

# **Characterization of Value Nutrition's 5-Androstenediol Product and its Proliferative Effects on the LNCaP Cell Line**

A Thesis

Submitted to the Faculty of

Worcester Polytechnic Institute

in partial fulfillment of the requirements for the

Degree of Master of Science

in

Biology and Biotechnology

by

---

Jaime L. Grouf

October 2007

APPROVED:

Jill Rulfs, Ph.D., Major Advisor

---

Theodore Crusberg, Committee Member

---

JoAnn Whitefleet-Smith, Committee Member

---

# TABLE OF CONTENTS

<b>TABLE OF CONTENTS</b> .....	<b>2</b>
<b>ABSTRACT</b> .....	<b>3</b>
<b>ACKNOWLEDGEMENTS</b> .....	<b>5</b>
<b>DEDICATION</b> .....	<b>6</b>
<b>INTRODUCTION</b> .....	<b>7</b>
<b>PROSTATE CANCER</b> .....	<b>7</b>
<b>PHYSIOLOGICAL ROLE OF ANDROGENS IN THE PROSTATE</b> .....	<b>8</b>
<b>ANDROGEN RECEPTOR: MODE OF ANDROGENIC ACTION AND PROSTATE CANCER DEVELOPMENT</b> .....	<b>11</b>
<b>ROLE OF ANDROGENS IN PROSTATE CANCER</b> .....	<b>13</b>
<b>ANDROGENIC-ANABOLIC STEROIDS (AAS): ELEVATED RISK OF PROSTATE CANCER?</b> .....	<b>17</b>
<b>MATERIALS AND METHODS</b> .....	<b>24</b>
<b>CELL CULTURE</b> .....	<b>24</b>
<b>MTT ASSAY</b> .....	<b>25</b>
<b>MTT ASSAY FOR ANDROGEN TREATMENT TIME DETERMINATION</b> .....	<b>25</b>
<b>MTT ASSAY FOR ANDROGEN CONCENTRATION DETERMINATION</b> .....	<b>26</b>
<b>DNA CONTENT ANALYSIS TO DETERMINE ANDROGEN TREATMENT TIME</b> .....	<b>26</b>
<b>DNA CONTENT ANALYSIS TO DETERMINE ANDROGEN CONCENTRATION</b> .....	<b>27</b>
<b>WESTERN BLOT FOR PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA)</b> .....	<b>28</b>
<b>ORGANIC EXTRACTIONS</b> .....	<b>29</b>
<b>REVERSE-PHASE HIGH PRESSURE LIQUID CHROMATOGRAPHY (RP-HPLC)</b> .....	<b>29</b>
<b>STATISTICAL ANALYSIS</b> .....	<b>30</b>
<b>RESULTS</b> .....	<b>31</b>
<b>TIME COURSE INVESTIGATION AND OPTIMAL ANDROGEN TREATMENT CONCENTRATION STUDY OF LNCAP CELLS</b> .....	<b>31</b>
<b>LNCAP AND HEPG2 EXPRESSION OF PCNA IN RESPONSE TO ANDROGEN TREATMENT</b> .....	<b>36</b>
<b>ANDROGEN DETECTION BY RP-HPLC IN LNCAP MEDIUM AFTER TREATMENT</b> .....	<b>40</b>
<b>ANDROGEN DETECTION BY RP-HPLC IN LNCAP MEDIUM AFTER HEPG2 CONDITIONED MEDIUM TREATMENT</b> .....	<b>54</b>
<b>DISCUSSION</b> .....	<b>57</b>
<b>REFERENCES</b> .....	<b>71</b>

## **ABSTRACT**

Prostate cancer (Pca) is the number one cancer in U.S. men and a leading cause of cancer-related deaths. The disease is initially dependent upon active male androgens for survival, such as testosterone or dihydrotestosterone. However, relatively inactive androgens like 5-androstenediol (5Adiol) have been considered to play larger roles in disease progression than previously considered. 5Adiol is synthesized in the prostate, is not affected by current anti-androgen therapies, and activates the androgen receptor in human prostate cancer cell lines more effectively than active male androgens.

The causal relationship between androgens and prostate cancer progression has significantly risen due to anabolic steroid abuse in competitive sports and recreational athletes. The evidence surrounding an association between androgen abuse and prostate cancer, as well as various other carcinomas, is of great concern within this demographic because of its preventability. Despite their prohibited use, anabolic products continue to be marketed and available for purchase as nutritional supplements. Surveys on current steroid user trends have documented high dosing regimens, extended periods of use, multiple self-diagnosed side effects, and unsafe injection practices.

Because few studies have investigated the effects of advertised 5Adiol products on prostate cancer progression, the goal of this study was to investigate whether Value Nutrition's 5Adiol product possesses comparable mitogenic stimulant activity to testosterone in the androgen-dependent cell line, LNCaP. It should be noted that while this compound is banned by the U.S. Drug Enforcement Administration and is no longer

sold through this company, alternative steroids continue to be marketed to consumers. Additional goals included determining whether this product would affect the proliferation of liver cells using the HepG2 cell line, if HepG2 metabolism of the product would play a role in the proliferation of LNCaP cells, and whether the LNCaP cells are capable of converting the 5Adiol product into testosterone. Multiple assays determined that the LNCaP cells were androgen responsive to the 5Adiol product, HepG2 proliferation was stimulated, and HepG2 metabolism of the 5Adiol product increased LNCaP mitogenicity, although significant differences were unattainable primarily due to the LNCaP cells reduced adhesion properties. Because reverse-phase HPLC was unable to detect compounds within 5Adiol even at high concentrations, the mitogenicity observed in both cell lines is unable to be correlated with a particular component in this product. While it is possible that 5Adiol is present at extremely low levels, contamination with other factors, hazardous or not, cannot be ruled out. In conclusion, these results suggest that the use of nutraceuticals have inherent risks for men at risk of developing prostate cancer. The mitogenic effects observed from small levels of unknown compounds in the tested 5Adiol product raise serious questions as to the legitimacy of allowing companies to market and self-regulate their own products.

## **ACKNOWLEDGEMENTS**

First and foremost, I need to thank my advisor, Jill Rulfs, without whom this work would not have been possible. I am also indebted to her for her optimism and reinforcement through the numerous hardships I encountered this year. I am also very grateful for my committee members, JoAnn Whitefleet-Smith and Ted Crusberg, for their assistance and guidance with my thesis work. JoAnn was a great help with data analysis and experimental design. I would also like to thank Dave Adams for his contributions towards my endeavors with tritium, and Liz Ryder who was always available for support and encouragement (and of course statistics!). Destin Heilman was an absolute lifesaver with his exuberance and willingness to assist me through every obstacle encountered with the HPLC, and Jessica Caron generously lent her time during my learning curve with the technique. I also need to thank Carol Butler and Eileen Dagostino for their friendship and help with everything encountered on a daily basis in the Biology Department. It is also of great importance that I thank the Biology Department not only for two years of financial support, but for also believing in my capabilities as a graduate student.

And last, but not least, I am so grateful for the unconditional love and encouragement of my family and friends. I want to especially thank my mother and father, because without them I would not be where I am today. And of course I want to thank Andrew for his never ending love and support.

## DEDICATION

*A man as grand as you must eventually leave this world, but cannot be forgotten; your spirit and memory continues to live on in the hearts of those who loved you.*

For Wilfred E. Dubey. A man of character and vitality. Someone to emulate and admire because of the goodness he wove into the fibers of my family. A flower enthusiast, he himself resembled a golden rose in full bloom, inspiring smiles, warmth, and love wherever he went. A patriotic veteran, nature-enthusiast, chef, and comedian, my grandfather was a delightful man. A vociferous advocate for higher education, I dedicate my thesis work to his memory.

# INTRODUCTION

## Prostate Cancer

Prostate cancer (Pca) is the single most commonly diagnosed cancer in men in the United States, as well as a leading cause of cancer-related deaths. In the year 2007 alone, the American Cancer Society estimates 218,890 new cases and 27,050 deaths [Society, 2007]. Pca is an incomparable cancer not only due to its morbidity but also because of the mortality rate associated with it [Claudio *et al.*, 2002]. In a majority of cases, prostate cancer is diagnosed as an androgen-dependent carcinoma initially combated with neoadjuvant therapy or prostatectomy surgery, if possible [Dehm and Tindall, 2006; Kent and Hussain, 2003]. If either fails to effectively fight the cancer, castration and androgen ablation therapy are employed, both of which aim to reduce/eliminate circulating androgens (C-19 sexual hormones responsible for masculinizing effects in the body), while ablation therapy also attempts to block the transcriptional activity of the androgen receptor (AR) [Dehm and Tindall, 2006; Heinlein and Chang, 2004; Kent and Hussain, 2003]. However, in almost all patients with metastatic or locally expansive disease, which is usually associated with poor differentiation, the cancer relapses as an untreatable adenocarcinoma that no longer relies upon androgens for its survival and development, ultimately resulting in death [Dehm and Tindall, 2006; Kent and Hussain, 2003]. Even if caught early, many cases have been observed to progress into a systemic untreatable malignancy [Kent and Hussain, 2003].

### Summary of Common In-Text Abbreviations:

**Pca:** prostate cancer

**5Adiol:** 5-Androstenediol

**DHEA:** dehydroepiandrosterone

**PSA:** prostate specific antigen

**AR:** androgen receptor

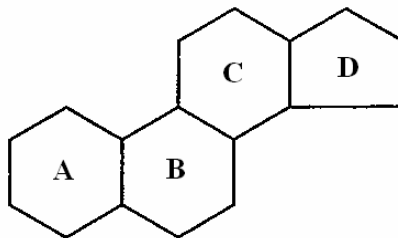
**T:** testosterone

**DHT:** dihydrotestosterone

**AAS:** androgenic-anabolic steroid

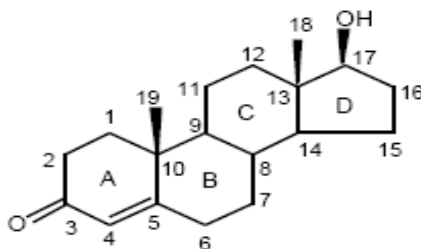
## Physiological Role of Androgens in the Prostate

All steroidal hormones contain a core structure characterized by a cyclopentanoperhydrophenanthrene nucleus, which contains three cyclohexane rings attached to one cyclopentane ring, designated A, B, C, and D (left to right) (Figure 1).



**Figure 1. Cyclopentanoperhydrophenanthrene nucleus of steroid structure [Hadley, 1996]**  
(Figure obtained from Endocrinology text, 4<sup>th</sup> edition, by Mac E. Hadley in 1996; see references for full citation).

The biological activity of steroids is conferred by a double bond between carbons 4 and 5 (C4 and C5) and a ketone group located at C3, both found within the A ring of the core nucleus (Figure 2).



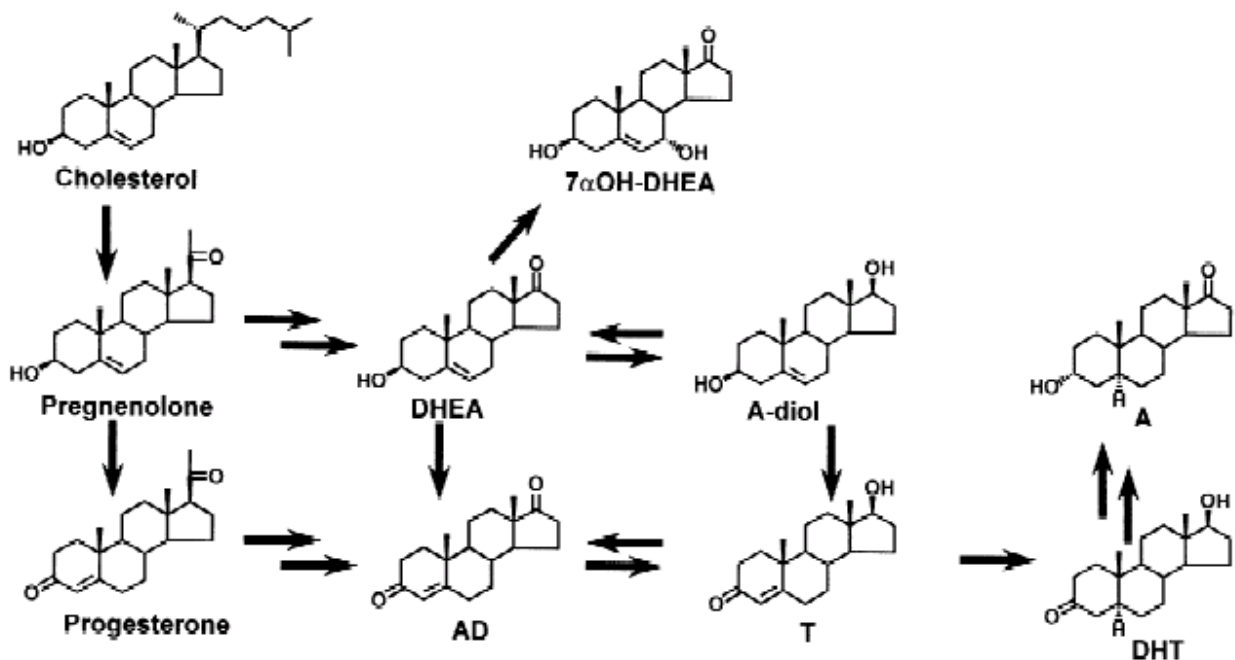
**Figure 2. Structure of testosterone [Hadley, 1996].**

Note the ketone group off C3 and the double bond at C4 which bestow biological activity to the molecule (Figure obtained from Endocrinology text, 4<sup>th</sup> edition, by Mac E. Hadley in 1996; see references for full citation).

Within the testes, androgen production occurs via an intricate and complex enzymatic process. Cholesterol and extracellular low-density lipoproteins are the major primary substrates for androgen synthesis within the Leydig cells [Hadley, 1996; Tentori and



Graziani, 2007]. The primary synthetic step in hormone production in the testes is the conversion of cholesterol to pregnenolone where the alkyl chain off of C17 is cleaved from the molecule (Figure 3). Dehydroepiandrosterone (DHEA), the most abundantly produced adrenal steroid, is then synthesized through the modification of C17 to form a ketone group. DHEA can then be metabolized to androstenedione or 5-androstenediol (5Adiol). Either of these metabolites can serve as substrates for testosterone (T) production. Testosterone can be converted to dihydrotestosterone (DHT) (Figure 3), the most biologically active androgen in the male body [George, 1997; Mizokami *et al.*, 2004; Tentori and Graziani, 2007; Zhu *et al.*, 2003].



**Figure 3. Biochemical pathway of androgen synthesis [Mitamura *et al.*, 2002].**

List of abbreviations: DHEA, dehydroepiandrosterone; AD, androst-4-ene-3,17-dione; A-diol, androst-5-ene-3 $\beta$ ,17 $\beta$ -diol; T, testosterone; A, androsterone; DHT, 5 $\alpha$ -dihydrotestosterone (Figure obtained from published data in 2002 paper by Mitamura *et al.*; see references for full citation).

With respect to the male anatomy, androgen synthesis occurs within the smooth endoplasmic reticulum of cells within the adrenal glands or gonads, with a much greater production occurring in the latter. In men, the testes are the primary source of androgens. Among their many functions, androgens control the anatomical and physiological development of the male urogenital system, sexual development and maturity, production and maintenance of secondary sex characteristics, and even play roles in fat metabolism, brain function, and bone and immune health [Hadley, 1996; Hartgens and Kuipers, 2004]. Specifically, T, the principle androgen in men, is endogenously produced and secreted by Leydig cells of the testes (an interstitial cell type) [Soronen *et al.*, 2004]. Within the human body, only 2-3% of blood T is active in an unbound form [Mottram and George, 2000]. The remaining T is bound to sex hormone binding protein (SHBP) in an inactive state. In the adult male and female body, physiological blood plasma levels of T are approximately 0.6  $\mu\text{g}/\text{dl}$  ( $\sim 20\text{mM}$ ) and 0.3  $\mu\text{g}/\text{dl}$  ( $\sim 10\text{mM}$ ), respectively [Mottram and George, 2000]. Physiological levels of DHT range between 0.8-4.1 nM [Horoszewicz *et al.*, 1983].

In target tissues like the prostate gland, whose primary function is to produce seminal fluid for ejaculation, T is converted to DHT. Both androgens bind the androgen receptor (AR), but while T regulates sexual development, maintains libido, and sexual function, DHT plays a pivotal role in embryonic & pubertal external virilization [Soronen *et al.*, 2004]. Furthermore, although T is more abundant in the circulation, DHT is known to be the more potent ligand of the AR, with a higher binding affinity [George, 1997; Mizokami *et al.*, 2004; Tentori and Graziani, 2007; Zhu *et al.*, 2003]. DHT is also a more

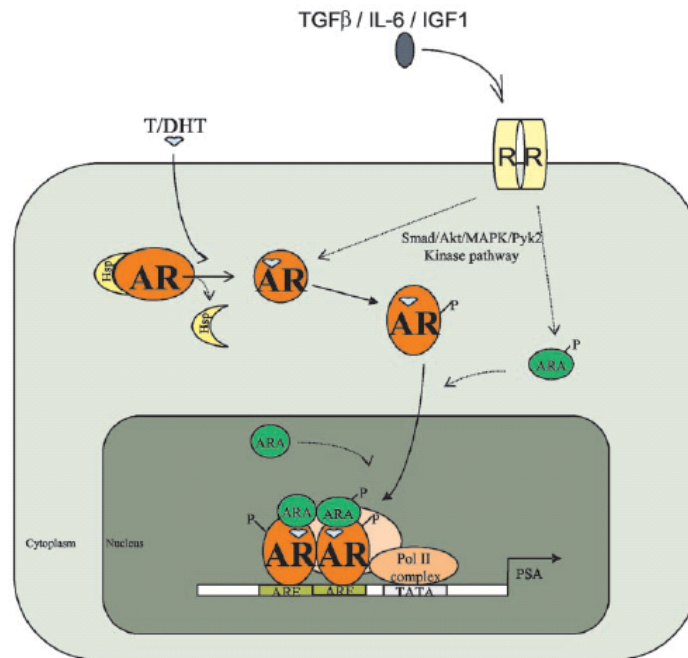
metabolically active ligand compared to T due to a slower dissociation rate from the AR and its induction of a receptor conformation that more readily resists degradation [Heinlein and Chang, 2004]. FW George in 1997 determined not only that DHT was the primary androgen in the prostate of rats, but also that the differential expression of T compared to DHT may be explained by the expression of 5 $\alpha$ -reductase, an enzyme that reduces T to DHT [George, 1997]. It appears as though cells express this enzyme as a mechanism to prevent T catabolism to less androgenically active steroids by the 17 $\beta$ -hydroxysteroid dehydrogenases.

### **Androgen Receptor: Mode of Androgenic Action and Prostate Cancer Development**

Androgens mediate their effects within cells and targeted tissues via signaling cascades initialized through ligand binding to the androgen receptor (AR). The AR is structurally characterized by four domains: a ligand-binding domain (LBD), a DNA-binding domain (DBD), a hinge region, and an amino-terminal domain. In addition to these four primary domains, the AR also contains two additional domains that function in its transcriptional activity, an N-terminal ligand-independent activation function-1 (AF) domain and a C-terminal ligand-dependent AF-2 domain [Agoulnik and Weigel, 2006; Heinlein and Chang, 2004].

The androgen receptor is a 110 kDa phosphoprotein and member of the nuclear receptor transcription factor superfamily [Agoulnik and Weigel, 2006; Lamb *et al.*, 2001]. It is a ligand-activated transcription factor located within the cytoplasm in an inactivated state (Figure 4). Upon activation, the AR separates from heat shock proteins, dimerizes, and

translocates to the nucleus. There it binds to androgen response elements (ARE's) in the cellular DNA, thereby initiating various signaling cascades which influence cellular growth and proliferation [Agoulnik and Weigel, 2006; Heinlein and Chang, 2004; Lamb *et al.*, 2001].



**Figure 4. Biological signaling cascade mediated by the AR within the prostate [Heinlein and Chang, 2004].**

Upon binding to its native ligands within the cytosol, the AR dissociates from heat-shock proteins to complex with AR coactivators (ARAs). The complex then translocates to the nucleus where it binds to ARE's in the DNA to activate the transcription of androgen-regulated genes. Additional signaling pathways (Smad, MAPK, etc.) can also be stimulated by growth factors to phosphorylate the AR, which upregulates its activity (Figure obtained from published work by Heinlein & Chang in a 2004 review article; see references for full citation).

Gene expression mediated by the AR is critical to prostate function, growth, and survival in both normal and malignant tissue [Balk, 2002; Dehm and Tindall, 2006]. Because androgenic activity is mediated through this receptor, the AR is a common target of androgen deprivation therapy [Agoulnik and Weigel, 2006; Balk, 2002]. AR functionality in Pca is supported by multiple studies using androgen-independent Pca; a form of Pca where androgen stimulation is sufficient but not required for proliferation and survival. In

androgen-independent Pca, the AR has been shown to be more stable in the absence of androgen compared to androgen-dependent Pca [Gregory *et al.*, 2001]. This stability is associated with a hypersensitivity to a range of androgen concentrations which were four-fold lower than those required to activate the AR in the androgen-dependent cell line, LNCaP [Gregory *et al.*, 2001]. Furthermore, upregulated transcription of the AR has been observed in androgen-independent tumors compared with androgen-dependent tumors [Balk, 2002]. A pivotal study by Chen *et al* in 2004 demonstrated increases in AR mRNA and protein in human tumor xenografts containing androgen-independent tumors derived from their parental dependent line [Chen *et al.*, 2004]. This group also demonstrated that the AR is necessary for the progression of hormone-dependence to independence using RNAi technology to knockdown AR expression in castrated mice.

### **Role of Androgens in Prostate Cancer**

From the initial ground-breaking discoveries of Huggins and Hodges in 1941, it was learned that castration drastically reduces endogenous androgen levels, and more importantly, that androgens regulate Pca activity [Huggins and Hodges, 1972]. In 1983, Horoszewicz *et al* developed a stable human cell line, named LNCaP, to model prostatic carcinoma [Horoszewicz *et al.*, 1983]. These cells were derived from a lymph node metastasis of a patient with Pca and have since become the paradigm for studying androgen-dependent Pca. They are characterized as having a mutated AR (T877A) which permits receptor binding promiscuity to both natural and unnatural ligands [Gregory *et al.*, 2001; Miyamoto *et al.*, 1998]. Horoszewicz's group demonstrated that the LNCaP cell line not only expresses the AR, but is also mitotically responsive to androgens,

specifically DHT, in a dose-dependent manner [Horoszewicz *et al.*, 1983]. Although attempting to prove a different aim, Lee *et al* also observed a dose-dependent proliferative response to DHT in the same cells [Lee *et al.*, 1995]. In a more recent study by Arnold *et al* in 2005, the proliferation of LNCaP cells treated with either DHEA, T, DHT, and estradiol was stimulated in both a time- and dose-dependent manner for all androgens [Arnold *et al.*, 2005].

Within *in vivo* studies, testosterone administration in rats has been shown both to increase the serum T level as well as stimulate hyperproliferative lesions in the prostate [Angelsen *et al.*, 1999]. An earlier study conducted in 1970 by Rosner and Macome demonstrated that human testes specimens obtained from men diagnosed with Pca were able to convert DHEA and pregnenolone into 5Adiol [Rosner and Macome, 1970]. Moreover, when incubated with 5Adiol, the testes were able to convert 5Adiol into T, which proves the ability of conversion to a more active steroid. Although rare, thirteen cases of Pca are reported to have been documented in patients receiving testosterone to combat symptoms of hypogonadism from Klinefelter syndrome [Bydder *et al.*, 2007]. In one particular case, the patient's serum T concentration was initially 2.1 nM with a prostate specific antigen (PSA) level of 1.7 µg/L. After seven years of T replacement therapy however, his T serum level had risen to 17 nM, the PSA level had elevated to 27.5 µg/L, and the patient was clinically diagnosed with prostatic adenocarcinoma [Bydder *et al.*, 2007]. Finally, documented cases of Pca development in bodybuilders exist, such as that reported by Roberts and Essenhigh in 1986, where prostate adenocarcinoma was reported in a bodybuilder who had used anabolic steroids for 20 years [Roberts and Essenhigh, 1986].

As previously mentioned, hormone-responsive Pca commonly recurs as a hormone-independent growth after androgen ablation therapy. Androgens that are relatively less metabolically active are not direct targets of ablation therapy. However, because they can still be converted to those which possess greater androgenic activity, androgen conversion was examined in one of the more significant studies to date by Mitamura *et al* [Mitamura *et al.*, 2002]. The main design was to incubate human prostate homogenate with dehydroepiandrosterone (DHEA), a less active androgen highly synthesized by the adrenal glands. Surprisingly, an androgen less focused upon in current literature, 5-androstenediol (5Adiol), was found to be the primary metabolite of DHEA in the human prostate. 5Adiol is known to be endogenously synthesized from DHEA in multiple tissues, including the prostate epithelium, and physiological levels are estimated to be approximately 5.2 nM [Mizokami *et al.*, 2004]. It had been previously demonstrated that 5Adiol activity is not blocked by current anti-androgen therapeutics [Chang *et al.*, 1999] and that it is capable of activating the AR in human Pca cells (LNCaP), [Miyamoto *et al.*, 1998] thereby indicating the importance of the findings from the Mitamura study.

Coupled with the aforementioned studies, in 2004, 5Adiol was recently shown by Mizokami's group to be a more potent activator of androgenic activity than DHT via the mutated AR in LNCaP cells [Mizokami *et al.*, 2004]. This activation is not proposed to be mediated through the binding affinity since DHT has a three-fold higher affinity for the AR compared to 5Adiol (DHT:  $K_d = 0.58$  nM; 5Adiol:  $K_d = 1.90$  nM), but rather through an increased ability to translocate the AR to the nucleus where it acts as a transcriptional activator of hormone-responsive elements in the DNA. Furthermore,

5Adiol stimulated proliferation more effectively in the LNCaP cell line at a 10-fold lower concentration than either T or DHT [Mizokami *et al.*, 2004]. Mizokami's group also observed that while the concentration of DHT decreased in human Pca tissue samples after hormone therapy, as compared to tissue from patients with benign prostate hypertrophy, the levels of 5Adiol remained unchanged although measurements before therapy in the same patients had not been performed [Mizokami *et al.*, 2004]. Providing validation to this finding, Higashi's group in 2006 used liquid chromatography-mass spectroscopy to determine that the level of 5Adiol was unchanged amongst patients within three groups: those with benign prostate hypertrophy, Pca that had been treated with 3-6 months of androgen deprivation therapy, or bladder cancer treated by radical cystoprostatectomy [Higashi *et al.*, 2006]. This is in comparison to DHT levels that were significantly lower in the Pca group versus the benign group, further indicating that 5Adiol is an active androgen unaffected by conventional ablation therapies. In slight contrast to these studies are the results from Nishiyama's lab in 2004 which analyzed Pca patient samples before and after anti-androgen therapy [Nishiyama *et al.*, 2004]. It was found that after six months of therapy, DHT levels were approximately 25% of the pre-therapeutic levels. Although levels were decreased, there is enough in the tissue to hypothetically sustain the cancer through therapy aimed at its destruction. This study also raises the question of developing additional drugs to target not only androgens, but the enzymes that are able to convert adrenal androgens to T or DHT in the prostate.



## **Androgenic-Anabolic Steroids (AAS): Elevated Risk of Prostate Cancer?**

Within the past sixty years, the role of androgens in Pca progression has become ever more compounded by steroid abuse. The use of performance enhancers was first documented during the 1950s in international weightlifting championships [Hartgens and Kuipers, 2004]. Since then androgens have been used abundantly by sport athletes (recreational and professional), bodybuilders, and high school students to accelerate muscle mass, enhance performance and strength, and improve appearance and self-esteem [Hartgens and Kuipers, 2004; King *et al.*, 1999; Mottram and George, 2000; Pallesen *et al.*, 2006; Parkinson and Evans, 2006]. Although the use of most steroids and androgens without a prescription was banned in the United States through the 2004 amendment of the Anabolic Steroid Control Act of 1990, anabolic products are still aggressively marketed and widely available for purchase. One reason for continued availability of anabolic steroids is linked to the 1994 Dietary Supplement Health and Education Act, which permits companies to sidestep regulations by the United States Food and Drug Administration as long as the products are not marketed to cure or prevent disease [Catlin *et al.*, 2000]. This leads to many androgenic-anabolic steroids (AAS) being marketed as nutritional supplements, which may be considered the most grave of mistakes. These products extend into a wide variety of substances referred to as ‘nutraceuticals’ which may have no drug benefits at all despite what the manufacturer attempts to convince the consumer. Of utmost concern however, are the potential implications of androgen use in the development of cancer and otherwise abnormal physiological changes in the body within this demographic, thereby making them a serious target of concern [Bryden *et al.*, 1995; Bydder *et al.*, 2007; Creagh *et al.*, 1988;

Froehner *et al.*, 1999; Martorana *et al.*, 1999; Overly *et al.*, 1984; Tentori and Graziani, 2007; Velazquez and Alter, 2004].

AAS are classified as synthetic derivatives of T [Hartgens and Kuipers, 2004; Mottram and George, 2000]. The androgenic effects of these drugs influence male properties such as aggression, strength, hair growth and sexual regulation [Hartgens and Kuipers, 2004]. The anabolic action of AAS achieves protein synthesis necessary for the production of muscle mass, while counteracting catabolism, or the degradation of proteins and muscle loss. Because complete separation of androgenic action from anabolic action has proven unattainable to date, health concerns over the use of these products primarily stems from the androgenic effects. Because T is inactivated by the liver when injected or administered orally, AAS are also altered with the goal of withstanding liver degradation. This is typically achieved via structural modifications such as methylations, alkylations, and esterifications which slows compound metabolism [Mottram and George, 2000]. Due to these issues, it is essential that users of AAS understand that despite marketing as anabolics or dietary supplements, these compounds do possess some androgenic activity and therefore must be used with caution. Most times the health risks associated with AAS are reversible, but with continuous use may lead to irreparable damage. These risks may include hypertension, carcinoma, infertility, gynecomastia, tendon damage, insulin resistance, and undesirable psychological conditions like mania, depression, and aggression [Hartgens and Kuipers, 2004; Mottram and George, 2000]. Additionally, moral and ethical opposition to AAS exists within professional sports because of the lending of unfair advantages to those athletes or teams who use them.

In addition to the plethora of research on an androgenic role in Pca development, androgens have also been linked to carcinomas of the kidneys, liver, and testes. One of the first diagnosed cases of renal malignancy associated with steroid use was reported by Prat *et al* (1977) in a middle aged man who had competed as a professional bodybuilder and had used steroids for multiple years [Prat *et al.*, 1977]. In 1995, two bodybuilders were diagnosed with renal carcinoma, one who had been using steroids for 20 months and the other for 6 years [Bryden *et al.*, 1995], and a fourth bodybuilder was diagnosed in 1999 who had been taking steroids for 15 years [Martorana *et al.*, 1999]. His cancer had progressed with metastatic lesions having formed in the lungs. There is at least one documented case of a form of testicular cancer found in a competitive weightlifter who had been using systematic high doses of steroids for five years [Froehner *et al.*, 1999]. Because of the rarity of his tumor, and the authors only knowing of three other cases of this kind, they state that there may be a more than causal link to androgens and cancer. More frequently documented however, are cases of hepatotoxicity in steroid abusers. An earlier report in 1984 documents hepatocellular carcinoma in a young bodybuilder who had consumed steroids for four years [Overly *et al.*, 1984]. His case was of interest to doctors because of the lack of any prior disease or condition that may have contributed to the malignancy. In 1988, another bodybuilder upon entering a hospital, admitted to having taken steroids for at least three years [Creagh *et al.*, 1988]. The patient unfortunately had a tumor, later diagnosed as a hepatocellular adenoma, which ruptured and hemorrhaged so badly that he lost his life. Further examination showed cardiac hypertrophy and testicular atrophy. Bagia *et al* (2000) also documented hepatic adenomas in a young bodybuilder [Bagia *et al.*, 2000]. Although in this case the tumors were

benign, they had previously undergone internal hemorrhaging as well. In 2005, two bodybuilders, one having used steroids for 15 years and the other for 6 months, displayed hepatic adenomas [Socas *et al.*, 2005]. The patient who had used steroids for 15 years also showed evidence of previous hemorrhaging within the tumor.

In addition to these cases where anabolic steroids are associated with cancer development, hepatocellular carcinoma has also been observed in patients diagnosed with Fanconi's anemia, a condition where androgen treatment is recommended when a suitable stem cell donor is unavailable [Velazquez and Alter, 2004]. Age does not discriminate in these patients, nor does the method of steroid administration (oral or parenteral). Finally, both liver toxicity [Kafrouni *et al.*, 2007] and the development of subdural haematomas in the brain [Alaraj *et al.*, 2005] have also been linked to anabolic steroid abuse in men.

AAS administration has also been shown to effect blood serum levels in men. Specifically, sublingual 5 $\alpha$ -Dihydrotestosterone administration has been shown to increase serum T and estradiol levels in men whereas oral intake does not [Brown *et al.*, 2002; Brown and McKenzie, 2006]. This is of great significance since multiple studies on hormonal response to AAS have administered drugs orally and not observed effects due to liver metabolism and clearance from the bloodstream. There are studies however that have used oral administration methods and found increases in serum androgen levels. For instance, when 4-androstenedione was administered orally, small significant increases in serum and free T levels were observed, whereas although not significant, oral intake of 4-androstenediol also increased T levels [Earnest *et al.*, 2000]. Furthermore, herbal

supplements containing 4-androstenediol, marketed to increase serum T levels, produced increases in serum free T levels as well as estradiol, androstenedione, and DHT [Brown *et al.*, 2001]. Broeder *et al* demonstrated that oral T supplementation significantly increased serum levels of estradiol, DHEA, and androstenedione as well as the participants lipid ratio risk (LDL:HDL) [Broeder *et al.*, 2000].

Perhaps most disturbing of all were the findings of a recent web survey conducted by Parkinson and Evans (2006) within an anonymous group of weightlifters, bodybuilders, recreational and professional sports athletes [Parkinson and Evans, 2006]. From this study, designed to elucidate current AAS trends amongst users, they found that 26% of participants admitted to starting AAS use in their teens, 60% admitted to administering a weekly dose of greater than or equal to 1000 mg of AAS, nearly 100% use both parenteral and oral administration to achieve the megadoses, 45% use steroids for 6+ months each year, and up to 13% practice unsafe injection practices including sharing needles or multi-dose vials and reusing needles. Compounding these figures, nearly all participants admitted to experiencing three or more subjective side effects such as acne, insomnia, edema, mood alterations, gynecomastia, and testicular atrophy. Yet, rather than stopping their doping regimens, 95% of participants took additional medications to alleviate these symptoms. For example, to reverse endogenous T suppression and testicular atrophy as well as attempt to revamp natural hormone production, 40% of participants self-administered human chorionic gonadotropin and clomiphene. Tamoxifan and other antiaromatase medication usage were also documented to block symptoms of gynecomastia. Additional medications taken to aid in appetite suppression or fat loss

included growth hormone, insulin, IGF-1, thyroid medications, ephedrine, caffeine, and clenbuterol [Parkinson and Evans, 2006].

Taken together, in addition to the multiple side effects of AAS, the evidence surrounding Pca stimulation is of great concern within this demographic because it can certainly be prevented. Few studies have investigated the effects of over-the-counter products advertised as 5Adiol on either Pca development or progression. 5Adiol is an androgen presumed to be relatively inactive, but from some of the literature described above, it may be worth investigating whether marketed 5Adiol products possesses comparable activity to some of the other metabolically active androgens, such as T or DHT. Because of the easy availability of AAS products over the internet from less than reputable sources, the main focus of this thesis was to analyze the effects of one marketed 5Adiol product (Value Nutrition's 5-Diol Powder; myvitanet.com) on the androgen-dependent Pca cell line, LNCaP. Whereas this product is no longer available through the company website, other AAS are still marketed to consumers. For all experiments using the 5Adiol product, it was assumed that the powder was comprised of pure 100% 5Adiol. The effects of this 5Adiol product were compared to those induced by T, which was purchased from Sigma-Aldrich Company, a well-known and reputable chemical company. In the first goal tested, it was hypothesized that 5Adiol and T will induce proliferation in the LNCaP cell line. Second, due to the role of the liver in androgen metabolism, links of androgen use to hepatic carcinoma, and previously unpublished data by our lab suggesting a mitogenic role in LNCaP proliferation from hepatic involvement [Nolan, 2002], it was predicated that conditioned medium from 5Adiol- and T-incubation with a hepatocellular

carcinoma cell line (HepG2) will stimulate a mitogenic response from LNCaP cells. It was also speculated that the HepG2 cells will not respond to androgen treatment. Finally, it was hypothesized that 5Adiol would be converted to a more metabolically active androgen, such as T by the LNCaP cell line.

# MATERIALS AND METHODS

## Cell Culture

Hepatocellular cancer cell line, HepG2 (HB-8065) and prostate carcinoma cell line, LNCaP clone FCG (CRL-1740) were purchased from the American Type Culture Collection (ATCC) (Rockville, MD). For MTT assays (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) only, cells were cultured in medium containing (1:1) DMEM:F12K supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA), and  $5 \times 10^{-5}$  M 2-mercaptoethanol (2-ME). Treatment medium for the MTT assays *only* was defined as described above but used 10% dextran-charcoal treated fetal bovine serum (DCT-FBS) in place of the 10% FBS. DCT-FBS contains reduced lipid levels for treatments so that the cells are cultured only in the presence of those lipids (androgens) supplemented back into the culture.

Standard medium for all other experiments required cells to be cultured in RPMI1640 with high glucose (2mM), supplemented with 10% FBS, 1% NEAA. Treatment medium *for all assays other than the MTT assay* was supplemented with a reduced concentration of 2% FBS. Cells were routinely fed 2-3 times per week and passaged either using 0.25% trypsin-EDTA or scraped from the culture surface at approximately 80% confluence. LNCaP cells were used between passages 3-9 and HepG2 between passages 3-10. All cell lines were maintained in humidified incubators at 37°C, 5% CO<sub>2</sub>.



## **MTT Assay**

According to Mosmann (1983), changes in cell number in response to androgen treatment were observed using MTT (thiazoyl blue, Sigma) with minor modifications to the original protocol [Mosmann, 1983]. This assay is based upon the reduction of MTT to a formazan crystal that can be solubilized and analyzed spectrophotometrically. Briefly, MTT was dissolved in 1X phosphate buffered saline (PBS) at 5 mg/mL. At the end of each treatment time period, MTT treatment medium (10% DCT-FBS) was removed from well of the 96-well assay plate and 100  $\mu$ L of MTT diluted 1:10 in treatment medium was added to each well and incubated for 3 hours in humidified 37°C, 5% CO<sub>2</sub> in the dark. 100  $\mu$ L of 0.1N or 0.4N HCl isopropanol (HCl-ISP) was then mixed into each well and shaken for 10 minutes. Wells were read using a Molecular Devices Spectramax 340 with a test wavelength of 570 nm and a reference wavelength of 630 nm.

## **MTT Assay for Androgen Treatment Time Determination**

To determine androgen treatment times (0, 6, 12, 24, 48, 72, or 96 hours), LNCaP cells were trypsinized and plated in 96-well flat-bottomed plates at a final concentration of 12,500 cells/mL (using a hemocytometer) in standard medium (10% FBS) and maintained in humidified 37°C, 5% CO<sub>2</sub>. At each respective time point, the standard medium was removed, treatment wells were rinsed with 1X PBS, and 200  $\mu$ L of treatment medium (10% DCT-FBS) was added for a 24 hour lipid starvation period. Wells in quadruplicate were then treated with either T (Sigma) or 5-Adiol (Value Nutrition's 5-Diol Powder; myvitane.com) dissolved in 100% ethanol at final well concentrations of 1 nM, 10 nM, or 100 nM. The 5Adiol product was assumed to contain

100% 5Adiol and was weighed according to 5Adiol's molecular weight. Control wells were treated with 100% ethanol. All treatment wells were assayed for increases in cellular proliferation compared to controls using the colorimetric MTT assay. Relative cell numbers were determined using a standard curve where cells/well (abscissa) was plotted against the absorbance readings at 570 nm (ordinate).

### **MTT Assay for Androgen Concentration Determination**

LNCaP cells were trypsinized and plated in quadruplicate in 96-well flat-bottomed plates as described above to determine androgen treatment concentrations. T or 5-Adiol dissolved in 100% ethanol was administered after 24 hrs of lipid starvation at final well concentrations of 1 nM, 10 nM, or 100 nM for 24 hours. Controls were treated with 100% ethanol. Relative cell numbers were calculated using a standard curve where cells/well (abscissa) was plotted against the absorbance readings at 570 nm (ordinate).

### **DNA Content Analysis to Determine Androgen Treatment Time**

LNCaP and HepG2 cell lines were removed from their culture surfaces, washed with 1X PBS, and re-plated in 24-well assay plates using treatment medium (2% FBS) at a final cell concentration of 5,000 cells/mL (using a hemocytometer for cell counting) for 24 hours in humidified 37°C, 5% CO<sub>2</sub>. T or 5Adiol dissolved in 100% ethanol was then added to wells in quadruplicate at final concentrations of 1 nM, 10 nM, or 100 nM for 12, 24, or 48 hours. Ethanol volume did not exceed 1%. Control wells were treated with 100% ethanol. At the start of each time period, tritiated thymidine (final concentration: 1 µCi/mL; specific activity: 61 Ci/mmol; MP Biomedicals, Inc.) was also added to each

well and incubated in humidified 37°C, 5% CO<sub>2</sub> for the specified length of treatment (*i.e.* 1 µCi/mL tritium and 10 nM 5Adiol were added at the same time for a 12 hour treatment period). After medium removal, cells were washed with a solution comprised of ice cold 10 mM sodium phosphate (Na<sub>3</sub>PO<sub>4</sub>), 150 mM sodium chloride (NaCl), pH 7.4 containing 10mM sodium pyrophosphate (Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>) and then the solution was aspirated. 100 µL of 1% sodium dodecyl sulfate (SDS) was added and cells were scraped from culture surface. Wells were then washed twice with 1 mL of ice cold water and combined with cell suspension. 100 µL was removed for protein determination and to the remaining volume 0.5 mL of 50% trichloroacetic acid (TCA, MP Biomedicals, Inc.) (f.c. 10%) was added, vortexed, and incubated on ice for 10 minutes. DNA was collected by filtration onto Whatman GF/C filters. Filters were washed with 2 mL 5% TCA and placed into a scintillation vial with 2 mL of scintillation fluid (Scintisafe Econo1, Fisher). Samples were read using a Beckman Coulter LS6500 Multi-Purpose Scintillation Counter. Optimal culture time was chosen dependent upon when maximum increase in DNA content occurred.

### **DNA Content Analysis to Determine Androgen Concentration**

Following the general protocol from the time determination section above, cells were cultured with 1 nM, 10 nM, or 100 nM of T or 5Adiol for 24 hours. At the start of the treatment period, 1 µCi/mL of tritiated thymidine was also added to the treatment medium (2%). After 24 hours, the DNA was collected and the labeled content was counted. Optimal androgen concentration was chosen pending which treatment promoted the maximal increase in DNA content during the treatment period.

### **Western Blot for Proliferating Cell Nuclear Antigen (PCNA)**

Cells were isolated from culture surface and washed with 1X PBS to remove exogenous proteins, and lysed via freezing at -20 °C. Sample volumes for gel loading were determined using the Coomassie Plus – The Better Bradford Assay Kit (Pierce) so that samples were normalized to total protein. This assay relies upon the binding of coomassie dye to proteins which causes a shift in absorption from 465 nm to 595 nm. Total protein concentration of the samples was estimated by reference to the absorbencies obtained using a standard curve for bovine serum albumin. 5X Laemmli tracking dye was added to all samples and diluted to a final concentration of 1X in PBS. Samples were boiled for 3-5 minutes at 100°C and loaded onto either a 10% polyacrylamide iGel or NuBlu gel (Life Therapeutics) and run at 40 mA for approximately 30-60 minutes in tris-glycine buffer (25 mM tris, 192 mM glycine, 1% SDS). Gel was blotted onto an Immobilon-P transfer membrane (PVDF) at 200 mA for 30 minutes. Membrane was blocked in 5% blocking buffer (dehydrated milk in 1X PBS) at least 1 hour, incubated overnight at 4°C with either mouse monoclonal anti-PCNA (2.5 µg/mL) or rabbit polyclonal anti-PCNA (1:1,000) (Calbiochem). The membrane was subsequently washed twice with 1X tris buffered saline (TBS), pH 7.4 followed by two washes with 1X TBS, 0.1% Tween 20, pH 7.4, each lasting at least 15 minutes. Washing was followed by incubation with appropriate secondary [goat anti-mouse IgG alkaline phosphatase conjugate (1:5,000) or goat anti-rabbit IgG alkaline phosphatase conjugate (1:10,000)] at 4°C for at least 1 hour. Pierce 1-Step NBT/BCIP solution was used for band development. Blots were analyzed by densitometry using Scion Image, a free image analysis program offered through Scion

Corporation (Frederick, MD; <http://www.scioncorp.com/>) in collaboration with the NIH. LNCaP blots were repeated in triplicate; HepG2 in duplicate.

### **Organic Extractions**

According to the method supplied by JoAnn Whitefleet-Smith [Whitefleet-Smith, 1984], cell culture medium samples from androgen treatments were mixed in a one phase solution of 2:1:0.8 methanol (MeOH):chloroform (CHCl<sub>3</sub>):water (H<sub>2</sub>O) (v:v) and mixed thoroughly. Phase separation was initiated by the addition of 1 volume of CHCl<sub>3</sub> and 0.2 volumes of H<sub>2</sub>O. The entire solution was mixed well and the organic layer was collected and evaporated to dryness under nitrogen using an N-EVAP 111 nitrogen evaporation setup with an OA-SYS heating system, set to 30°C.

### **Reverse-Phase High Pressure Liquid Chromatography (RP-HPLC)**

RP-HPLC was performed according to a documented method for testosterone analysis [Navajas *et al.*, 1995]. Briefly, a Hewlett-Packard HP 1100 series liquid chromatograph was used to analyze 20 µL sample injections at either 240 or 254 nm (wavelength specified within text) on a C18 250 X 4.6 mm reversed-phase analytical column. A flow rate of 1.2 mL/min was maintained in a mobile phase comprised of 1 mM phosphoric acid and acetonitrile. The starting acetonitrile concentration of 15% was increased to 30% in 10 minutes, 35% in 16.5 minutes, and sustained at 35% until 28 minutes where initial conditions were reinstated.

## **Statistical Analysis**

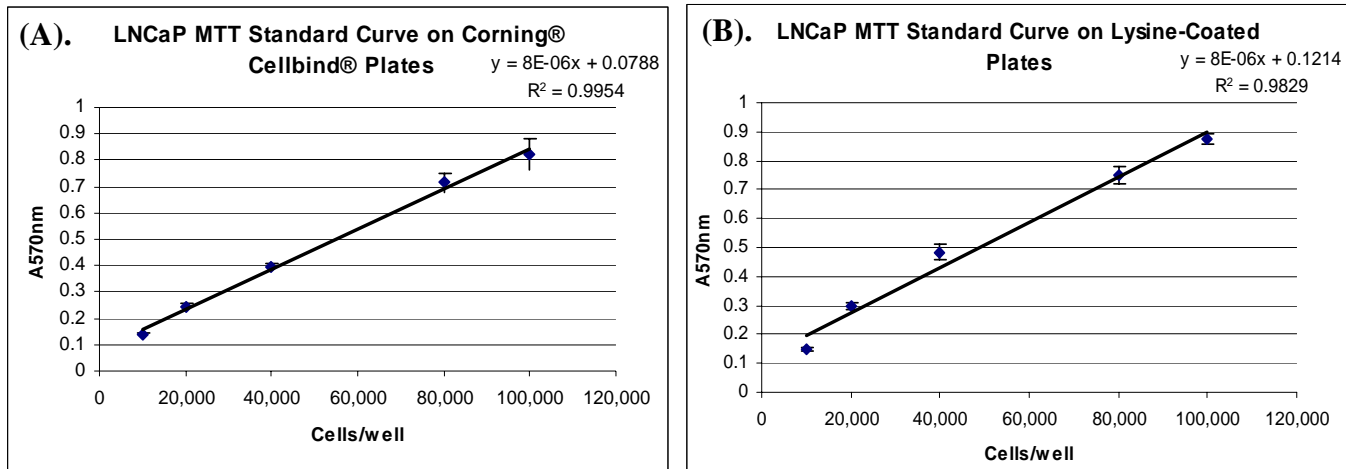
Sigmaplot v.9 software was used to perform statistical tests. Dependent upon the experimental design, a one-way ANOVA, two-way ANOVA, or randomized blocked two-way ANOVA was used (chosen test is stated within text), considering  $p < 0.05$  to be significant. If significant differences were found, the Holm-Sidak post-hoc analysis was performed.

# RESULTS

## **Time Course Investigation and Optimal Androgen Treatment Concentration Study of LNCaP Cells**

Initial studies to determine the optimal length of LNCaP treatment with either 5Adiol or T were explored using the MTT assay. Since the MTT assay is normalized by cell number, the most important factor necessary for optimization of the assay is cell adherence to the culture substrata. The earliest MTT trials were inconclusive due to inter-experimental variability. Macroscopic observation suggested the variability was a result of cell loss during solvent addition and removal. Poly-lysine culture surface treatment or culture on Corning<sup>®</sup> Cellbind<sup>®</sup> plates (Figure 5) were used to enhance cell attachment.

Although these changes appeared to reduce experimental variability (Figure 5), cells continued to detach during solvent changes. However, the assay did appear to work more efficiently at lower cell densities (20-40,000 cells/well), perhaps due to a reduced cell-cell and an increased cell-substrate attachment at lower densities (data not shown). Thus, successive assays were carried out at lower cell densities.

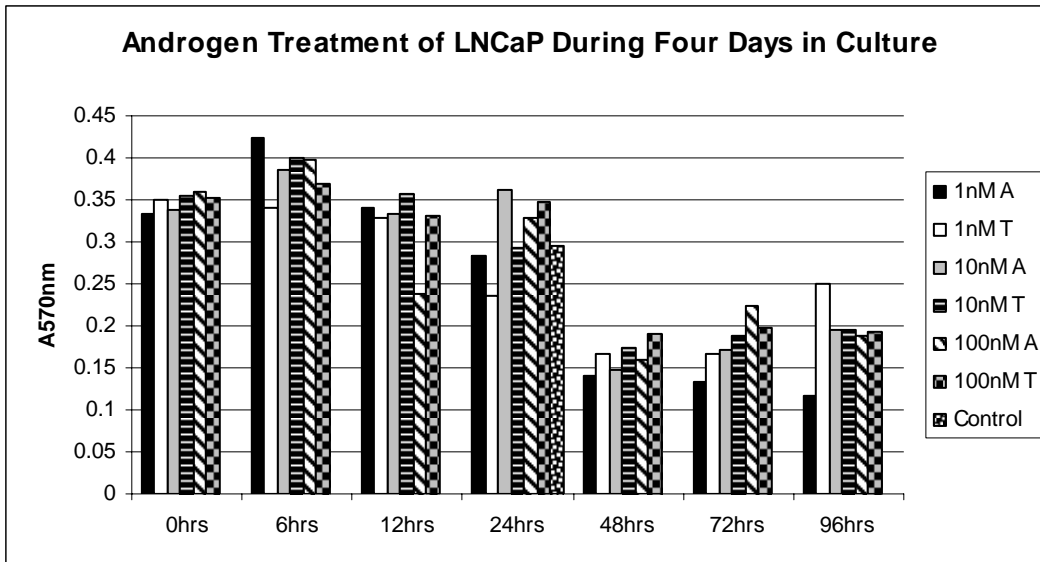


**Figure 5. Absorbance of MTT crystals at 570 nm from LNCaP samples.**

The MTT standard curve was derived from cells plated on either Corning® Cellbind® plates (A) or poly-lysine coated plates (B), respectively. Standard curves ranged from 10,000-100,000 cells/well. Data shown are mean  $\pm$  standard error of the mean.

In one time course study of LNCaP cells treated with a single dosage of androgen over a range of concentrations, the data supported an optimal treatment time period between 0-24 hours (Figure 6). At 48 hours of treatment, there was a significant drop in viable cell number ( $p < 0.05$ ; two-way ANOVA) which continued to be evident at all subsequent time points. Perhaps androgen treatment has a negative impact on cellular proliferation or viability after two days in culture. Because cells continued to detach from the culture wells, further interpretation of these data were speculative at best.



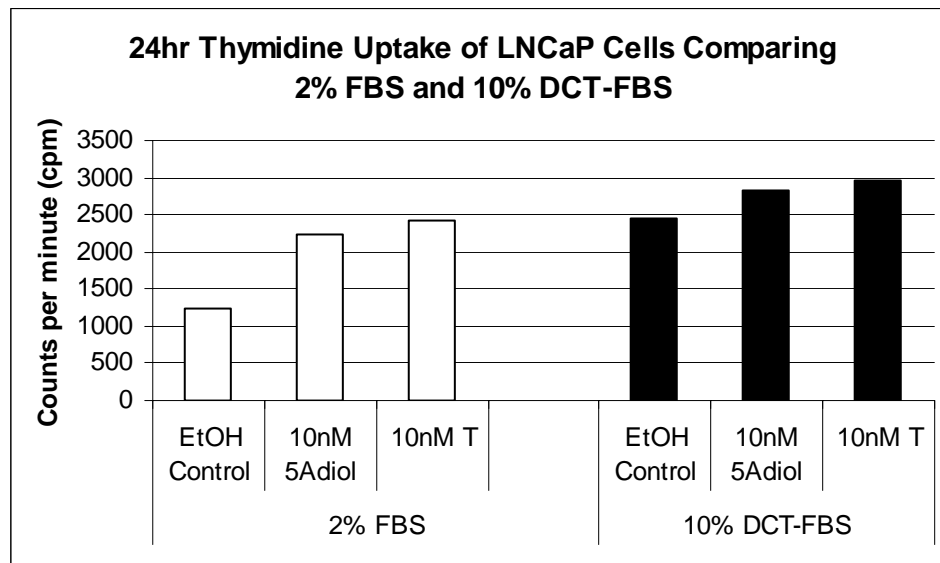


**Figure 6. LNCaP proliferation over four days in culture in response to either 5 $\alpha$ Diol or T treatment.** Cells were plated at a density of 2,500 cells/well on poly-lysine coated plates. MTT crystals were dissolved with HCl-ISP. Control data are shown for 24 hours, but absent for all other time points. Groups are averaged from triplicate wells from each treatment within this single experiment.

Centrifugation of the LNCaP cells prior to solvent addition/removal, use of alternative culture medium, plating cells onto a larger culture surface area, and increased time between plating and treatment did not significantly improve the situation. Therefore, an alternative assay system was used in subsequent studies. The use of tritiated thymidine to measure DNA replication was used as the second measure of cellular proliferation because collected data can be normalized to either cell number or total protein concentration within samples.

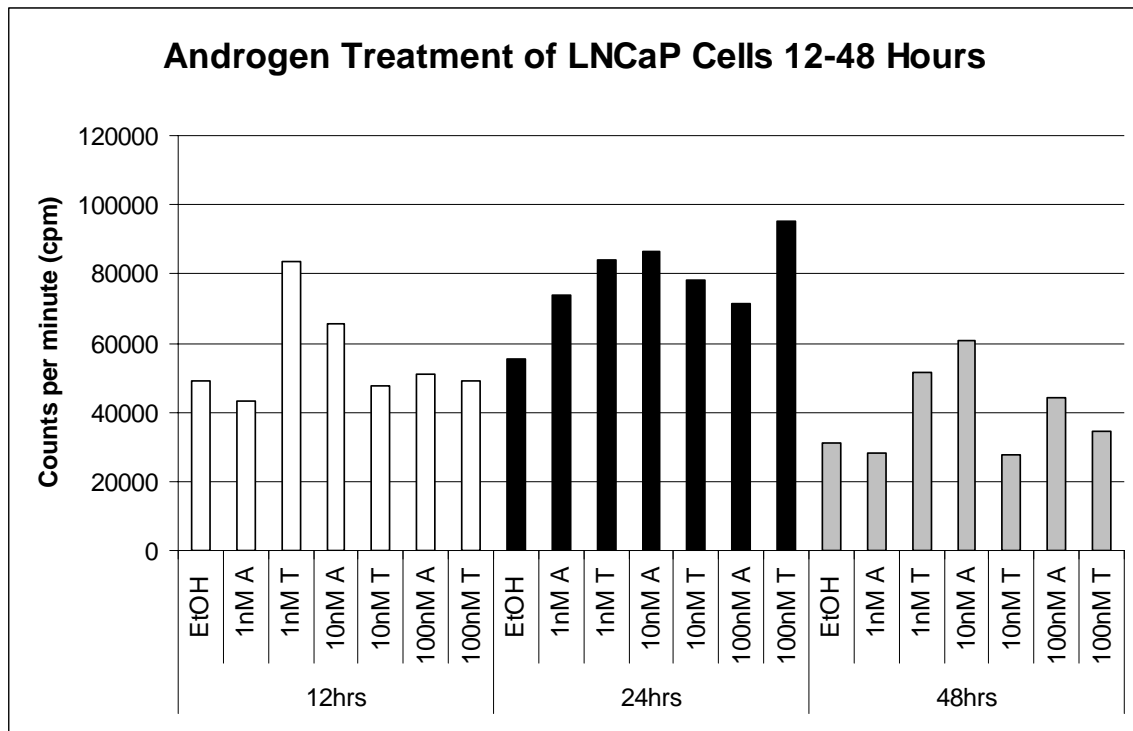
Additionally, because cells plated in DCT-FBS did not appear mitotically robust, an investigation was performed to determine whether the use of 5% FBS in culture medium could improve cell adhesion over 24 hours. Indeed, higher cell counts were obtained in medium containing 5% FBS (data not shown) than in 10% DCT-FBS medium. Therefore,

2% FBS was investigated to minimize the amount of lipids in the culture medium without sacrificing cell viability (Figure 7). Higher cell densities (100-200,000 cells/well) were plated in the attempt to mitigate subsequent cell loss, and a single dose of 10 nM 5Adiol or T was administered for 24 hours. Differences due to treatment were more readily evident in 2% FBS compared to 10% DCT-FBS (Figure 7). Only two of four trials showed a similar trend of responsiveness to steroid treatment compared to a vehicle control [ethanol (EtOH) only] for the 2% FBS group. In the other two trials there was either a decrease or no change in cell number as a result of treatment. The 10% DCT-FBS group responses to treatment varied with every trial. It is likely that cell attachment continued to contribute to inter-assay variability and confound interpretation of results. However, for whatever advantage it conferred to cellular adhesion, culture medium containing 2% FBS was used throughout the remaining assays since DCT-FBS contains fewer lipids, which may be necessary for cell attachment and proliferation.



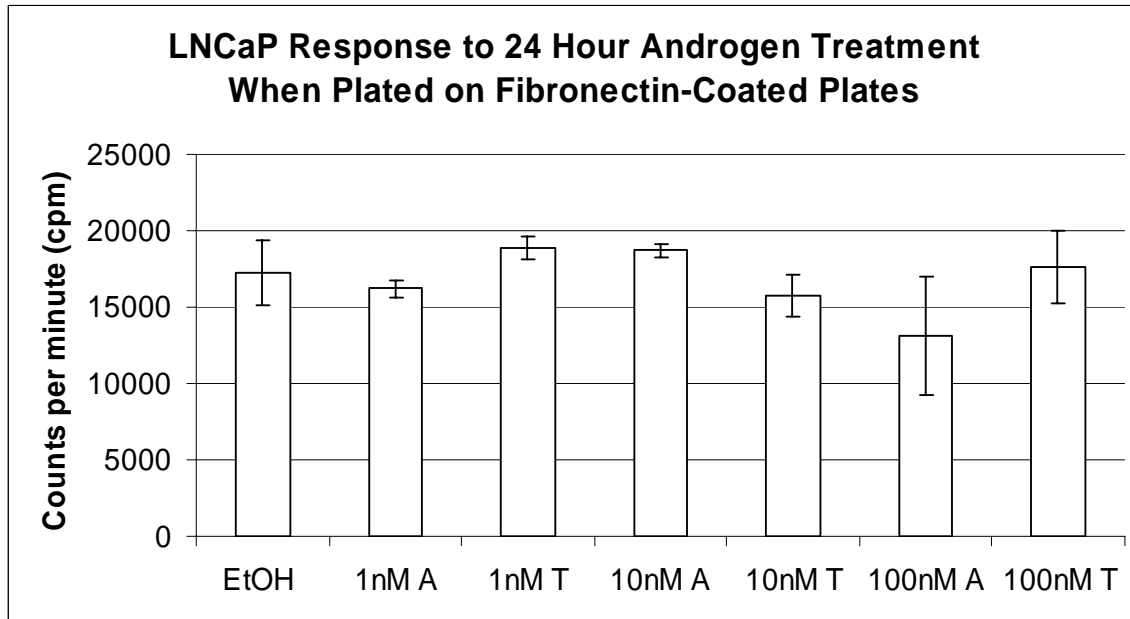
**Figure 7. LNCaP proliferation in response to treatment with 10 nM 5Adiol or T.** Cells were plated in culture medium containing either 2% FBS (100,000 cells/well) or 10% DCT-FBS (200,000 cells/well). The experiment was repeated four times. This figure is representative of the data trend that was repeatable in two of the four trials. Groups are expressed as the average of each treatment from triplicate well. Standard error is not shown because this experiment was performed once.

A time course study of LNCaP cells using tritiated thymidine at 12, 24, and 48 hours showed an optimal time point of 24 hours with an apparent increase in proliferation at all doses compared to vehicle control (Figure 8). This was in keeping with initial observations using the MTT assay (Figure 6). Additionally, as seen in the MTT data, a decrease in viable cell number was observed at 48 hours. Although there was significant variability, the data from this experiment suggested that a concentration of 10 nM be used in further trials since 10 nM 5Adiol supported the greatest increase in cell number at each time point when compared to 1 nM or 100 nM 5Adiol.



**Figure 8. LNCaP proliferative response to a single androgen dosage using tritiated thymidine.** A single dose treatment with either 1, 10, or 100 nM 5Adiol or T was administered for 12, 24, or 48 hours in culture. Cells were plated at a density of 100,000 cells/well in culture medium containing 2% FBS. Groups are expressed as the average from triplicate wells. Standard error is not shown because this experiment was performed once.

In a final attempt to mitigate the problem of cell detachment, BD Biocoat™ fibronectin-coated plates were used to enhance cell adhesion (Figure 9).



**Figure 9. LNCaP proliferative response to 24 hours of androgen treatment.**

Cells were plated at a density of 100,000 cells/well in culture medium containing 2% FBS on BD Biocoat™ plates. Data shown are mean  $\pm$  standard error of the mean.

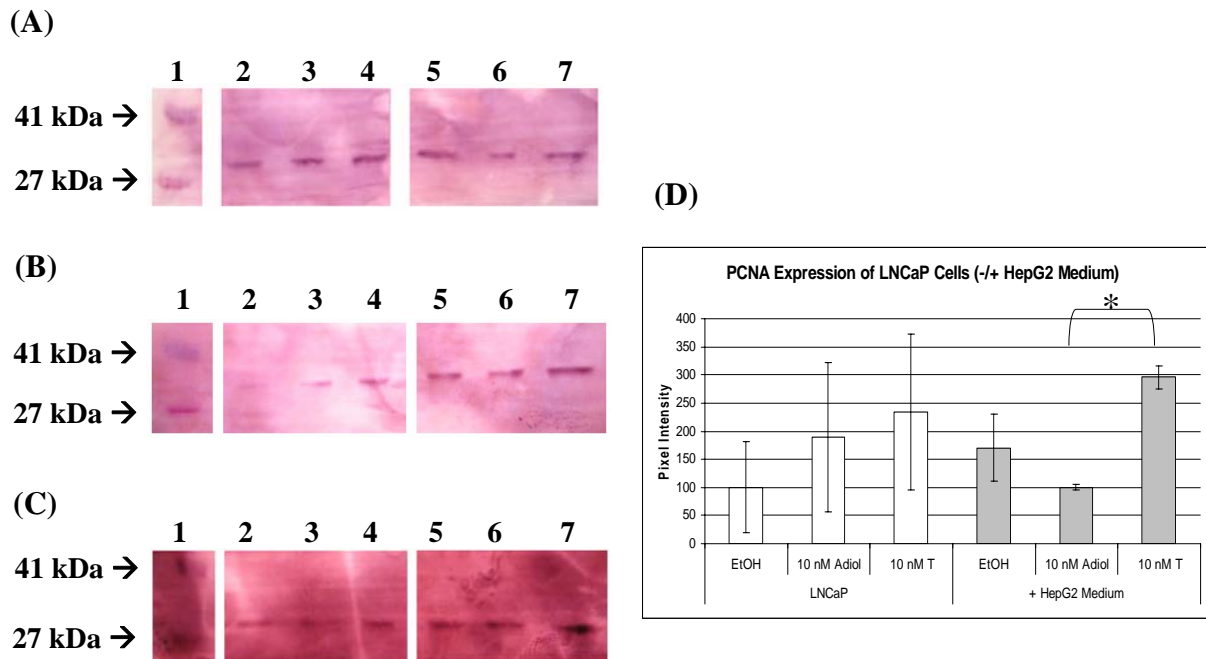
When observed macroscopically on the fibronectin-coated plates cell attachment seemed vastly improved compared to prior methods. However, the differences among all treatments were indistinguishable from the control (one-way ANOVA,  $p = 0.474$ ;  $p < 0.05$  considered significant). Since changes in focal attachment using fibronectin have been shown to have an effect on signal transduction pathways, this approach was not pursued. Thus, all future trials were conducted using 10 nM androgen over a 24 hour time period on standard-treated tissue culture plastic.

### **LNCaP and HepG2 Expression of PCNA in Response to Androgen Treatment**

Proliferating cell nuclear antigen (PCNA) is a 35 kDa auxiliary protein of DNA polymerase  $\delta$  [Bravo *et al.*, 1987]. Its nuclear concentration directly correlates with DNA replication, thereby making it an optimal protein for proliferative studies. As a secondary

validation to the tritiated thymidine assay, the expression of PCNA in response to 24 hours of 10 nM androgen treatment in the LNCaP and HepG2 cell lines was explored (Figures 10 & 11). Furthermore, the proliferative response of LNCaP cells after 24 hours of culture with conditioned medium from HepG2 cells treated with either 10 nM of 5Adiol or T for 24 hours was also investigated.

Immunoblot analysis of LNCaP cell samples revealed a positive proliferative response to androgen treatment compared to vehicle only (Figures 10A-D), although one blot did not show as strong of a response to 5Adiol (Figure 10C). While both androgens were able to stimulate proliferation, T consistently proved to have a greater effect than 5Adiol. This finding contradicts those found by Mizokami *et al* where picomolar levels of 5Adiol were found to induce greater proliferative effects than testosterone [Mizokami *et al.*, 2004]. This might be explained by later experiments using reverse-phase high performance liquid chromatography (RP-HPLC), which suggest that this 5Adiol product by Value Nutrition may not actually contain 5Adiol.



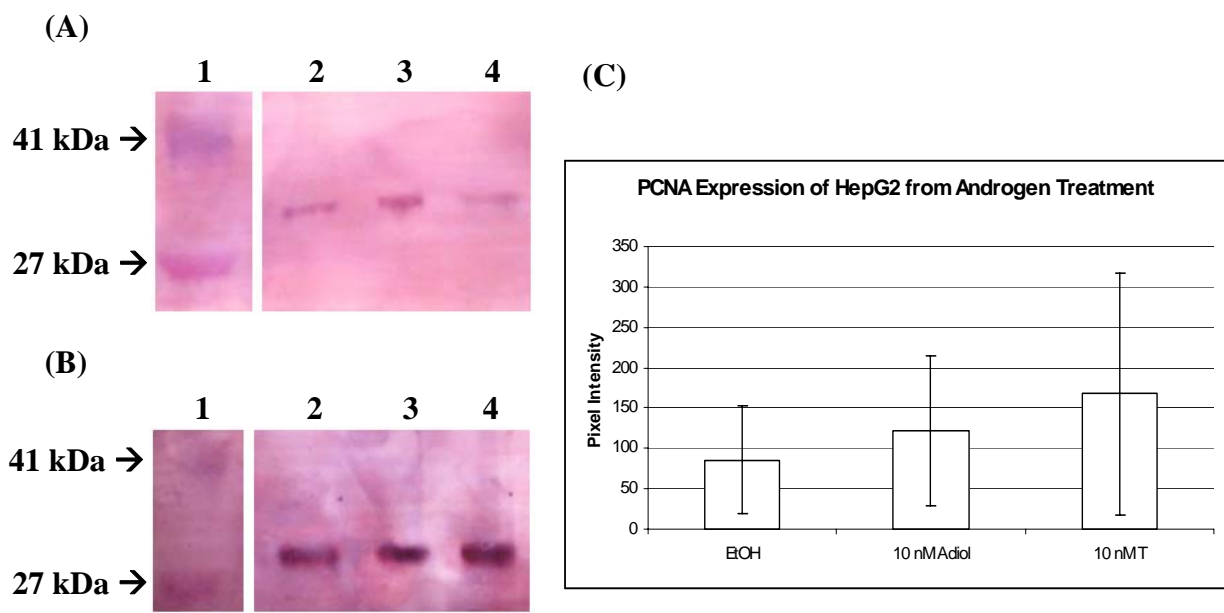
**Figure 10. Western blot of PCNA expression in LNCaP cells after 24 hours of 10 nM androgen treatment.**

(A-C) First, second, and third PCNA blots from LNCaP samples, respectively. Lanes are as follows: 1 – Marker, 2 – LNCaP EtOH control, 3 – LNCaP 10 nM 5Adiol, 4 – LNCaP 10 nM T, 5 – LNCaP with HepG2 EtOH control medium, 6 – LNCaP with HepG2 10 nM 5Adiol medium, 7 – LNCaP with HepG2 10 nM T medium. Sample loading was normalized to total protein ( $\mu\text{g}/\text{mL}$ ). (D) Graphical representation of the compiled band intensities from each blot as analyzed by densitometry. Groups are mean  $\pm$  standard error of the mean; \* indicates statistical difference between groups.

Graphical representation of the LNCaP data is shown in Figure 10D. The band intensity from each blot was measured using Scion Image. To compare relative differences between groups, the values from each blot were averaged together and analyzed using a randomized blocked two-way ANOVA. Although an androgen response is seen by observing the blots subjectively, significant differences were not found between androgen treatment of LNCaP cells compared to the control ( $p = 0.106$ , with  $p < 0.05$  considered significant). However, the general trend from the blots supports the tritiated thymidine assay conclusion that LNCaP cells are androgen responsive to both 5Adiol and T compared to vehicle after 24 hours of treatment.

To analyze the effect of hepatic androgen metabolism on Pca progression, HepG2 cells were treated with EtOH, 5Adiol, or T for 24 hours. The conditioned medium was then harvested, spun down to pellet any cell debris, and the supernatant was added to a flask of LNCaP cells and allowed to incubate for 24 hours. As shown in Figures 10A-C; lanes 5-7, two of the three blots showed a stimulated growth response of LNCaP cells to HepG2 conditioned media from 5Adiol and T treatments (Figures 10B&C), whereas the third blot showed an inhibition of their mitogenic capability (Figure 10A). Similar to what was found with the androgen treatment of LNCaP cells, the HepG2 conditioned medium from treatment with T also induced a greater level of proliferation in the LNCaP cells compared to HepG2 conditioned medium from 5Adiol treatment. Statistical analysis by the randomized blocked two-way ANOVA detected significant differences between the treatment groups, but not between treatments and control ( $p = 0.026$ , with  $p < 0.05$  considered significant). These data suggest that hepatic metabolism of androgens may play a role in the acceleration of prostate cancer development and maintenance.

To determine whether the HepG2 cells respond to androgen treatment as a mitogenic stimulus, the expression of PCNA was explored (Figures 11A-B). Interestingly, the HepG2 cells were responsive to 5Adiol, although the response to T was conflicting. Statistical analysis by a randomized blocked two-way ANOVA did not find significant differences between the androgen treatment groups and/or the ethanol control ( $p = 0.515$ , where  $p < 0.05$  is considered significant).



**Figure 11. Western blot of PCNA expression in HepG2 cells after 24 hours of 10 nM androgen treatment.**

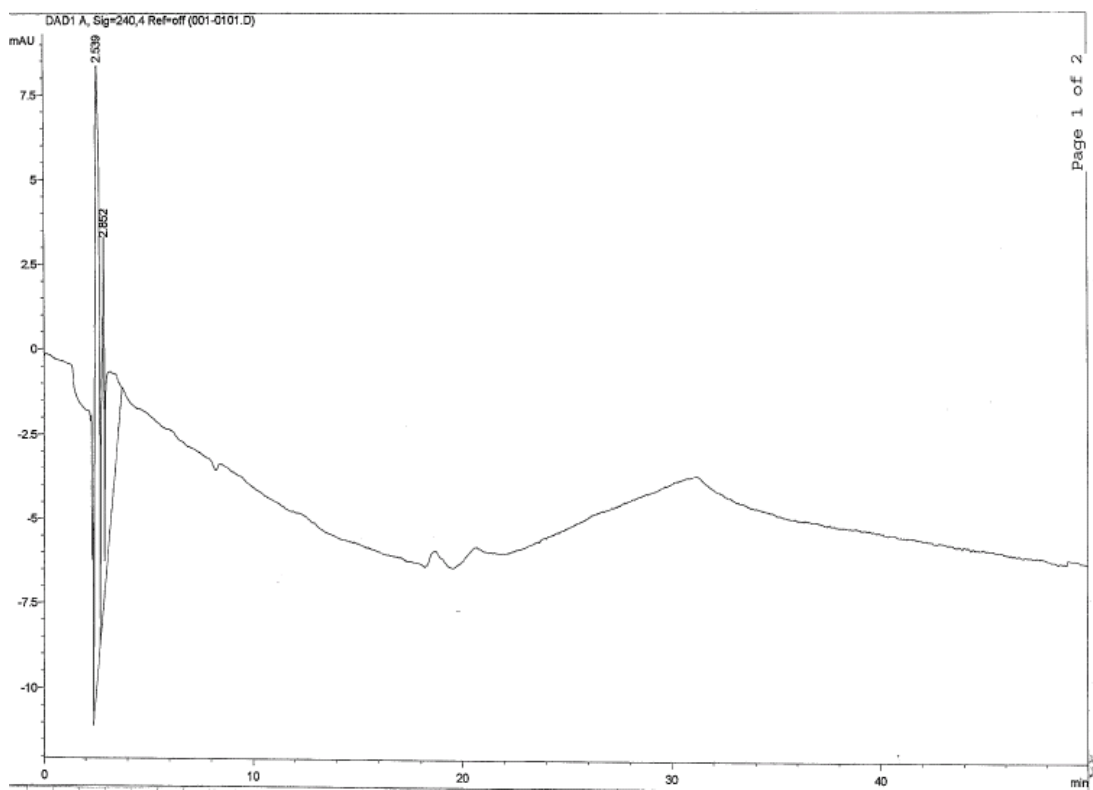
(A) First and (B) second PCNA blot of HepG2 samples. Lanes are as follows: 1 – Marker, 2 –EtOH control, 3 –10 nM 5Adiol, 4 – 10 nM T. Sample loading was normalized to total protein ( $\mu\text{g}/\text{mL}$ ). (C) Graphical representation of compiled band intensities from both PCNA blots after densitometry analysis. Data shown are mean  $\pm$  standard error of the mean.

### Androgen Detection by RP-HPLC in LNCaP Medium after Treatment

Initial investigations into androgen conversion by LNCaP cells using RP-HPLC were not able to reveal any significant peaks in the androgen stocks at concentrations in the nM or low  $\mu\text{M}$  range (data not shown) at 240 nm. Using a Thermo Genesys 10uv spectrophotometer, the minimum detectable concentrations at 240 nm were 1 mM for 5Adiol (0.652 AU) and 100  $\mu\text{M}$  for T (0.757 AU), so concentrated stocks were made. Furthermore, to optimize the sample analyses, the solvent conditions of the column were changed from water to phosphoric acid and the injection volume was adjusted from 10 to 20  $\mu\text{L}$ . After these adjustments were made, a baseline from MeOH was collected (Figure 12A) and a retention time for T at 22 minutes at 240 nm was successfully obtained



(Figure 12B) despite futile attempts to detect a peak for 5Adiol even at a 1 mM concentration (Figure 12C).



**Figure 12A. Methanol baseline profile at 240 nm.**

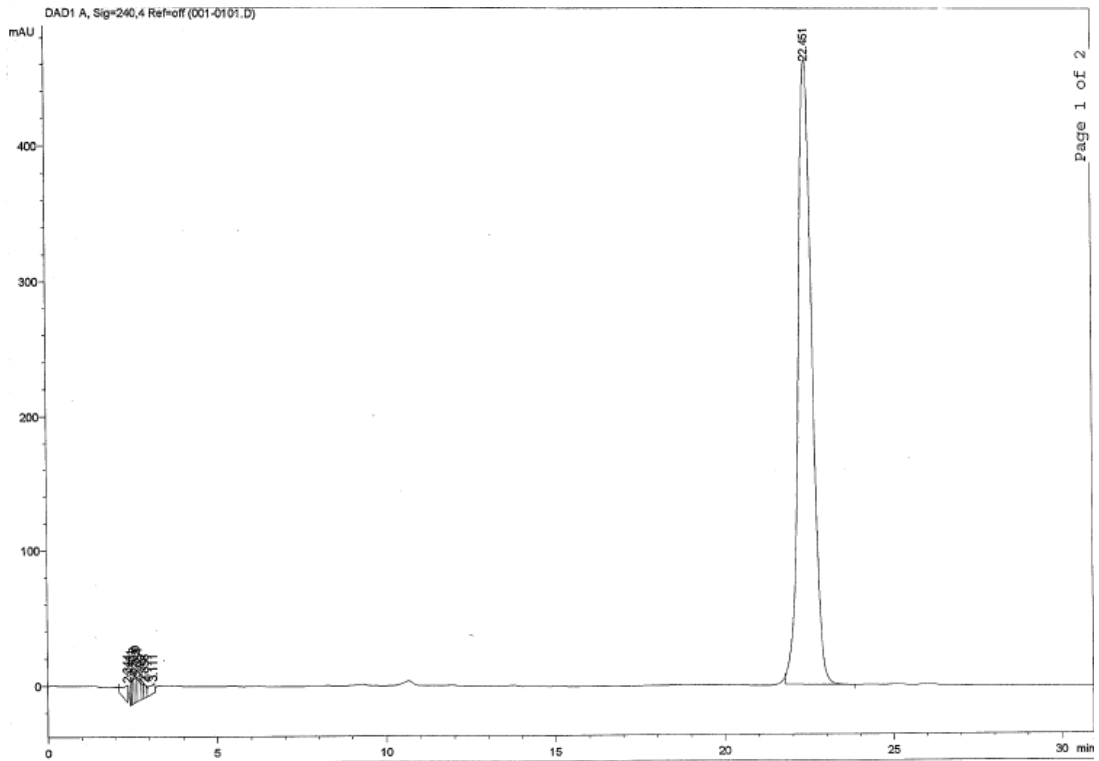


Figure 12B. T profile at 240 nm.

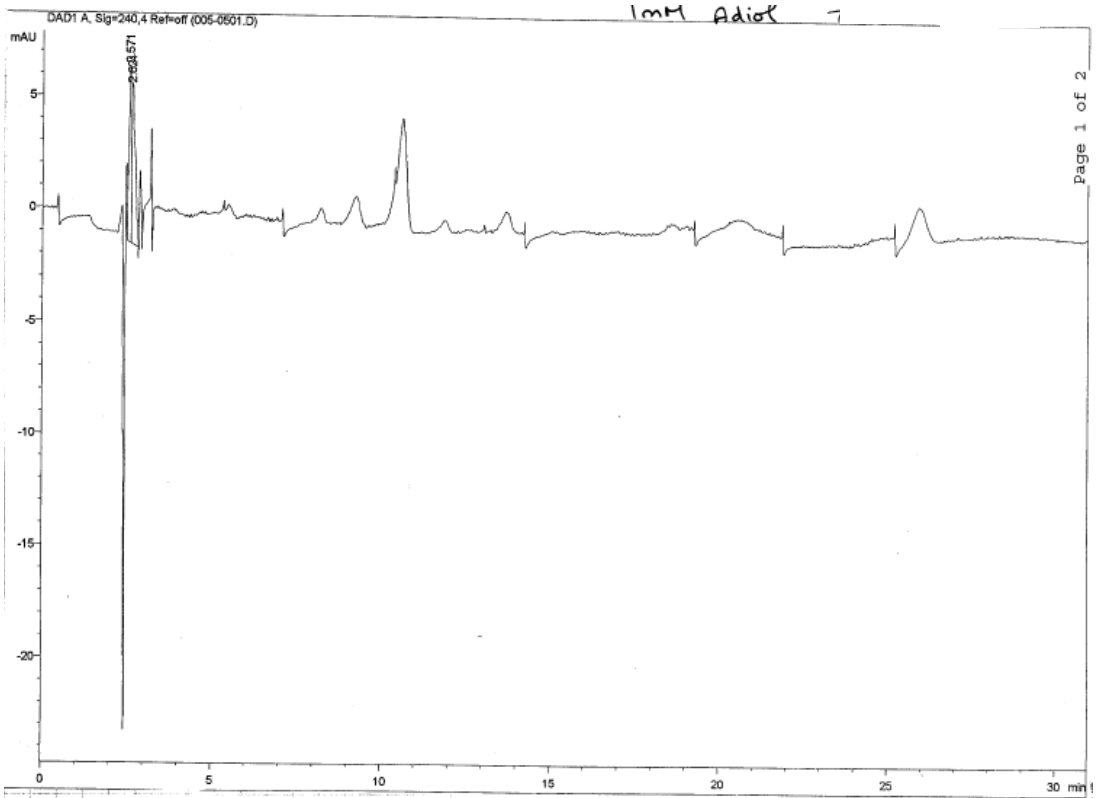


Figure 12C. 5Adiol profile at 240 nm.

Because it was unknown whether a different  $\lambda_{\max}$  was the cause of undetectable peaks from the 5Adiol stock, the wavelength was increased from 240 nm to 254 nm and both T & 5Adiol were reanalyzed (Figures 12D & 12E, respectively). Significant peaks for 5Adiol were not detected, allowing for the conclusion that wavelength was not an issue. Furthermore, the altered wavelength did not have a diminishing effect on the absorbance of T, thus remaining samples were tested at 254 nm in the attempt to detect any 5Adiol peaks in later analyses.

An alternative 5Adiol supplement (AST Science Performance Nutrition) was also tested at 254 nm (Figure 12F) to elucidate if there were any detectable components in another 5Adiol product. Despite the detection of one peak at 9.8 minutes, it was of an insignificant amount considering the estimated stock concentration was 100 mM. Questions were raised as to whether the 5Adiol product used for cell treatments was a true androgenic compound or a false marketing campaign by the manufacturer despite the proliferative response it seemed to elicit.

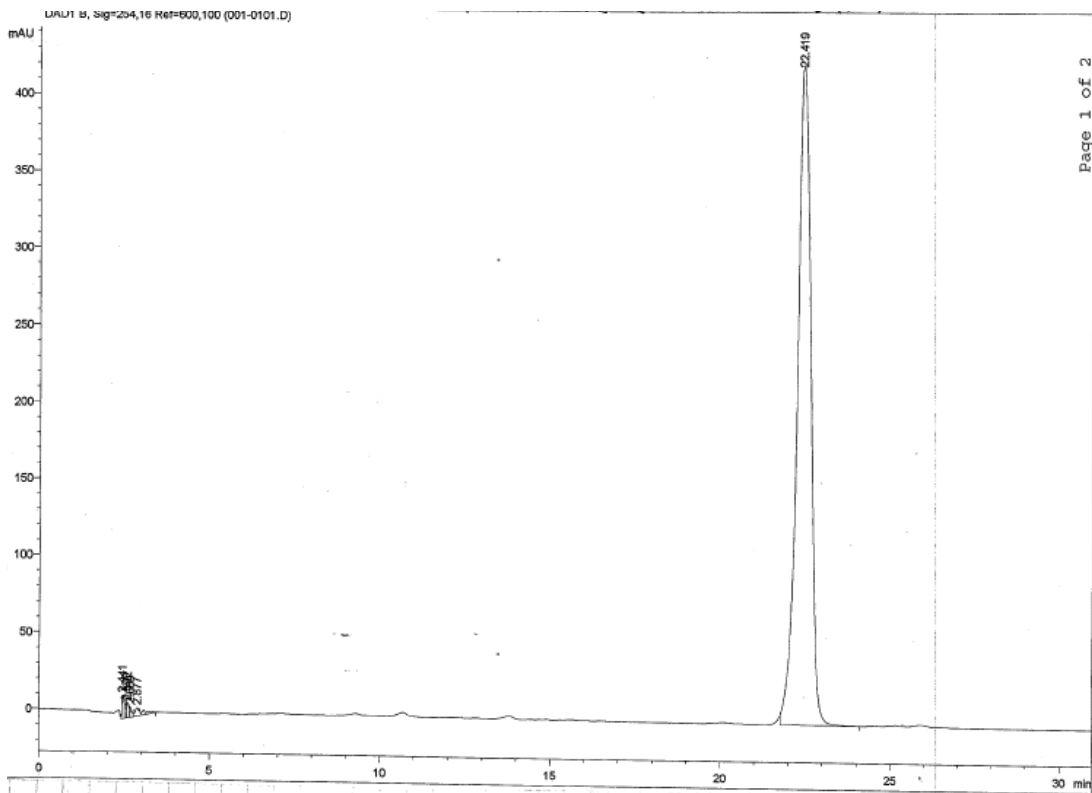


Figure 12D. T profile at 254 nm.

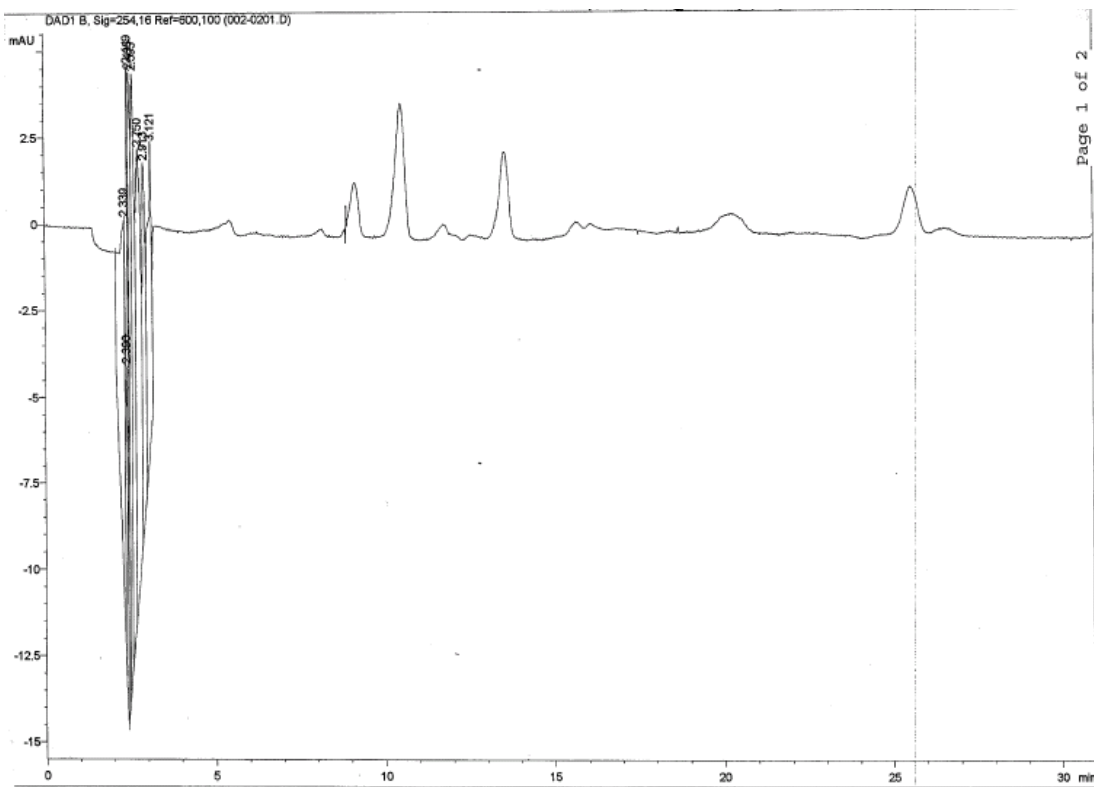
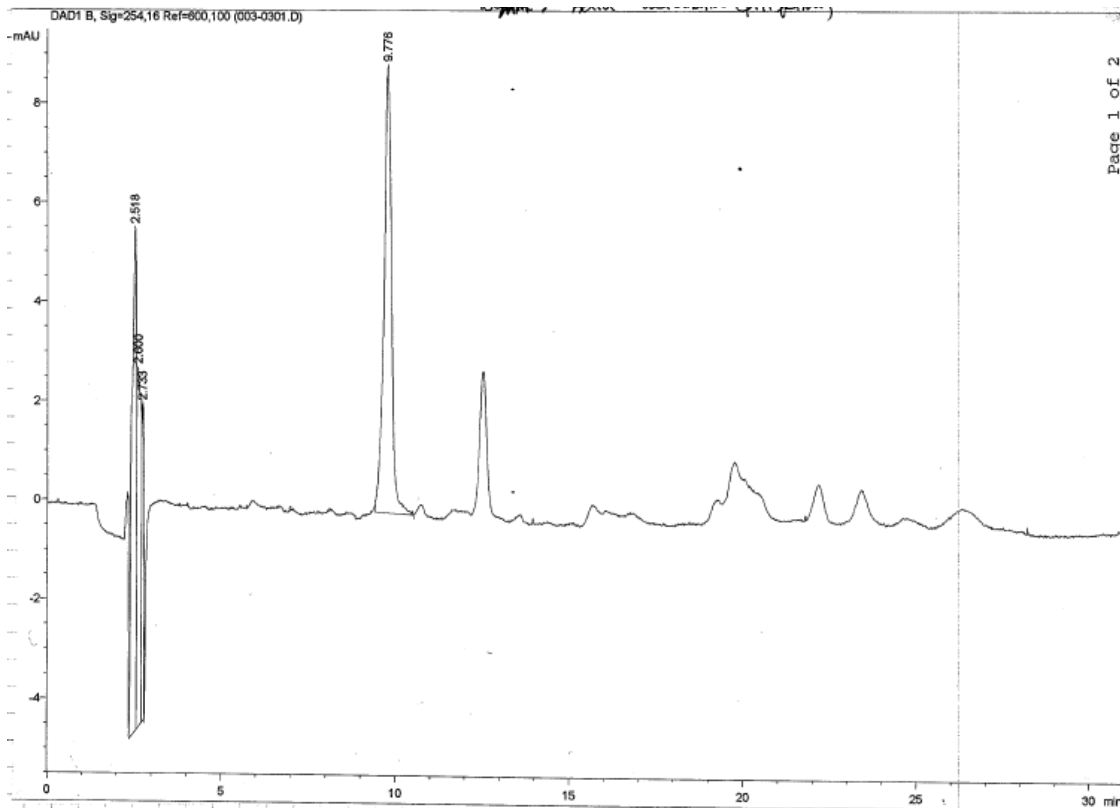


Figure 12E. 5Adiol profile at 254 nm.



**Figure 12F. Alternative 5Adiol product profile at 254 nm.**

Because the cell medium samples from androgen treatments would need to be organically extracted to concentrate the hydrophobic elements (*i.e.* steroids) in the medium, the extraction efficiency was determined by using the stock solution of 1 mM T at 254 nm (Figures 13A&B).

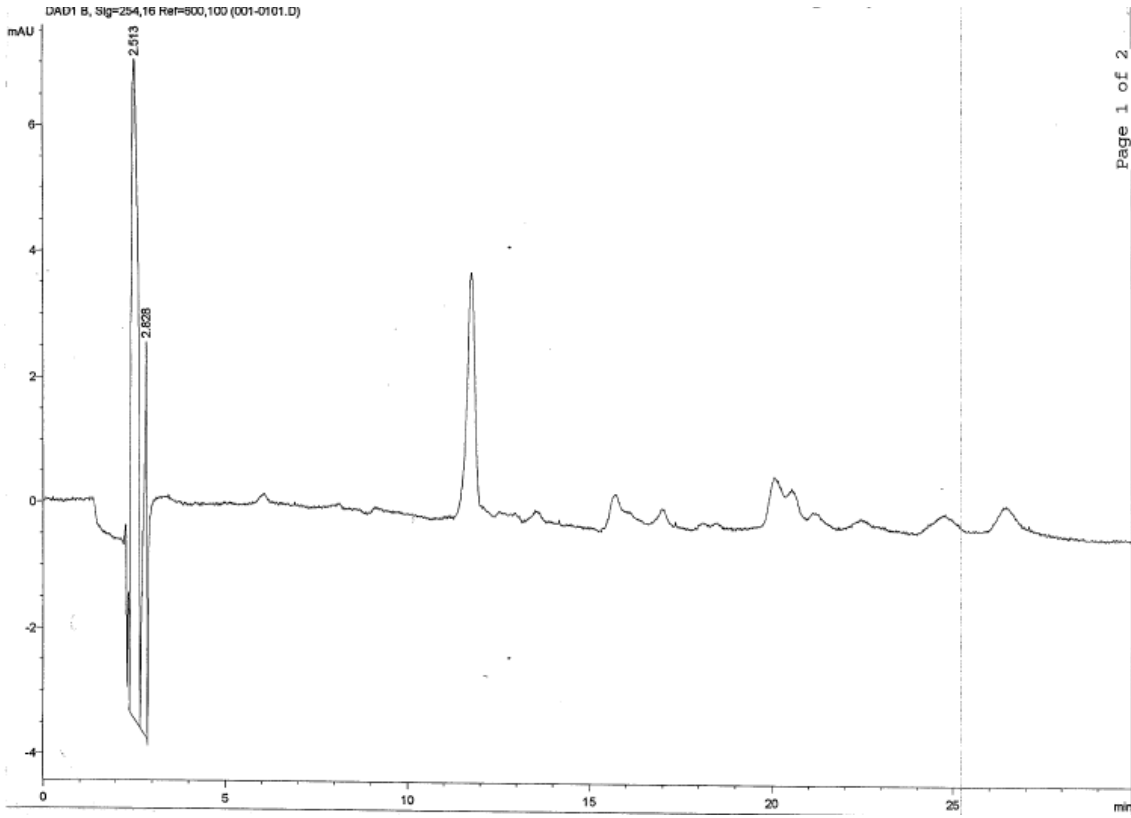


Figure 13A. Methanol baseline after organic extraction, measured at 254 nm.

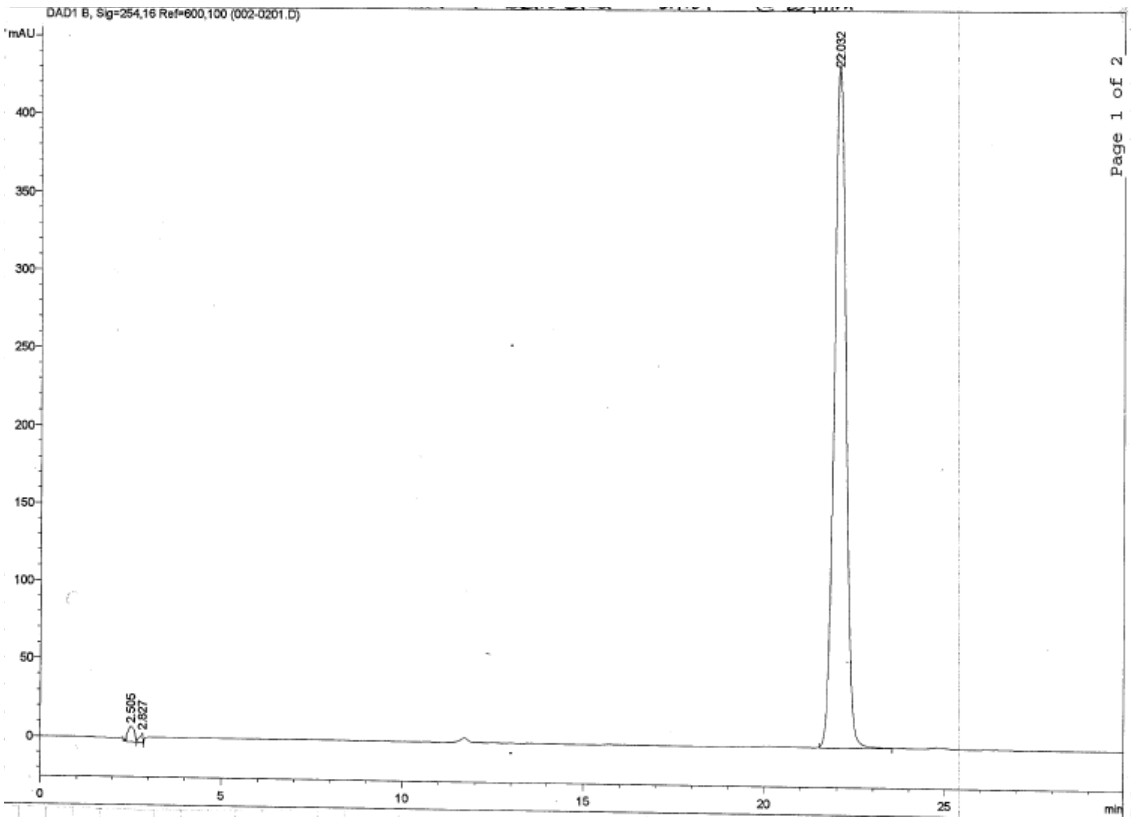
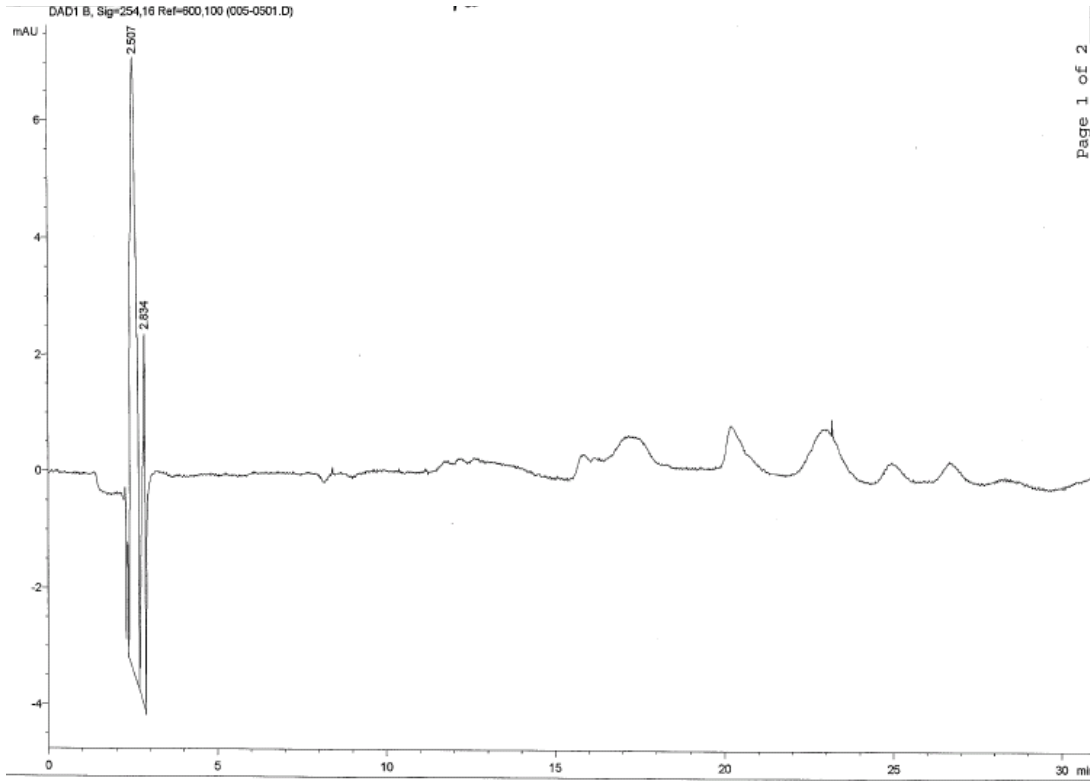


Figure 13B. 1 mM T profile after organic extraction, measured at 254 nm.

Since the efficiency was calculated at 83.5% (no extraction: area = 11704; post-extraction: area = 9777.9), 2% FBS medium controls were extracted and analyzed (Figures 14A-E).



**Figure 14A. Methanol baseline after extraction, measured at 254 nm.**

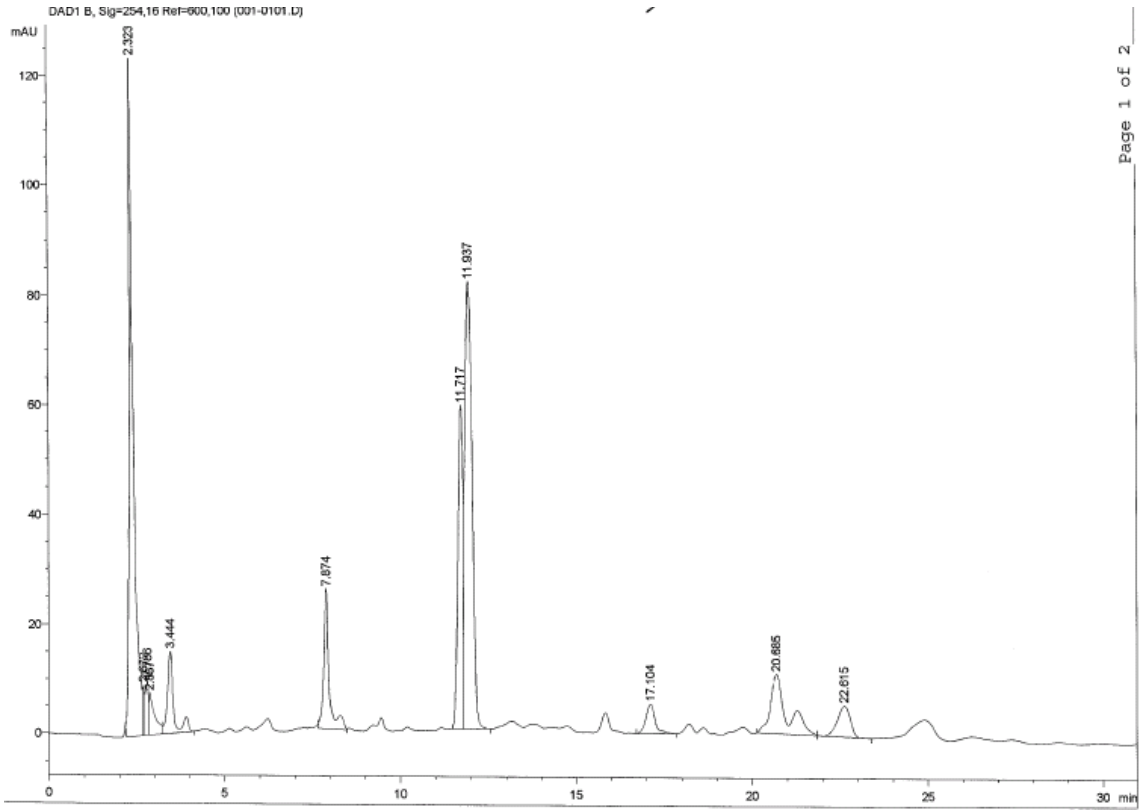


Figure 14B. 2% FBS medium control after extraction, measured at 254 nm.

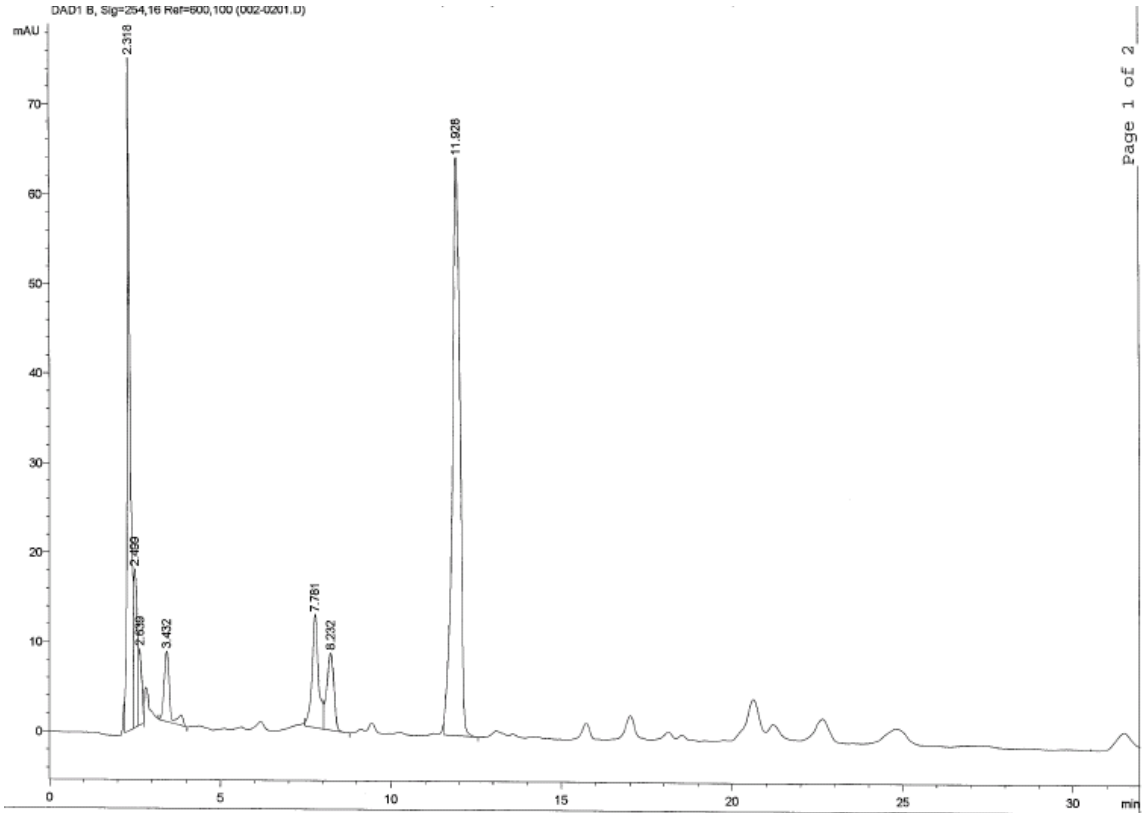


Figure 14C. 2% FBS + 10 nM 5Adiol medium control after extraction, measured at 254 nm.



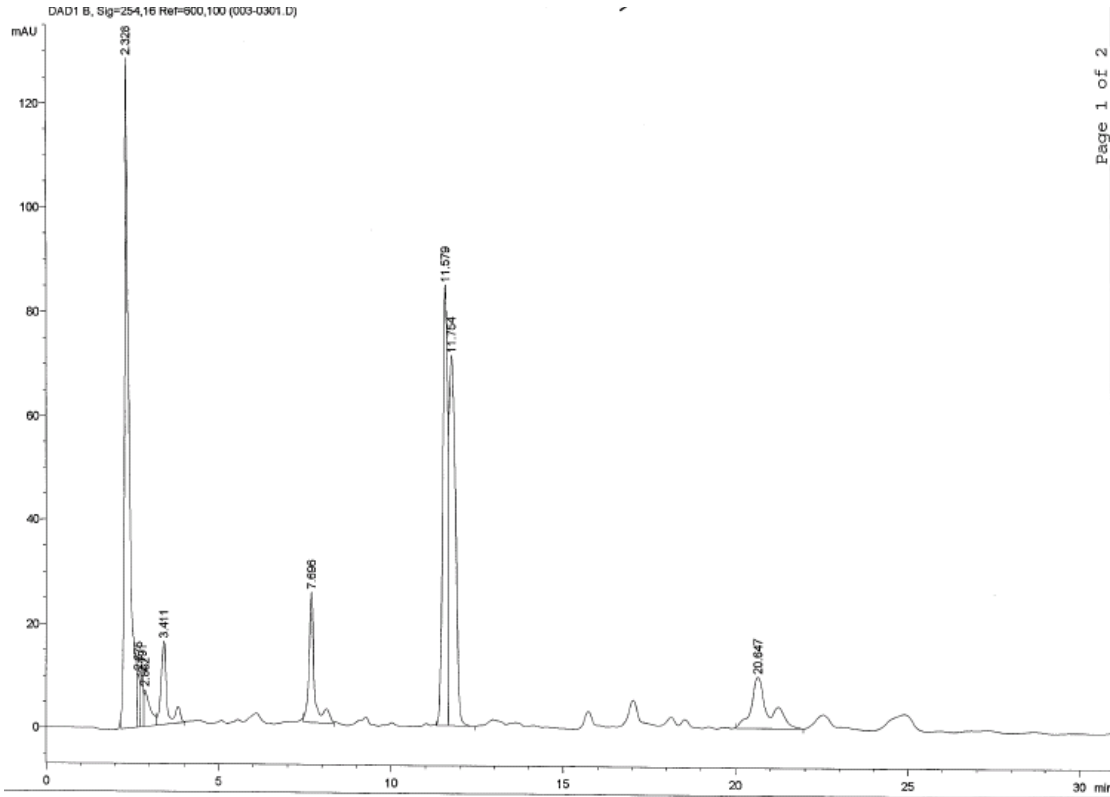


Figure 14D. 2% FBS + 10 nM T medium control after extraction, measured at 254 nm.

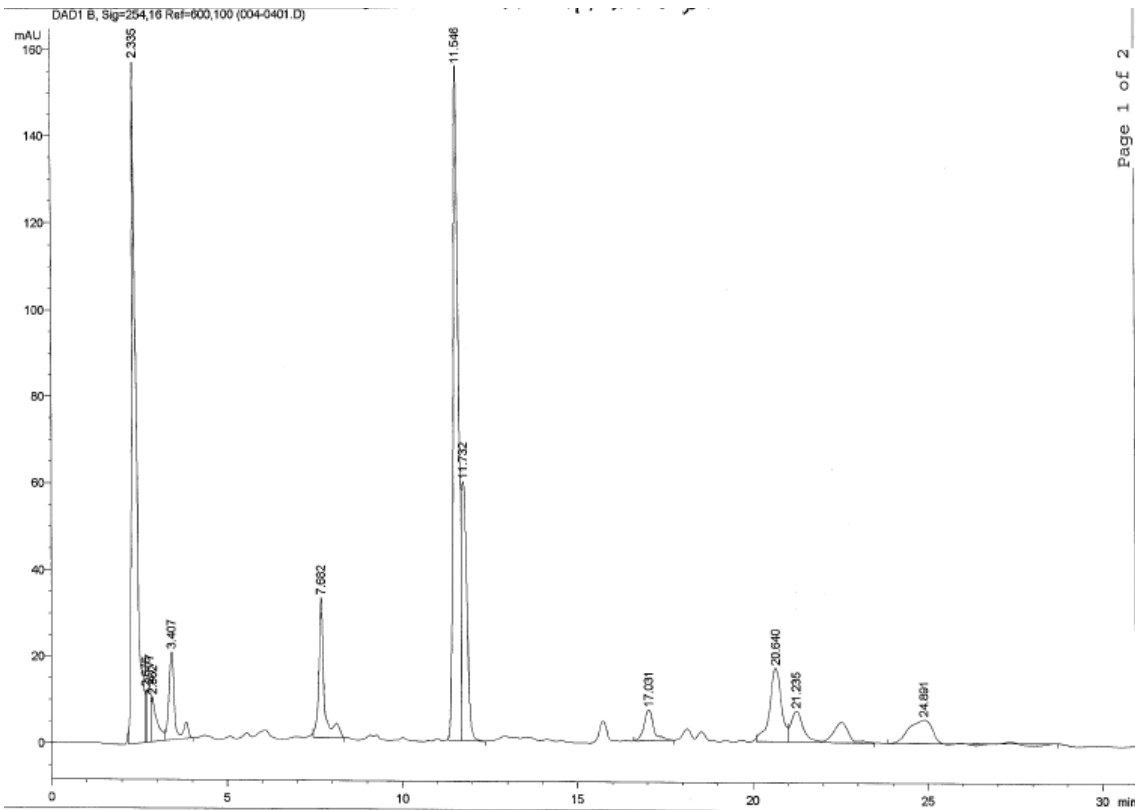


Figure 14E. 2% FBS + 10 nM 5Adiol and T medium control after extraction, measured at 254 nm.

Examination of the 2% FBS medium controls concentrated in 50  $\mu$ L MeOH showed multiple peaks. However, the chromatogram profiles of each control were extremely similar. Although the peak heights did vary between controls, and retention times may or may not have been assigned by the HPLC dependent upon the detection threshold, the peak profiles were consistent in each sample. Two peaks in particular, eluted around 11.5 minutes, were consistent in both time and peak area throughout the analysis. These were initially considered to be phenol red from the medium, however, their presence in a DMSO sample (data not shown) contradicted this hypothesis since DMSO contains no phenol red. Thus, these compounds are unknown contaminants. Overall, these combined data revealed the possibility that even concentrating the androgens in a smaller volume may not yield detectable androgen peaks.

Before moving on with cell treatment medium analysis, sample filtering was ruled out as a possible reason for undetectable 5 $\alpha$ -Diol peaks due to the globular proteins in FBS (data not shown). Prior to analysis on the column, LNCaP medium samples were extracted, evaporated under nitrogen and concentrated in 80  $\mu$ L of MeOH (Figures 15A-E).

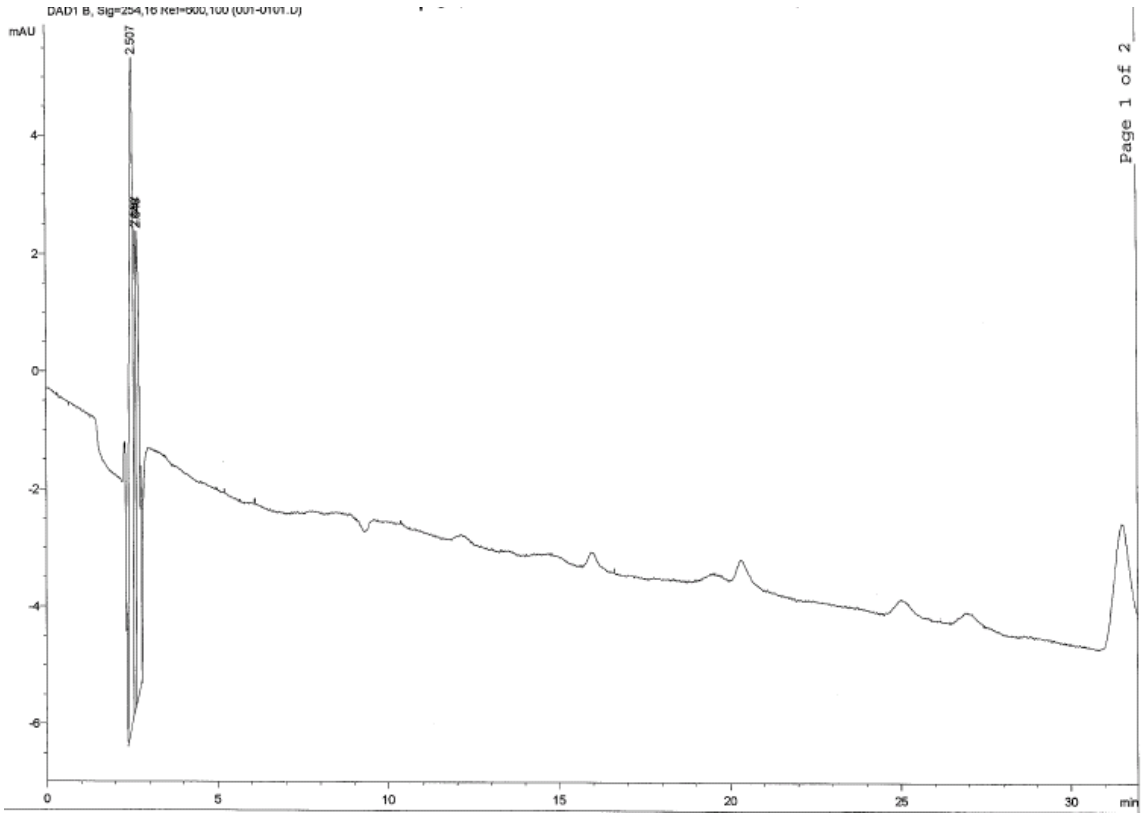


Figure 15A. Methanol baseline after extraction, measured at 254 nm.

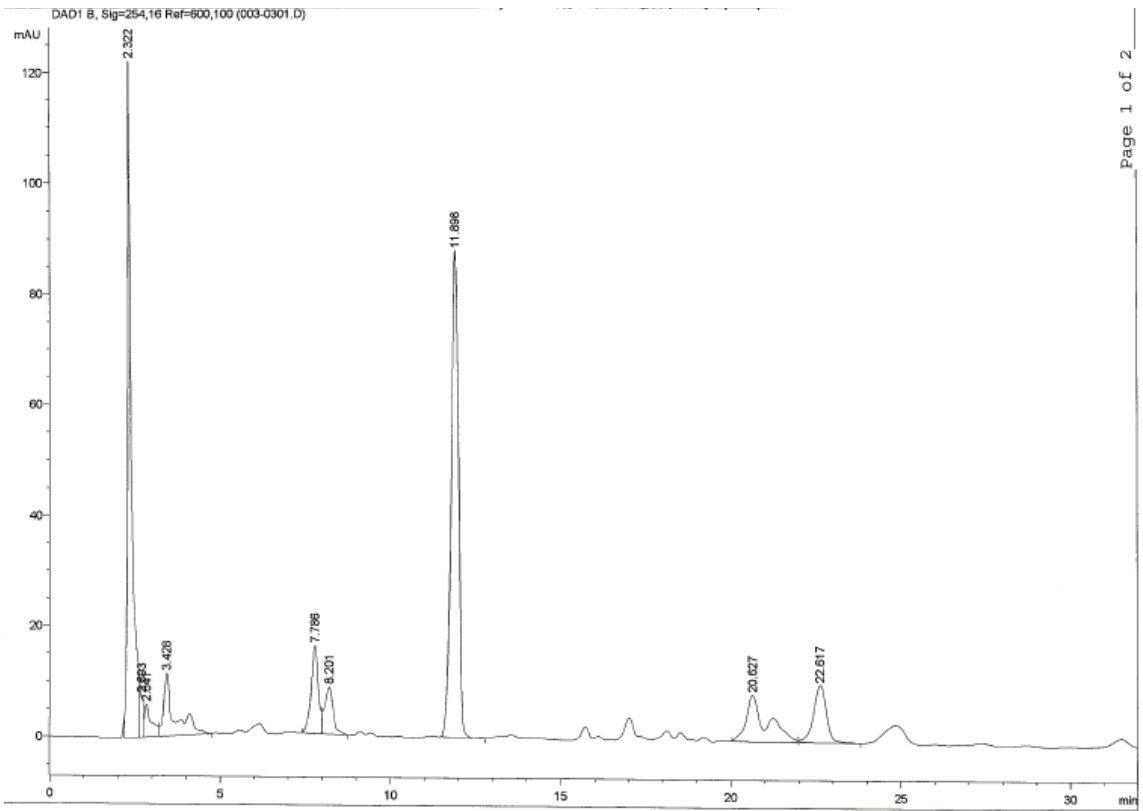


Figure 15B. LNCaP no treatment control measured at 254 nm.

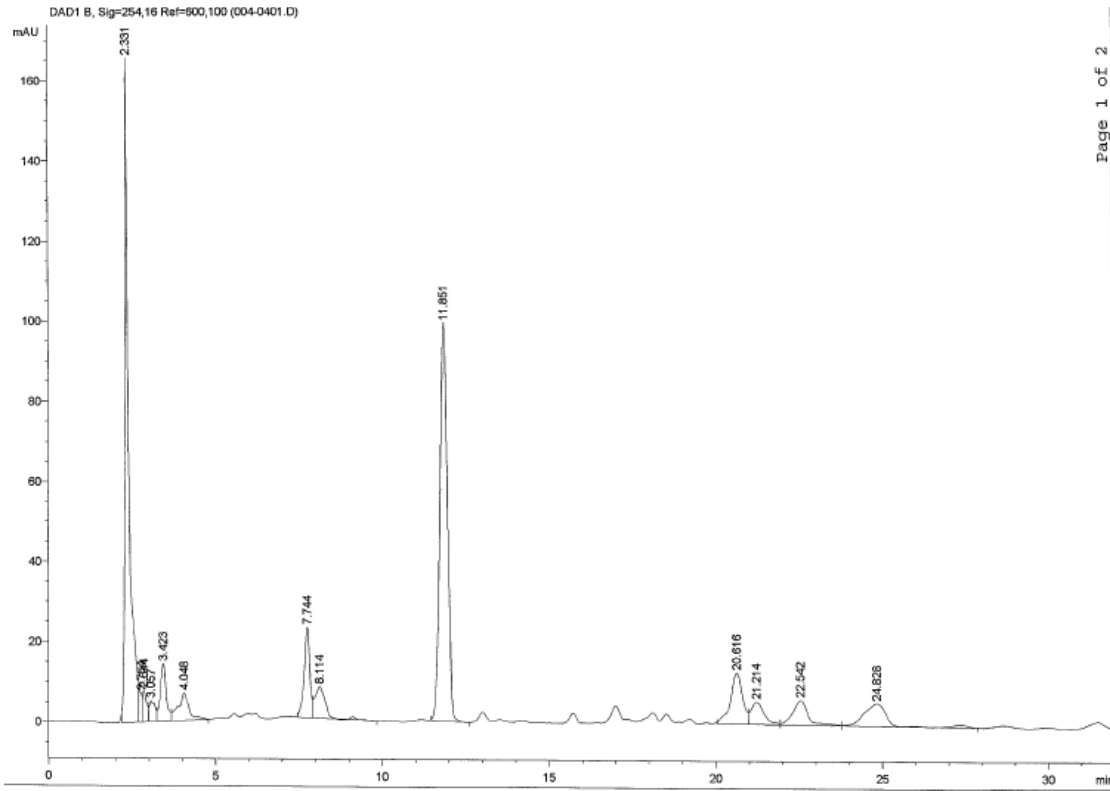


Figure 15C. LNCaP EtOH control measured at 254 nm.

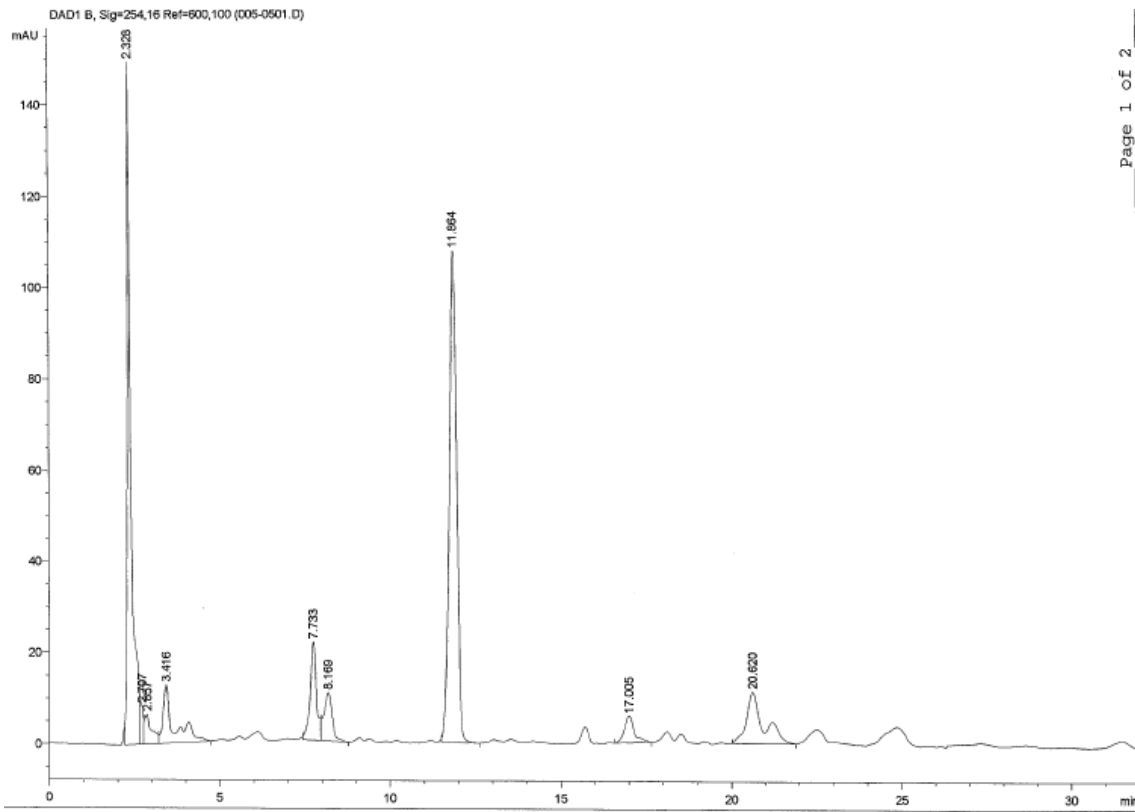
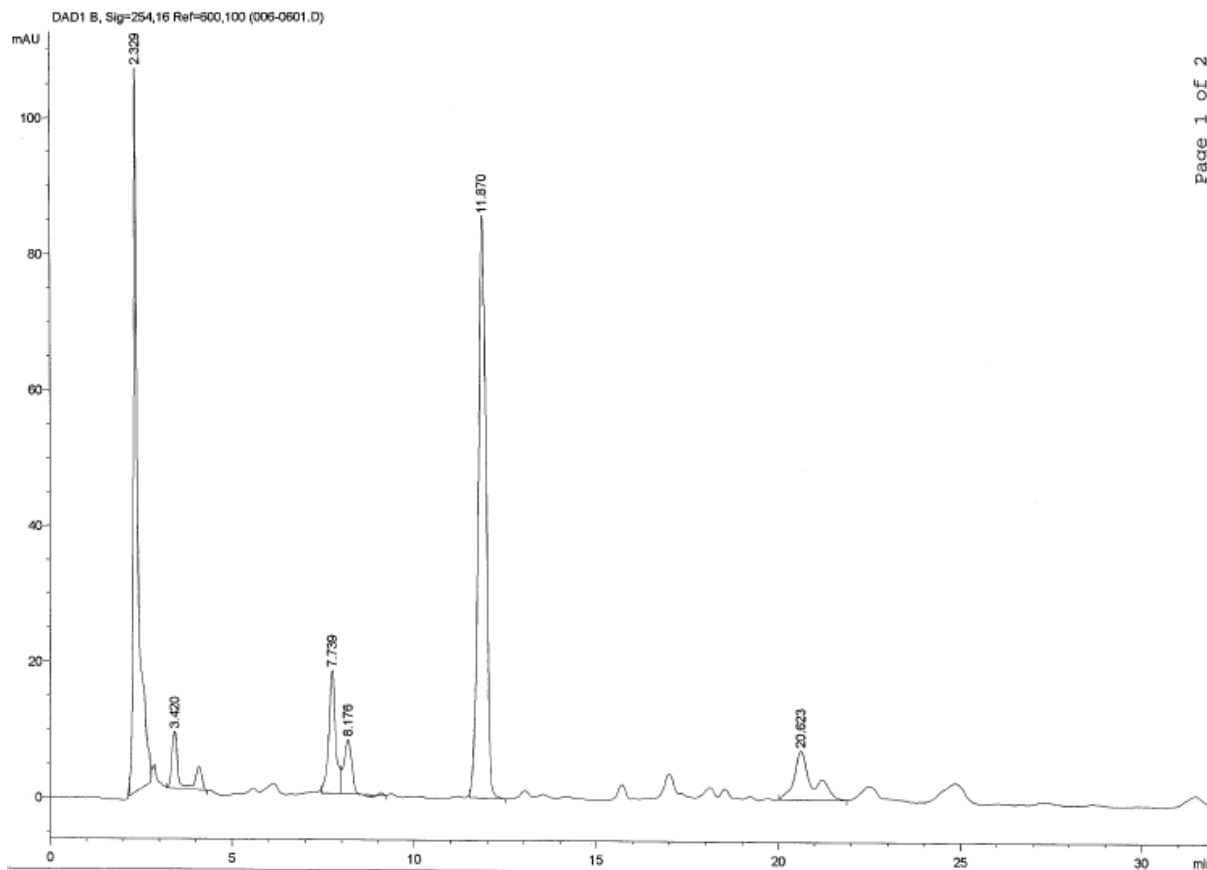


Figure 15D. LNCaP sample treated with 10 nM 5Adiol measured at 254 nm.

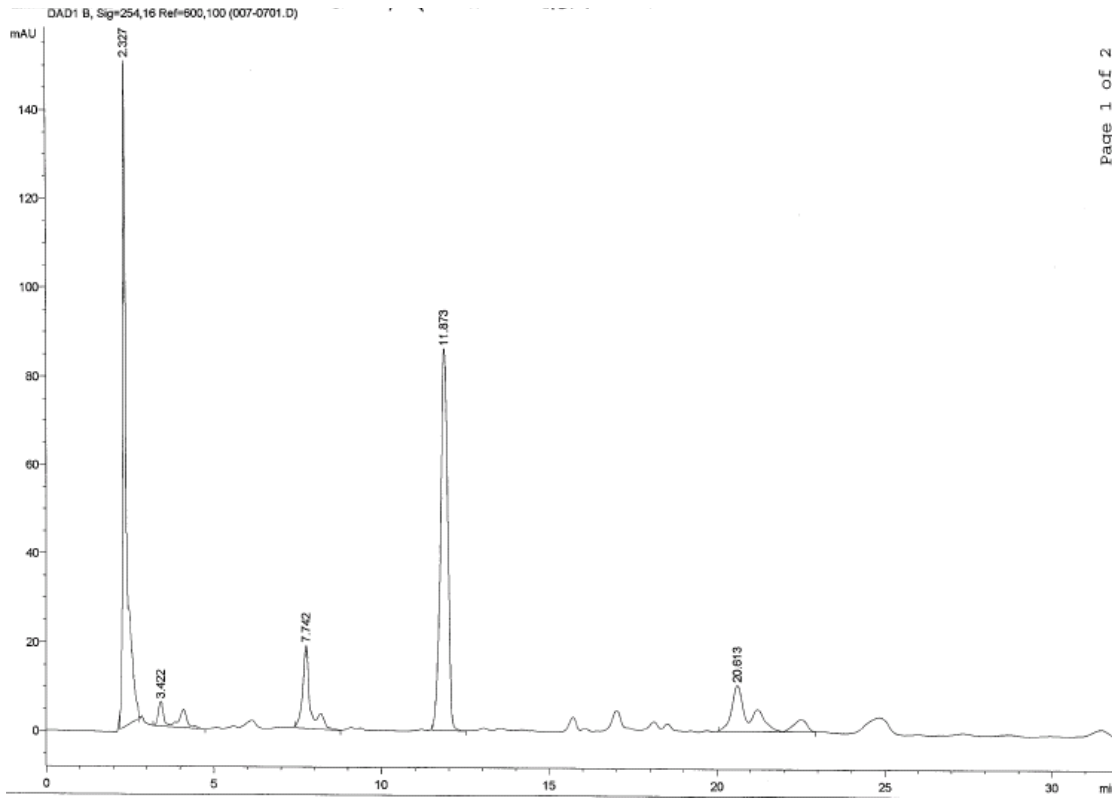


**Figure 15E. LNCaP sample treated with 10 nM T measured at 254 nm.**

Although peaks were seen in each sample, none appeared to be significant among the various treatments because the profiles were very consistent, mimicking what had been seen in the 2% FBS medium controls. Again, the one peak of a significant concentration was at 11 minutes, but its significance is unknown since it consistently appeared in each sample and was observed in a DMSO sample, as mentioned previously. Taken together, it appeared as though the detection capability of the HPLC spectrophotometer was not sufficient for the analysis of these samples or alternatively, that there are no androgens present in the 5Adiol product.

## Androgen Detection by RP-HPLC in LNCaP Medium after HepG2 Conditioned Medium Treatment

LNCaP medium samples from culture with conditioned medium from HepG2 androgen treatments were also analyzed (Figures 16A-D) at 254 nm. These samples were extracted, evaporated under nitrogen and concentrated in 80  $\mu$ L of MeOH prior to analysis on the column.



**Figure 16A. LNCaP sample cultured with HepG2 no treatment control conditioned medium.**

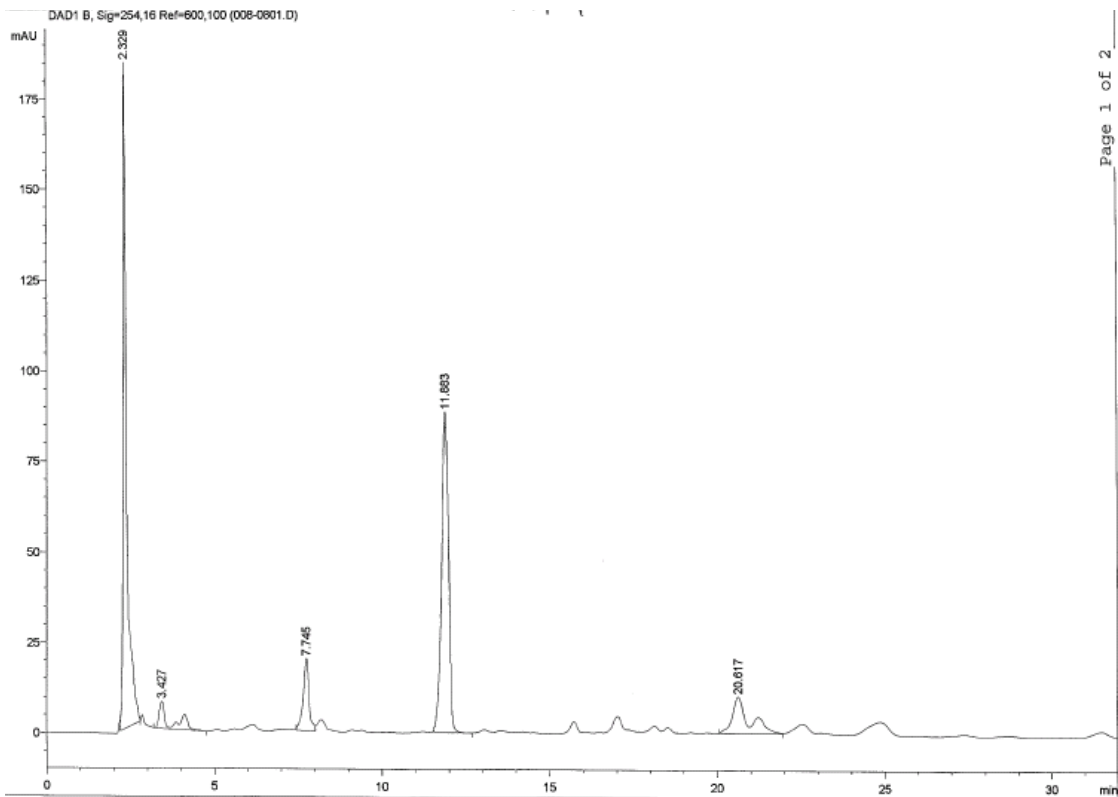


Figure 16B. LNCaP sample cultured with HepG2 EtOH control conditioned medium.

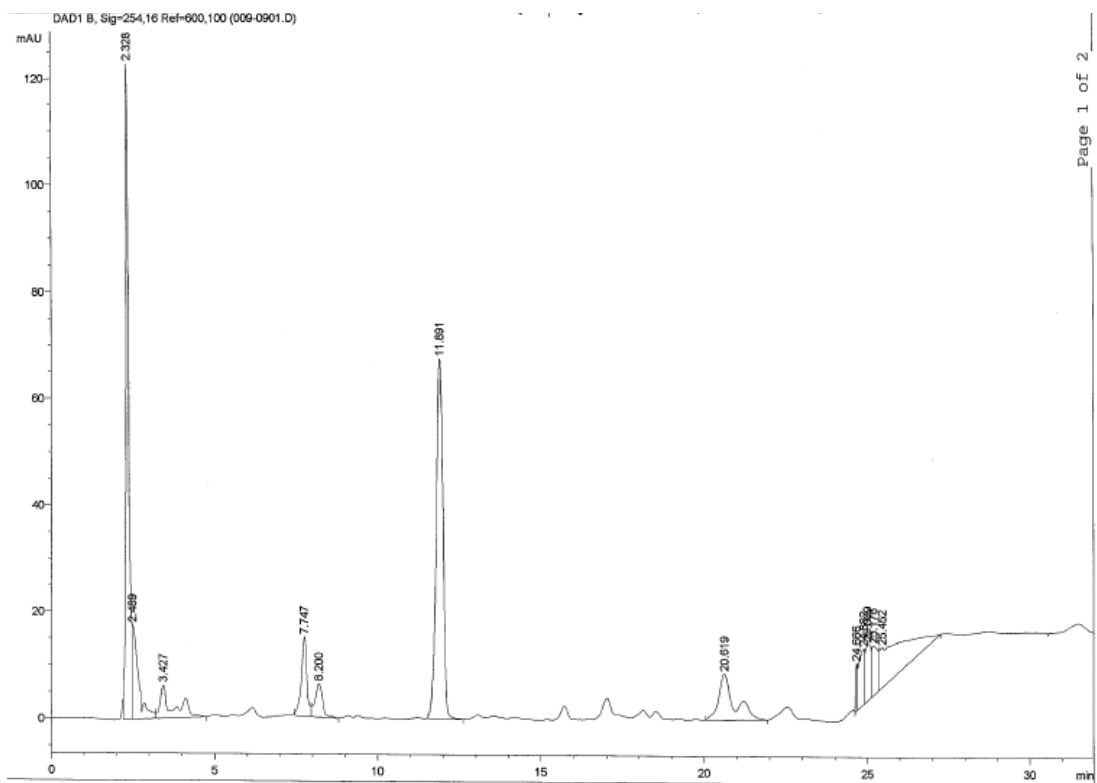
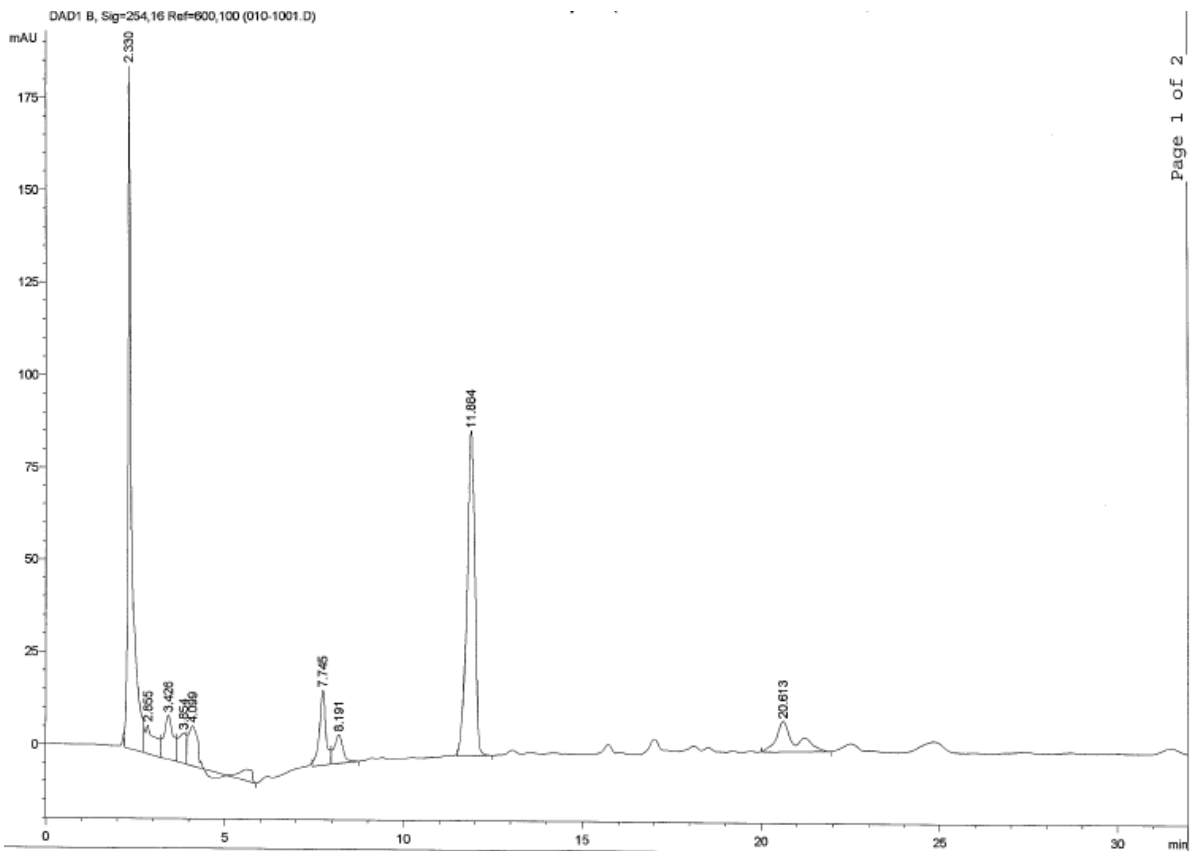


Figure 16C. LNCaP sample cultured with HepG2 conditioned medium from 10 nM 5Adiol treatment.



**Figure 16D. LNCaP sample cultured with HepG2 conditioned medium from 10 nM T treatment.**

Reiterating what had been seen with the LNCaP medium samples, detected peaks were equivalent between all samples despite any observable changes in peak heights. These findings supported the conclusion that the detection system of the HPLC was not sensitive enough for the analysis of this 5Adiol product.



## DISCUSSION

As AAS become more popular in the world of competitive sports, the ever-increasing availability of these products quickly outpaces the resources of government agencies responsible for their regulatory control. With an almost nonexistent guarantee of quality for such products, multiple links of their use with cancer development, and contamination with regulated substances or mislabeled components, the use of these substances is a growing cause of concern [Bagia *et al.*, 2000; Bryden *et al.*, 1995; Bydder *et al.*, 2007; Catlin *et al.*, 2000; Delbeke *et al.*, 2002; Froehner *et al.*, 1999; Hartgens and Kuipers, 2004].

The major aim of this study was to investigate the effects of a 5Adiol product (Value Nutrition 5-Diol Powder) marketed over the internet, on the androgen-dependent prostate cancer cell line, LNCaP. Intraprostatic androgen conversion has been proposed to play a role in prostate cancer progression to androgen-independent growth, so this aim was important in assessing the risk of this and similar products [Nishiyama *et al.*, 2007]. Treatment concentration range (1-100 nM) was chosen based upon the known 5.2 nM physiological level in the body [Mizokami *et al.*, 2004]. Although the tested androgen range in this effort may not accurately mimic what is found in men using this substance for anabolic purposes, it would be uniquely difficult to do so considering the wide range of concentrations used by these individuals [Parkinson and Evans, 2006]. An additional objective of this study was to evaluate the effects of this product on a liver cell line (HepG2) since the liver plays a role in androgen metabolism. Because liver metabolism also affects serum hormonal levels, which ultimately affect other organs in the body such

as the prostate, the medium from HepG2 treatment with 5Adiol was also used to elucidate any effects of the hepatic metabolism of this product on LNCaP cells. Since the 5Adiol was purchased from a non-reputable source over the internet, the effects of this product were compared to those induced by testosterone, which was purchased from the chemical company, Sigma-Aldrich.

The major finding of this study was that LNCaP cells were stimulated to undergo mitosis in a dose- and time-dependent manner in response to treatments with either 5Adiol or testosterone. A dose-dependent response of LNCaP cells to dihydrotestosterone was demonstrated by Horoszewicz *et al* in 1983 [Horoszewicz *et al.*, 1983] as well as by Mizokami *et al* in 2004, where LNCaP cells were treated with testosterone, dihydrotestosterone, dehydroepiandrosterone, and 4-androstenedione over a range of concentrations for four days [Mizokami *et al.*, 2004]. Importantly, they observed a mitogenic response where cell proliferation increased 20% with 100 pM 5Adiol treatment when compared to cells treated with 100 pM of testosterone or dihydrotestosterone [Mizokami *et al.*, 2004]. At a 10 nM dose after 4 days of treatment, the proliferative trend was 5Adiol>testosterone>dihydrotestosterone, although the authors didn't include a statistical analysis of the observed difference.

The data presented here suggest that treating cells with either the tested 5Adiol product or testosterone resulted in an increased rate of proliferation relative to untreated controls. Despite the case of several different assays and multiple repetitions, both inter-and intra-experimental variability prevented robust statistical analysis of the data. However, the

trends observed are consistent with those of Mizokami *et al* even though their studies were carried out at very different doses. How the addition of picomolar quantities of substances, which are present at nanomolar levels in the normal physiologic state, would have an effect on cells is unclear. However, the demonstration that testosterone precursors even at significantly reduced levels may be mitogenic is alarming.

Most of the variability in this work appeared to be related to LNCaP adhesion to the culture substrata, which caused great variability in the data due to the large loss of viable cells during solvent changes. The LNCaP cell line is characterized as a loosely adherent culture due to a reduced expression of the  $\alpha6\beta4$  integrin [Bonaccorsi *et al.*, 2000], focal adhesion kinase [Slack *et al.*, 2001], insulin growth factor (IGF) receptor [Reiss *et al.*, 2001], and tyrosine phosphatase (an immunoglobulin adhesion molecule) [Hellberg *et al.*, 2002], which all play critical roles in cellular adhesion and motility. Culture surface modifications less likely to cause significant biological changes within the cells were attempted, which included net charge through the use of poly-lysine, more oxygen groups using a Corning® Cellbind® product which stabilizes the surface for improved attachment, centrifugation to force the cells to the dish during solvent changes, and alterations in plating density or surface area. However, because these treatments did not appear to effectively improve the situation, the use of a fibronectin-coated surface was examined. Despite the drastically improved attachment to the culture wells, no significant differences were found, and so this avenue wasn't investigated further for two reasons: (i) few groups that research androgenic effects in prostate cancer use surface modifications, and (ii) extracellular matrix (ECM) proteins such as fibronectin are known players in

signal transduction pathways that affect biological processes involved in cellular division. Where this study was dependent upon cell proliferation as a means for determining treatment response to androgens, an additional mitogenic variable was not desirable.

Although the majority of research groups involved in androgen studies do not to utilize surface modifications to improve adhesion, some research has been completed using collagen and other ECM proteins to improve attachment and observe effects mediated on the LNCaP phenotype [Arnold *et al.*, 2005; Fong *et al.*, 1992; Guo *et al.*, 1994]. Whereas the proliferation of LNCaP cells plated on ECM was found to occur in a time- and dose-dependent manner following treatment with dehydroepiandrosterone, testosterone, dihydrotestosterone, or estradiol [Arnold *et al.*, 2005], previous work [Fong *et al.*, 1992; Guo *et al.*, 1994] observed that LNCaP proliferation was either attenuated or not affected by ECM modification to the culture environment when compared to growth on plastic or collagen. Fong and colleagues logically concluded that the environment in which the LNCaP cells are cultured determines their cellular response, differentiated function, and morphological response to androgens. Where the LNCaP cell line was derived from a metastatic lesion of Pca [Horoszewicz *et al.*, 1983], the reduced attachment dependence of these cells would allow them to be transported via the lymphatic or circulatory systems to metastatic sites. This lack of attachment dependence is, in fact, a hallmark of transformation and related to metastatic potential. Thus, modifications to the culture environment aimed at improving adhesion may in fact alter the native phenotype and/or genotype of the cells, making firm conclusions or comparisons theoretical at best.

As a solution to the attachment issue, 2% FBS was added to the medium rather than use 10% DCT-FBS. Although most groups studying androgen treatment on prostate cancer use DCT-FBS [Arnold *et al.*, 2005; Aspinall *et al.*, 2004; Chen *et al.*, 2004; Fong *et al.*, 1992; Gregory *et al.*, 2001; Guo *et al.*, 1994; Horoszewicz *et al.*, 1983; Lee *et al.*, 1995; Miyamoto *et al.*, 1998; Umekita *et al.*, 1996], it is unclear whether these groups have observed the same issues encountered in this study, or whether they acknowledge the issue of LNCaP adherence. For comparative reasons between this study and others, as well as biological rationale (*i.e.* cells are only treated with the androgens supplemented in the medium), dextran-charcoal treated FBS would have been preferred. However, it is unclear whether the charcoal treatment of DCT-FBS also stripped the culture of necessary factors for both adhesion and cell division, thereby preventing the cells from completing either native function. Furthermore, it cannot be ruled out whether passage number or alternative culture conditions used in this study may have selected for a certain phenotype/genotype or initiated cellular differentiation or other modifications which amplified the non-adherent characteristics of this cell line. Despite these arguments however, 2% FBS was used for whatever advantages it may have conveyed to the cells.

Also giving pause for speculation is the cytotoxicity observed in both MTT and tritiated thymidine uptake assays at 48 hours and beyond. It is possible that because medium changes were not performed during the single dosage treatments, cells began to apoptose due to a lack of nutrients or a change in the culture pH. It may also be possible that these androgens were metabolized to a cytotoxic product in the LNCaP cell line. Speculations aside, other investigators have observed cytotoxic effects or growth arrest in LNCaP cells

due to higher nanomolar and micromolar level treatments using dihydrotestosterone [Horoszewicz *et al.*, 1983; Lee *et al.*, 1995]. In particular, Horoszewicz *et al.* observed greater cytotoxicity when basal medium with 5% FBS was supplemented with dihydrotestosterone compared with basal medium plus 5% DCT-FBS and dihydrotestosterone. This seems logical if dihydrotestosterone can independently induce cytotoxic effects, whereas such effects would be compounded by normal serum, which already contains steroids. It may be possible that a similar phenomenon occurred with the 5 $\alpha$ -DHT and testosterone treatment in this study, where length of treatment was somehow toxic for cell viability.

Hepatic metabolism of the tested androgens may be an arguable factor in prostate cancer progression, suggested by the significantly increased PCNA expression in LNCaP cells when cultured with HepG2 androgen-conditioned medium. To the best of our knowledge, this is one of the first observations into the mitogenic effects of androgenic liver metabolism on prostate cancer. The liver is known to express key enzymes in androgen metabolism, namely the 5 $\alpha$ -reductases and 17 $\beta$ -hydroxysteroid dehydrogenases (17HSD) [Andersson *et al.*, 1995; Soronen *et al.*, 2004; Vihko *et al.*, 2006]. Whereas the 17HSD are known to inactivate active estrogens and androgens, such as testosterone to androstenedione, the reductases catalyze the opposite reaction, where steroids are reduced to a more active metabolite. The expression of both types of enzymes permits the liver to have a regulatory role in both local and systemic steroid metabolism as well as serum hormone levels. It can be postulated that in malignant transformation, alterations in these

enzyme's expression patterns would have drastic effects upon the progression of a malignancy that responds to an androgenic stimuli.

Although solid conclusions cannot be made regarding the response of the HepG2 cell line to androgen treatment, due to the small sample size and observed variability in the response to testosterone, it appears as though androgen treatment with 5 $\alpha$ -DHT may stimulate the proliferation of this cell line. However, this data is contradicted by one attempt to study HepG2 proliferation by tritiated thymidine uptake, which did not show any significant differences between androgen treatments (data not shown). It is likely that more repetitions would clarify this response. Despite the hypothesis that proliferation would not be stimulated in response to androgen treatment, the response of the HepG2 cells to 5 $\alpha$ -DHT was unknown at the start of this study. This was primarily due to a limited number of investigations having been previously conducted that evaluated the role of androgens, or the expression of the androgen receptor, in liver cancer *in vitro*. Lim *et al* determined in 1997 that 25 nM dihydrotestosterone induced proliferation of HepG2 cells [Lim *et al.*, 1997]. However, a very recent study in 2007 by Jie *et al* demonstrated that androgen receptor mRNA levels are expressed at lower concentrations in HepG2 cells compared to other hepatocellular carcinoma cell lines, and furthermore, that HepG2 treatment with 100 nM dihydrotestosterone elicits only a modest increase in proliferation [Jie *et al.*, 2007]. These results may be attributed to the fact that translational expression of the androgen receptor was not investigated, and the modest increase in proliferation may be due to cytotoxic effects of higher concentrations of dihydrotestosterone, which has been seen with prostate cancer cell lines [Horoszewicz *et al.*, 1983; Lee *et al.*, 1995].

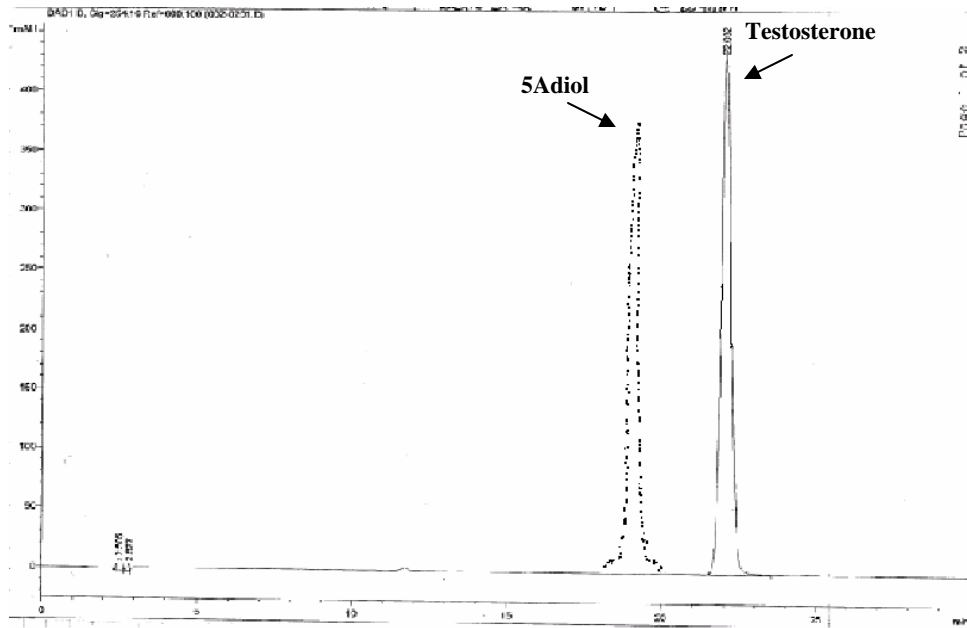
If the androgen receptor is only weakly translated in the HepG2 cell line, it is possible that the effects observed in our study were related to androgen regulation of the insulin-like growth factor-1 receptor (IGFR). This protein was shown to be upregulated in prostate cancer by androgen treatment [Pandini *et al.*, 2005], and is a known vital component of growth, apoptotic resistance, and invasiveness in malignant growths. Pandini *et al* also showed that the androgen-induced upregulation is mediated through kinase activation of the androgen receptor, which is an alternative pathway to ligand binding activation for this receptor.

The hypothesis regarding 5 $\alpha$ -Dihydrotestosterone conversion to testosterone was unable to be tested due to the limited detection capability of the reverse-phase high performance liquid chromatography (RP-HPLC) spectrophotometer. The concentrations tested were likely too small to be observed, thus making conclusions impossible. It is interesting to note that attempts to concentrate the stock 2% FBS medium control containing 10 nM testosterone did not result in discernable peaks in the chromatogram. Although these efforts changed the initial concentration of 10 nM to 334 nM, the final concentration was still 10-fold lower than the calculated maximum detection limit of 5  $\mu$ M. Sample filtering was also ruled out as a possible means of androgen entrapment prior to column analysis.

Concentration of urine samples for analysis by RP-HPLC was sufficient for the detection of testosterone at a lower limit of 69 nM [Navajas *et al.*, 1995]. Because of this study by Navajas *et al*, it was proposed that 10 nM of androgen treatment in our study would have been detectable on the HPLC system used here, but this was obviously not successful.



In contrast to the relative ease of testosterone detection, the inability to detect significant peaks for multiple 5Adiol products even at high concentrations (1 mM and 100 mM) is very disconcerting. These levels were well above what should have been the detection limits when calculated using the product information (250mg of 5Adiol twice daily, which approximates to a serum level of 157  $\mu$ M at any given time, assuming a blood volume of 5.5 L). The prior knowledge that 5Adiol was undetectable at high concentrations led to the assumption that it would not be detected in the medium controls or LNCaP treatment samples since those were supplemented with a concentration 10,000-fold lower than the 1 mM stock. However, the retention time of 5Adiol was still deduced in order to propose where the compound theoretically would've come off the column (Figure 17). Based upon the compound structure and polarity, 5Adiol would be expected to elute prior to testosterone using the same RP-HPLC system. This is theorized because of its slightly more polar structure which contains two hydroxyl groups compared to the structure of testosterone which contains one hydroxyl and one ketone group (Figure 3). However, because these molecules are so similar in structure, and extrapolating from relative retention times in another solvent system, the theoretical elution of 5Adiol would likely be expected within a few minutes of testosterone (3-5 minutes maximum).



**Figure 17. Theoretical elution peak of 5Adiol with respect to testosterone.**

Despite not being able to detect significant peaks that may have been attributed to any one particular compound from the 5Adiol supplement, it is interesting to speculate about the mitogenic stimulus of LNCaP cells due to treatment with this product. This issue is of great concern because a compound(s) within the product that maximally absorbed at 254 nm was detected (through the use of an alternative spectrophotometer). It is impossible to propose exactly what compound or combination thereof might have been the cause of action without further analysis. However, possibilities may include human growth hormone or an alternative growth factor such as IGF, epidermal growth factor (EGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), or a protein such as insulin, lectin, or other plant hormone. Furthermore, it is entirely possible that 5Adiol or other androgens are components of Value Nutrition's 5Adiol product and that they are simply present at very low quantities within the powder. However, to determine this, the product needs to be analyzed by a more sensitive technique with standard controls for

comparison. Analysis of picomolar levels of androgens has been successful using mass spectroscopy, liquid chromatography, gas chromatography, or a combination thereof, so perhaps these are viable alternatives [Higashi *et al.*, 2006; Mitamura *et al.*, 2002; Nishiyama *et al.*, 2007; Titus *et al.*, 2005]. Additionally, Mizokami *et al* has suggested that 5Adiol is more effective than testosterone even at doses in the picomolar range [Mizokami *et al.*, 2004].

Future studies from this investigation are limitless. First and foremost, the mitogenic effects of 5Adiol and testosterone should be repeated to confirm the results reported herein. However, one critical necessity is a 5Adiol standard. Although 5Adiol itself is not sold through any of the chemical companies we have contacted (VWR, Fisher, JT Baker/Mallinckrodt, Pfizer, FMC Corp, Genentech, Eastman chemical Company, Dow Chemical, Bristol-Myers Squibb, BASF, DuPont, and J&J), ethynyl androstenediol is available through Sigma Aldrich. It would be difficult to synthesize 5Adiol from this product, but it is possible. More likely however, is the prospect of using dehydroepiandrosterone to manufacture the compound, which was exactly what was done by Higashi *et al* to obtain a 5Adiol standard [Higashi *et al.*, 2006].

Future studies should include determining the effects of these androgens on prostate specific antigen (PSA) expression in prostate cancer, which is the current primary diagnostic marker for this malignancy. The potential route of androgen receptor activation in LNCaP and HepG2 cells (ligand or kinase) is also a worthwhile investigation. Only a minority of patients express a mutated androgen receptor phenotype

which conveys activation via ligand promiscuity, modeled in the LNCaP cell line.

However, a majority of prostate cancer patients are found to have increased kinase signaling that alternatively activates the receptor [Chen *et al.*, 2004].

Androgen receptor expression is also critical in future analyses because it has been shown to be hypersensitive to 5 $\alpha$ -Dihydrotestosterone compared to dihydrotestosterone and testosterone [Mizokami *et al.*, 2004]. Impairment of prostate tumor growth in mice has been demonstrated when expression of the androgen receptor is knocked down by RNAi [Chen *et al.*, 2004]. Moreover, increased expression of the androgen receptor is correlated with the elevation in prostate cancer grade; thus, a more aggressive malignancy is linked to elevated expression of the receptor [Nishiyama *et al.*, 2007; Wako *et al.*, 2007].

Furthermore, the androgen receptor has been proposed to play a pivotal role in prostate cancer progression to an androgen-independent carcinoma [Umekita *et al.*, 1996]. It was demonstrated by Umekita and colleagues that if the LNCaP cell line is grown in androgen-depleted medium for a significant amount of time (2 years), the dependency upon androgens for growth and survival is lost as the cells transform into a malignancy that no longer depends on steroids. This mimics what is seen in men receiving androgen ablation therapy, where the amount of circulating androgens is reduced for a period of time during which many cases recur as an androgen-independent phenotype.

Interestingly, however, Umekita *et al* implanted these androgen-independent LNCaP cells into castrated male mice and found that if androgens were administered, the cancer regressed. This continued for some time until the cancer later recurred as a hormone-dependent growth again. These results are fascinating because they suggest that prostate

cancer may have the ability to undergo transient dependency upon androgens and furthermore, that androgen-independence is simply a misnomer. In combination, these studies seem to suggest that the malignancy continues to respond to androgens and that it may just simply adapt to its environmental surroundings in order to survive [Chen *et al.*, 2004; Mizokami *et al.*, 2004; Nishiyama *et al.*, 2007; Umekita *et al.*, 1996; Wako *et al.*, 2007]. Whether this is accomplished through a modification in the androgen receptor to confer a hypersensitivity to the diminished serum/intracellular levels of androgens, an upregulation in the expression of the androgen receptor, or the transient death/survival of internal populations of cellular clones that vary in their androgen response, this all certainly stimulates interest to delve deeper into the complexity of prostate cancer.

If the androgen receptor and PSA expression were to be analyzed in future studies, they should be analyzed at both the transcriptional (by real time RT-PCR or northern blots) and translational (western blots or immunocytochemistry) levels. Androgen receptor expression should also be observed in response to androgen treatment within the HepG2 cell line as well as IGFR expression in both LNCaP and HepG2 cells. The use of primary hepatocyte cultures may also be interesting to study whether benign tissue metabolism of androgens elicits the same effects on LNCaP, thus mimicking what would be seen in men using these types of supplements who have prostate cancer, but normal liver tissue.

Finally, no matter whether primary or transformed hepatocellular lines are used, it would also be of importance to analyze the regulation, expression, and kinetic parameters of the  $5\alpha$ -reductases and  $17\beta$ HSD enzymes in LNCaP and HepG2 cells in response to androgen treatment.

In conclusion, these results, despite the variability, suggest that the use of nutraceuticals marketed as 5Adiol have the potential for disastrous results in men who are at a higher risk of developing prostate cancer. The risk of mitogenic effects, especially from small levels of unknown compounds in the tested 5Adiol product of this study, raises serious questions as to the legitimacy and validity of permitting companies to market and self-regulate their own products. These questions are further compounded by the documented cases of cancer development or neoplastic lesions associated with men who abuse such supplements [Alaraj *et al.*, 2005; Bagia *et al.*, 2000; Bryden *et al.*, 1995; Bydder *et al.*, 2007; Creagh *et al.*, 1988; Froehner *et al.*, 1999; Kafrouni *et al.*, 2007; Martorana *et al.*, 1999; Overly *et al.*, 1984; Prat *et al.*, 1977; Socas *et al.*, 2005; Velazquez and Alter, 2004]. In combination with alarming surveys like those completed by Parkinson and Evans in 2006, which seek to elucidate current trends in steroid abuse by men, this societal problem is not likely to fade anytime soon [Parkinson and Evans, 2006].

Furthermore, as doping scandals involving professional athletes continues throughout the world (*i.e.* Mark Maguire, Barry Bonds, and Floyd Landis), the influence of steroid abuse on young minds has the potential to expand because of their admiration for athletic role models. Education and marketing campaigns are the keys to combating unhealthy use of AAS. People from all demographics need to be exposed to the alarming facts of steroid use if we are to start to rectify this entirely preventable problem.

## REFERENCES

- Agoulnik IU, Weigel NL (2006): Androgen receptor action in hormone-dependent and recurrent prostate cancer. *J Cell Biochem* 99:362-72.
- Alaraj AM, Chamoun RB, Dahdaleh NS, Haddad GF, Comair YG (2005): Spontaneous subdural haematoma in anabolic steroids dependent weight lifters: reports of two cases and review of literature. *Acta Neurochir (Wien)* 147:85-7; discussion 87-8.
- Andersson S, Geissler WM, Patel S, Wu L (1995): The molecular biology of androgenic 17 beta-hydroxysteroid dehydrogenases. *J Steroid Biochem Mol Biol* 53:37-9.
- Angelsen A, Falkmer S, Sandvik AK, Waldum HL (1999): Pre- and postnatal testosterone administration induces proliferative epithelial lesions with neuroendocrine differentiation in the dorsal lobe of the rat prostate. *Prostate* 40:65-75.
- Arnold JT, Le H, McFann KK, Blackman MR (2005): Comparative effects of DHEA vs. testosterone, dihydrotestosterone, and estradiol on proliferation and gene expression in human LNCaP prostate cancer cells. *Am J Physiol Endocrinol Metab* 288:E573-84.
- Aspinall SR, Stamp S, Davison A, Shenton BK, Lennard TW (2004): The proliferative effects of 5-androstene-3 beta,17 beta-diol and 5 alpha-dihydrotestosterone on cell cycle analysis and cell proliferation in MCF7, T47D and MDAMB231 breast cancer cell lines. *J Steroid Biochem Mol Biol* 88:37-51.
- Bagia S, Hewitt PM, Morris DL (2000): Anabolic steroid-induced hepatic adenomas with spontaneous haemorrhage in a bodybuilder. *Aust N Z J Surg* 70:686-7.
- Balk SP (2002): Androgen receptor as a target in androgen-independent prostate cancer. *Urology* 60:132-8; discussion 138-9.
- Bonaccorsi L, Carloni V, Muratori M, Salvadori A, Giannini A, Carini M, Serio M, Forti G, Baldi E (2000): Androgen receptor expression in prostate carcinoma cells suppresses alpha6beta4 integrin-mediated invasive phenotype. *Endocrinology* 141:3172-82.
- Bravo R, Frank R, Blundell PA, Macdonald-Bravo H (1987): Cyclin/PCNA is the auxiliary protein of DNA polymerase-delta. *Nature* 326:515-7.
- Broeder CE, Quindry J, Brittingham K, Panton L, Thomson J, Appakondur S, Breuel K, Byrd R, Douglas J, Earnest C, Mitchell C, Olson M, Roy T, Yarlagadda C (2000): The Andro Project: physiological and hormonal influences of androstenedione supplementation in men 35 to 65 years old participating in a high-intensity resistance training program. *Arch Intern Med* 160:3093-104.
- Brown GA, Martini ER, Roberts BS, Vukovich MD, King DS (2002): Acute hormonal response to sublingual androstenediol intake in young men. *J Appl Physiol* 92:142-6.

Brown GA, McKenzie D (2006): Acute resistance exercise does not change the hormonal response to sublingual androstenediol intake. *Eur J Appl Physiol* 97:404-12.

Brown GA, Vukovich MD, Martini ER, Kohut ML, Franke WD, Jackson DA, King DS (2001): Endocrine and lipid responses to chronic androstenediol-herbal supplementation in 30 to 58 year old men. *J Am Coll Nutr* 20:520-8.

Bryden AA, Rothwell PJ, O'Reilly PH (1995): Anabolic steroid abuse and renal-cell carcinoma. *Lancet* 346:1306-7.

Bydder SA, Joseph DJ, Weinstein S, Stuckey BG (2007): Prostate cancer following testosterone replacement in Klinefelter syndrome. *ANZ J Surg* 77:93-4.

Catlin DH, Leder BZ, Ahrens B, Starcevic B, Hatton CK, Green GA, Finkelstein JS (2000): Trace contamination of over-the-counter androstenedione and positive urine test results for a nandrolone metabolite. *Jama* 284:2618-21.

Chang HC, Miyamoto H, Marwah P, Lardy H, Yeh S, Huang KE, Chang C (1999): Suppression of Delta(5)-androstenediol-induced androgen receptor transactivation by selective steroids in human prostate cancer cells. *Proc Natl Acad Sci U S A* 96:11173-7.

Chen CD, Welsbie DS, Tran C, Baek SH, Chen R, Vessella R, Rosenfeld MG, Sawyers CL (2004): Molecular determinants of resistance to antiandrogen therapy. *Nat Med* 10:33-9.

Claudio PP, Zamparelli A, Garcia FU, Claudio L, Ammirati G, Farina A, Bovicelli A, Russo G, Giordano GG, McGinnis DE, Giordano A, Cardi G (2002): Expression of cell-cycle-regulated proteins pRb2/p130, p107, p27(kip1), p53, mdm-2, and Ki-67 (MIB-1) in prostatic gland adenocarcinoma. *Clin Cancer Res* 8:1808-15.

Creagh TM, Rubin A, Evans DJ (1988): Hepatic tumours induced by anabolic steroids in an athlete. *J Clin Pathol* 41:441-3.

Dehm SM, Tindall DJ (2006): Molecular regulation of androgen action in prostate cancer. *J Cell Biochem* 99:333-44.

Delbeke FT, Van Eenoo P, Van Thuyne W, Desmet N (2002): Prohormones and sport. *J Steroid Biochem Mol Biol* 83:245-51.

Earnest CP, Olson MA, Broeder CE, Breuel KF, Beckham SG (2000): In vivo 4-androstene-3,17-dione and 4-androstene-3 beta,17 beta-diol supplementation in young men. *Eur J Appl Physiol* 81:229-32.

Fong CJ, Sherwood ER, Braun EJ, Berg LA, Lee C, Kozlowski JM (1992): Regulation of prostatic carcinoma cell proliferation and secretory activity by extracellular matrix and stromal secretions. *Prostate* 21:121-31.



- Froehner M, Fischer R, Leike S, Hakenberg OW, Noack B, Wirth MP (1999): Intratesticular leiomyosarcoma in a young man after high dose doping with Oral-Turinabol: a case report. *Cancer* 86:1571-5.
- George FW (1997): Androgen metabolism in the prostate of the finasteride-treated, adult rat: a possible explanation for the differential action of testosterone and 5 alpha-dihydrotestosterone during development of the male urogenital tract. *Endocrinology* 138:871-7.
- Gregory CW, Johnson RT, Jr., Mohler JL, French FS, Wilson EM (2001): Androgen receptor stabilization in recurrent prostate cancer is associated with hypersensitivity to low androgen. *Cancer Res* 61:2892-8.
- Guo Y, Pili R, Passaniti A (1994): Regulation of prostate-specific antigen gene expression in LNCaP human prostatic carcinoma cells by growth, dihydrotestosterone, and extracellular matrix. *Prostate* 24:1-10.
- Hadley ME (1996): "Endocrinology." Upper Saddle River, NJ: Prentice Hall.
- Hartgens F, Kuipers H (2004): Effects of androgenic-anabolic steroids in athletes. *Sports Med* 34:513-54.
- Heinlein CA, Chang C (2004): Androgen receptor in prostate cancer. *Endocr Rev* 25:276-308.
- Hellberg CB, Burden-Gulley SM, Pietz GE, Brady-Kalnay SM (2002): Expression of the receptor protein-tyrosine phosphatase, PTPmu, restores E-cadherin-dependent adhesion in human prostate carcinoma cells. *J Biol Chem* 277:11165-73.
- Higashi T, Takayama N, Kyutoku M, Shimada K, Koh E, Namiki M (2006): Liquid chromatography-mass spectrometric assay of androstenediol in prostatic tissue: influence of androgen deprivation therapy on its level. *Steroids* 71:1007-13.
- Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Chu TM, Mirand EA, Murphy GP (1983): LNCaP model of human prostatic carcinoma. *Cancer Res* 43:1809-18.
- Huggins C, Hodges CV (1972): Studies on prostatic cancer. I. The effect of castration, of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *CA Cancer J Clin* 22:232-40.
- Jie X, Lang C, Jian Q, Chaoqun L, Dehua Y, Yi S, Yanping J, Luokun X, Qiuping Z, Hui W, Feili G, Boquan J, Youxin J, Jinquan T (2007): Androgen activates PEG10 to promote carcinogenesis in hepatic cancer cells. *Oncogene* 26:5741-51.
- Kafrouni MI, Anders RA, Verma S (2007): Hepatotoxicity associated with dietary supplements containing anabolic steroids. *Clin Gastroenterol Hepatol* 5:809-12.

- Kent EC, Hussain MH (2003): Neoadjuvant Therapy for Prostate Cancer: An Oncologist's Perspective. *Rev Urol* 5 Suppl 3:S28-37.
- King DS, Sharp RL, Vukovich MD, Brown GA, Reifenrath TA, Uhl NL, Parsons KA (1999): Effect of oral androstenedione on serum testosterone and adaptations to resistance training in young men: a randomized controlled trial. *Jama* 281:2020-8.
- Lamb DJ, Weigel NL, Marcelli M (2001): Androgen receptors and their biology. *Vitam Horm* 62:199-230.
- Lee C, Sutkowski DM, Sensibar JA, Zelner D, Kim I, Amsel I, Shaw N, Prins GS, Kozlowski JM (1995): Regulation of proliferation and production of prostate-specific antigen in androgen-sensitive prostatic cancer cells, LNCaP, by dihydrotestosterone. *Endocrinology* 136:796-803.
- Lim IK, Joo HJ, Choi KS, Sueoka E, Lee MS, Ryu MS, Fujiki H (1997): Protection of 5alpha-dihydrotestosterone against TGF-beta-induced apoptosis in FaO cells and induction of mitosis in HepG2 cells. *Int J Cancer* 72:351-5.
- Martorana G, Concetti S, Manferrari F, Creti S (1999): Anabolic steroid abuse and renal cell carcinoma. *J Urol* 162:2089.
- Mitamura K, Nakagawa T, Shimada K, Namiki M, Koh E, Mizokami A, Honma S (2002): Identification of dehydroepiandrosterone metabolites formed from human prostate homogenate using liquid chromatography-mass spectrometry and gas chromatography-mass spectrometry. *J Chromatogr A* 961:97-105.
- Miyamoto H, Yeh S, Lardy H, Messing E, Chang C (1998): Delta5-androstenediol is a natural hormone with androgenic activity in human prostate cancer cells. *Proc Natl Acad Sci U S A* 95:11083-8.
- Mizokami A, Koh E, Fujita H, Maeda Y, Egawa M, Koshida K, Honma S, Keller ET, Namiki M (2004): The adrenal androgen androstenediol is present in prostate cancer tissue after androgen deprivation therapy and activates mutated androgen receptor. *Cancer Res* 64:765-71.
- Mosmann T (1983): Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65:55-63.
- Mottram DR, George AJ (2000): Anabolic steroids. *Baillieres Best Pract Res Clin Endocrinol Metab* 14:55-69.
- Navajas R, Imaz C, Carreras D, Garcia M, Perez M, Rodriguez C, Rodriguez AF, Cortes R (1995): Determination of epitestosterone and testosterone in urine by high-performance liquid chromatography. *J Chromatogr B Biomed Appl* 673:159-64.

Nishiyama T, Hashimoto Y, Takahashi K (2004): The influence of androgen deprivation therapy on dihydrotestosterone levels in the prostatic tissue of patients with prostate cancer. *Clin Cancer Res* 10:7121-6.

Nishiyama T, Ikarashi T, Hashimoto Y, Wako K, Takahashi K (2007): The change in the dihydrotestosterone level in the prostate before and after androgen deprivation therapy in connection with prostate cancer aggressiveness using the Gleason score. *J Urol* 178:1282-8; discussion 1288-9.

Nolan JM (2002): Effect of 5-androstenediol on testosterone responsive prostate cells.: "Biology and Biotechnology." Worcester: Worcester Polytechnic Institute, pp 1-19.

Overly WL, Dankoff JA, Wang BK, Singh UD (1984): Androgens and hepatocellular carcinoma in an athlete. *Ann Intern Med* 100:158-9.

Pallesen S, Josendal O, Johnsen BH, Larsen S, Molde H (2006): Anabolic steroid use in high school students. *Subst Use Misuse* 41:1705-17.

Pandini G, Mineo R, Frasca F, Roberts CT, Jr., Marcelli M, Vigneri R, Belfiore A (2005): Androgens up-regulate the insulin-like growth factor-I receptor in prostate cancer cells. *Cancer Res* 65:1849-57.

Parkinson AB, Evans NA (2006): Anabolic androgenic steroids: a survey of 500 users. *Med Sci Sports Exerc* 38:644-51.

Prat J, Gray GF, Stolley PD, Coleman JW (1977): Wilms tumor in an adult associated with androgen abuse. *Jama* 237:2322-3.

Reiss K, Wang JY, Romano G, Tu X, Peruzzi F, Baserga R (2001): Mechanisms of regulation of cell adhesion and motility by insulin receptor substrate-1 in prostate cancer cells. *Oncogene* 20:490-500.

Roberts JT, Essenhight DM (1986): Adenocarcinoma of prostate in 40-year-old body-builder. *Lancet* 2:742.

Rosner JM, Macome JC (1970): Biosynthesis of 5-androstenediol by human testis in vitro. *Steroids* 15:181-93.

Slack JK, Adams RB, Rovin JD, Bissonette EA, Stoker CE, Parsons JT (2001): Alterations in the focal adhesion kinase/Src signal transduction pathway correlate with increased migratory capacity of prostate carcinoma cells. *Oncogene* 20:1152-63.

Socas L, Zumbado M, Perez-Luzardo O, Ramos A, Perez C, Hernandez JR, Boada LD (2005): Hepatocellular adenomas associated with anabolic androgenic steroid abuse in bodybuilders: a report of two cases and a review of the literature. *Br J Sports Med* 39:e27.

Society AC (2007): *Cancer Facts & Figures 2007*: "Atlanta: American Cancer Society."

- Soronen P, Laiti M, Torn S, Harkonen P, Patrikainen L, Li Y, Pulkka A, Kurkela R, Herrala A, Kaija H, Isomaa V, Vihko P (2004): Sex steroid hormone metabolism and prostate cancer. *J Steroid Biochem Mol Biol* 92:281-6.
- Tentori L, Graziani G (2007): Doping with growth hormone/IGF-1, anabolic steroids or erythropoietin: is there a cancer risk? *Pharmacol Res* 55:359-69.
- Titus MA, Schell MJ, Lih FB, Tomer KB, Mohler JL (2005): Testosterone and dihydrotestosterone tissue levels in recurrent prostate cancer. *Clin Cancer Res* 11:4653-7.
- Umekita Y, Hiipakka RA, Kokontis JM, Liao S (1996): Human prostate tumor growth in athymic mice: inhibition by androgens and stimulation by finasteride. *Proc Natl Acad Sci U S A* 93:11802-7.
- Velazquez I, Alter BP (2004): Androgens and liver tumors: Fanconi's anemia and non-Fanconi's conditions. *Am J Hematol* 77:257-67.
- Vihko P, Herrala A, Harkonen P, Isomaa V, Kaija H, Kurkela R, Pulkka A (2006): Control of cell proliferation by steroids: the role of 17HSDs. *Mol Cell Endocrinol* 248:141-8.
- Wako K, Kawasaki T, Yamana K, Suzuki K, Jiang S, Umezu H, Nishiyama T, Takahashi K, Hamakubo T, Kodama T, Naito M (2007): Expression of androgen receptor through androgen-converting enzymes is associated with biological aggressiveness in prostate cancer. *J Clin Pathol*.
- Whitefleet-Smith J (1984): Investigation of the Toxins Produced by Marine Dinoflagellates: "Biochemistry." Madison, WI: University of Wisconsin-Madison, pp 156-158.
- Zhu YS, Cai LQ, You X, Cordero JJ, Huang Y, Imperato-McGinley J (2003): Androgen-induced prostate-specific antigen gene expression is mediated via dihydrotestosterone in LNCaP cells. *J Androl* 24:681-7.