that of nanosuspensions in rats.

6.7 Pharmacodynamic Evaluations from NS-160, NS-500 and Oral Suspension.

6.7.1 Short-term Toxicity Evaluations of Single Dose of the NS-160, NS-500 and Oral Suspension of DES.

Two key clotting factors, fibrinogen and ATIII, and rat blood clotting time were evaluated after a single, 7 mg dose of nanosuspensions and oral suspensions. A comparative sham control study was also performed. The mean percent changes of ATIII and fibrinogen levels were compared to those of the sham control. The levels of fibrinogen and ATIII levels were significantly reduced for the oral suspension as compared to the two NS-160 and NS-500 groups. Also, the rat blood clotting time was significantly shortened for the oral suspension while the levels remained unaffected for the NS-160 and NS-500 formulations. Thus, the NS-160 and NS-500 formulations could potentially reduce the thromboembolic complications. Based on these results the long-term toxicity of DES was evaluated after multiple dosing.

6.7.2 Long-term Toxicity Evaluations of Multiple Dosing of the NS-160, NS-500 and Oral Suspension.

The long-term multiple dosing experiments were conducted to investigate if NS-160 and NS-500 had the potential lower toxicity as compared to the oral suspension. Same protocols of short-term toxicity evaluation on the fibrinogen,

ATIII and rat blood clotting time were used. The fibrinogen and the ATIII levels were significantly lower for the oral suspension as compared to the NS-160 and NS-500. The potential safety with ATIII was found to be better with the NS-160 group as compared to the NS-500 group. The rat blood clotting times were reduced significantly in the oral group but were maintained for 28 days for the NS-160 and NS-500 groups.

Thus the NS-160 and NS-500 groups could potentially reduce the cardiovascular toxicity and thromboembolic complications of DES.

6.8 NS-160 Efficacy Study in a MDA PCa 183 Xenograft Prostate Tumor Mouse Model

This is the first efficacy study of DES performed in the PCa 183 xenograft prostate tumor mouse model. The NS-160 at a dose of 47 mg/kg given once in two weeks yielded significantly greater tumor suppression and maintained the PSA levels over a period of 28 days as compared to the oral DES suspension which showed tumor progression and elevated levels of PSA on the same dose basis. The PSA levels increased significantly in all the groups control, NS-10 mg/kg, NS-23.5 mg/kg and oral suspension except for the NS-47 mg/kg group. Thus the novel subcutaneous NS DES formulations show a better efficacy profile as compared to the conventionally delivered oral DES.

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