Characterization of a Novel Endogenous Steroid, Estradienolone (ED), in Human Pregnancy: Isolation of its Conjugated Form

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Abstract

Preterm birth is the major clinical issue in maternal-child health in the developed and developing world. Even though multiple factors have been identified as causes of spontaneous preterm labour, the specific events leading to preterm birth are still poorly understood. Our group has previously identified a novel endogenous steroid, estradienolone (ED), and reported that the plasma and placental levels of ED remain high during pregnancy and decrease in association with term and preterm labour. Since ED appears to have progestin-like properties and is a natural ligand of estrogen receptor-related orphan receptors, ED may play a role in the maintenance of pregnancy or its decrease in the initiation of parturition. To fully analyze the unique biological properties of ED, availability of large quantities of ED by chemical synthesis is required. Although mass spectrometric analysis revealed an estradienolone structure, details such as position of double bonds and absence of other subgroups are necessary before chemical synthesis can be attempted. The objective of the current project was to prepare sufficient quantities of pure ED for detailed structural analysis. Early studies focused on purifying unconjugated ED from hydrolysed pregnancy urine since it is an abundant source of sulfate-conjugated ED. However, the yield of unconjugated ED was low probably due to its relatively unstable nature. Several methodologies were therefore developed for the isolation of ED sulfate as it is a more stable form of ED. Availability of sufficient quantities of ED or ED sulfate are critical for defining the detailed structure (using methods such as nuclear magnetic resonance), of this novel steroid.

Résumé

Les naissances prématurées sont un problème clinique majeur en maternité/pédiatrie dans les pays développés et en voie de développement. Bien que de nombreux facteurs de risques d'accouchement prématuré spontané aient été identifiés, les évènements spécifiques conduisant à une naissance avant terme sont encore mal compris. Notre groupe a identifié précédemment un nouveau stéroïde endogène, l'estradienolone (ED), et a montré que les niveaux placentaires et du plasma d'ED restent élevés pendant la grossesse et chutent en association avec le travail prématuré ou à terme. Puisqu'ED possède des caractéristiques semblables à la progestérone et est un ligand naturel des récepteurs orphelins associés à l'œstrogène (ERR), il est possible qu'ED joue un rôle dans le maintien de la grossesse. Sa diminution entrainerait alors le déclenchement de la parturition. Afin de finir de caractériser les propriétés biologiques uniques d'ED, il nous faut le synthétiser chimiquement en grandes quantités. Cependant, bien que l'analyse par spectrométrie de masse ait indiqué qu'ED a une structure d'estradienolone, la position des liaisons doubles et la présence ou absence d'autres sous-groupes restent à déterminer avant de pouvoir effectuer une telle synthèse chimique d'ED. L'objectif de ce projet était donc de préparer des quantités suffisantes d'ED pur en vue de réaliser une analyse structurale détaillée. Nous avons d'abord essayé de purifier ED non conjugué après hydrolyse d'urine de femme enceinte, puisque c'est une source importante d'ED conjugué à un groupement sulfate (sulfate d'ED). Le faible rendement d'ED non conjugué (probablement dû à sa nature relativement instable) m'ont conduit à développer d'autres techniques pour l'isoler le sulfate d'ED car cette forme est plus stable. La disponibilité de quantités suffisantes de sulfates d'ED ou d'ED non conjugué est essentielle pour l'obtention de la structure détaillée de ce nouveau stéroïde (en employant des méthodes telles que la résonance magnétique nucléaire).

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Abbreviations

°C	celsius
µL	microlitre
^{3}H-ES	^{3}H-estrone sulfate
^{3}H-PS	^{3}H-pregnenolone sulfate
ACTH	adrenocorticotropic hormone
Ca2+	calcium
CAP	contraction-associated protein
cm	centimetre
COX	cyclooxygenase
cpm	counts per minute
CRH	corticotropin-releasing Hormone
CRH-R	corticotropin-releasing hormone-receptor
DAG	diacylglycerol
DCC	dextran coated charcoal
DHEAS	dehydroepiandrosterone
DNA	deoxyribonucleic acid
ED	estradienolone
ER	estrogen receptor
ERRE	ERR-response elements
ERRs	estrogen-receptor-related-receptors
ER-α	estrogen-receptor α
GC-MS	Gas Chromatography-Mass Spectrometry
H2O	water
HCl	hydrochloric acid
HPLC	high pressure liquid chromatography
HRE	hormone response elements
IP3	inositol-1,4,5-triphosphate
L	litre
LC/MS/MS	Liquid Chromatography/Mass Spectrometry/ Mass Spectrometry
LPS	late pregnancy serum
M	molar
MAP	mitogen-activated protein
min	minute
mL	mililitre
mRNA	messenger ribonucleic acid
MTBP	methyltrienolone (R1881) binding protein
N	normal
N2	nitrogen
NaCl	sodium chloride
NH4OH	ammonium hydroxide
NMR	Nuclear Magnetic Resonance

NP	non-pregnant
OT	oxytocin
OTR	oxytocin receptor
PG	prostaglandin
PGHS	prostaglandin H synthase
PGT	prostaglandin transport protein
PIP2	phosphatidylinositol-4,5-bisphophate
PKC	protein kinase C
PLCβ	phospholipase Cβ
PR	progestrone-receptor
PR-A	progestrone-receptor A
PTS	protein-tracer solution
SHBG	sex hormone binding globulin
UV	ultra violet

CHAPTER 1:

INTRODUCTION

Introduction

Preterm birth, defined as childbirth at less than 37 completed weeks of gestation, is the major clinical issue in maternal-child health in the developed and developing world. Although pre-term birth occurs in 5% to 15% of pregnancies, it accounts for 75% of neonatal death.[1] Premature infants are at higher risk of chronic neurological, metabolic and respiratory disorders due to the interruption of fetal organ development. In later life, preterm children also have a higher rate of motor and sensory impairment, learning difficulties and behavioural problems. The short-term cost of initial hospitalization and the long-term cost of medical and educational services make preterm birth the major financial burden to the family and society. According to a survey done by the government of United States of America, in 2005, more than US$26.2 billion has been spent on medical and educational services associated with preterm birth.[2]

Even though multiple factors have been identified as causes of spontaneous preterm labour, the specific events leading to most of the preterm birth cases are still poorly understood due to the lack of knowledge of the mechanism of human parturition. Hence, there is still no effective therapy to prevent preterm birth. Diagnosis and management of preterm labour is one of the major challenges facing the medical profession today.

1.1. Rationale for the Current Study

Estradienolone (ED) is a little-known endogenous steroid that was first identified by Philip and Murphy in pregnant women.[3,4] It was considered to be an isomer of estradiol and could rearrange to estradiol under alkali conditions. Along with its unique structure and biological properties, it was observed that its plasma and placental level remained high during pregnancy, and decreased dramatically in association with term and preterm labour. Accumulated circumstantial evidence suggested that ED was a steroid of considerable potency and that there may be a direct relationship between ED levels and labour status.

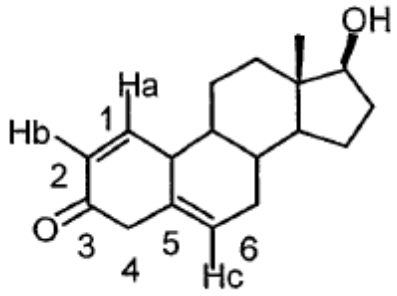
In order to fully investigate the biological effects of ED in human parturition, large quantities of ED are needed. This requires chemical synthesis of ED which in term demands the knowledge of precise structural detail of ED. Based on the previous Gas Chromatography – Mass Spectrometry (GC-MS) data and the known physiological

properties of ED, the proposed structure of ED was deduced to be 17β-hydroxy-[1,5]-estradien-3-one (Fig. 1). The positions of its two double bonds have been assigned to C_1-C_2 and C_5-C_6 of the A and B rings, respectively.[4] However, the chemically synthesized 17β-hydroxy-[1, 5]-estradien-3-one displayed properties different from those of the endogenous ED. There is the possibility that the bonds exist in one of the other three conformations or that there may be a reactive side group attached. Hence, the main objective of the present study was to extract and purify ED in large-scale quantities to perform Nuclear Magnetic Resonance (NMR) to determine ED's molecular structure. Due to the fact that the free ED is relatively unstable [4], the extraction and purification of its conjugated form, ED-sulfate, was also attempted.

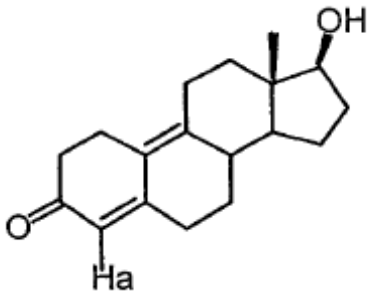
The literature review below provides an overview of the current understanding of the mechanism leading to parturition and how ED may play a role in this process. The discovery of the ED and its unique structural and functional properties will also be discussed.

1.2. Endocrinology of Parturition

Parturition occurs when the diverse maternal and fetal factors interact to initiate the expulsion of the fetus from the pregnant uterus. During pregnancy, the uterus is maintained in a relative functional quiescence in order to accommodate the fetus. The onset of labour is associated with co-ordinated contraction of high intensity and frequency, resulting in the effacement and dilation of the uterine cervix, finally leading to the membrane rupture and fetus expulsion.[5] The endocrine system plays an important role in maintaining the uterine quiescence during pregnancy and in initiating uterine activity at labour. Extensive studies in animal models have demonstrated that there is considerable diversity in the endocrine mechanisms employed by different species in the onset and course of labour. In mammals, such as the sheep, parturition is initiated by the activation of the fetal pituitary-adrenal axis, with increased fetal cortisol secretion, followed by the conversion of placental progesterone to estrogen. This results in a progesterone withdrawal and an increase in estrogen level in the maternal circulation. The change in progesterone/estrogen ratio together with the uterotonic stimulants (prostaglandins and oxytocin) lead to uterine activation.[6,7] Even though the same pathways are operational at the end of pregnancy, the level of circulating progesterone



1. Proposed ED Structure:
17β-hydroxy-[1,5]-estradien-3-one



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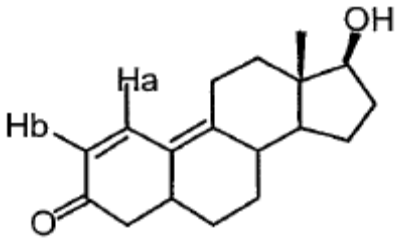

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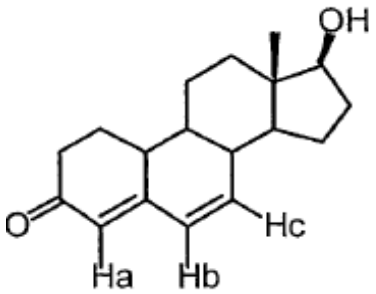

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Figure 1A: Proposed ED structure (1) and alternative structure (2-4)

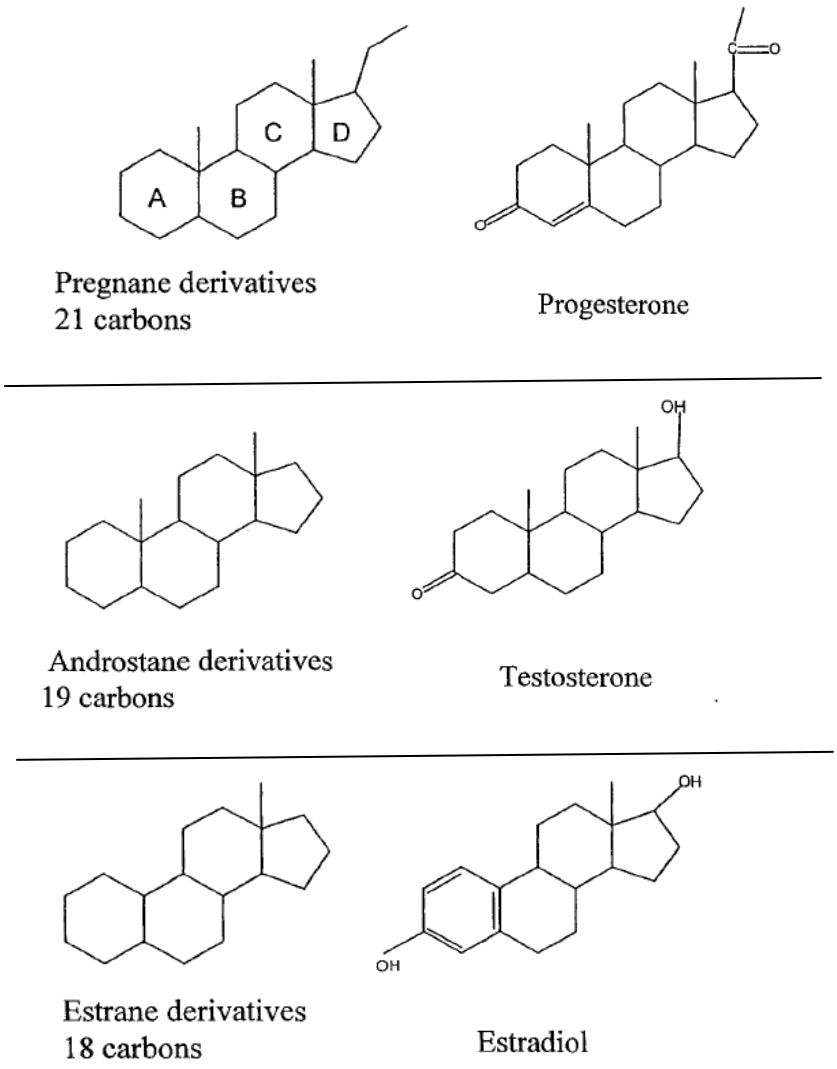


Figure 1B: Major sex hormone structures and their relation to ED

does not decrease with the onset of labour in the human.[8,9] The endocrine signals that initiate the human parturition remain elusive primarily due to the lack of adequate animal models and the ethical difficulties in human experimentation.

Researchers have been working for decades in order to figure out the endocrine signalling cascades in human parturition. Corticotropin-releasing hormone (CRH), progesterone, estrogen, uterotonins (prostaglandin and oxytocin) are considered to be the key players in the endocrine signalling cascade and have been demonstrated to be tightly associated with maintenance of pregnancy and the onset of labour.

1.2.1 Corticotropin-releasing hormone (CRH)

Corticotropin-releasing hormone is expressed in the hypothalamus; however it is also produced in the placenta and secreted into the maternal blood and fetal membranes during pregnancy.[10,11] The production of CRH by the placenta is restricted to primates.[12,13] CRH acts primarily by binding to the CRH type 1 receptor (CRH-R1), which is present in the maternal pituitary/myometrium and the fetal pituitary/adrenal glands.[14] It stimulates pituitary release of adrenocorticotropic hormone (ACTH), which leads to cortisol secretion from the adrenals. The level of CRH in the maternal circulation is regulated by a high-affinity binding protein known as CRH-Binding Protein (CRHBP). CRH bound to the CRHBP is biologically inert as evidenced by the binding protein's ability to inhibit the stimulatory effects of CRH on the pituitary ACTH secretion.

Previous studies have shown a tight correlation of placental-derived CRH level and the timing of birth. Maternal plasma CRH level begins to rise at the second trimester and reaches the peak at term. However, it declines rapidly within 24 hours after parturition.[15] The elevation of the placental CRH level is moderated by the circulating CRHBP and the desensitization of CRH-R. At term, the circulating CRHBP level decreases thereby increasing the "free" CRH.[16] A high concentration of CRH drives the rise in maternal ACTH level, and in turn promotes the production of cortisol and dehydroepiandrosterone sulfate (DHEAS).[17,18] An increase in the fetal cortisol level has been shown to have a stimulatory effect on the placental conversion of progesterone to estrogen in sheep[6,7] and DHEAS also provides a substrate for placental estrogen synthesis. Moreover, CRH has been reported to have both priming and potentiating action on uterine myometrial contractile activity by interacting with the uterotonins, such as

oxytocin and prostaglandin F2α.[19,20] All the above-noted actions plus the desensitization effect of CRH-R favour parturition by inducing contraction. Hence, a high CRH level during pregnancy is considered to have an association with a greatly increased risk of preterm labour.

1.2.2 Progesterone

Progesterone is synthesized in the placenta using the circulating cholesterol in the maternal blood as precursor. In most species, progesterone plays a critical role in the maintenance of pregnancy by keeping myometrial relaxation and the functional withdrawal of circulating progesterone initiates the labour.[21,22] Progesterone functions to support the pregnancy by down-regulating the expression of contraction-associated protein (CAP) genes and inhibiting the formation of gap junctions within the myometrium.[23] [24] These actions of progesterone are mediated by the progesterone receptors (PR) and can be blocked by progesterone antagonists, such as mifepristone (RU 486).[25,26] There are three known PR isoforms: progesterone receptor-A (PR-A), progesterone receptor-B (PR-B) and progesterone receptors-C (PR-C). PR-B is the most abundant form and is responsible for most of the actions of progesterone.[26]

In the human, however, the maintenance of pregnancy is less dependent on progesterone and the level of circulating progesterone dose not fall with the onset of labour.[8,9] Some studies have argued that the progesterone synthesis and metabolism occurs near the site of action, and change of the local concentration of progesterone might not be reflected in the plasma.[27] The current postulated theory for this phenomenon is that the progesterone withdrawal and estrogen activation in human parturition are caused by the changes in the sensitivity of the myometrium to progesterone and estrogen via alteration in the rate of expression of their receptors, instead of the changes of their serum levels.[28] During gestation, relatively high levels of plasma progesterone and PR-B inhibit the expression of estrogen receptor alpha (ER-α) thereby reducing the myometrial estrogen sensitivity. At term, the myometrial sensitivity to progesterone changes through altering the relative ratio of PR-A to PR-B. PR-A becomes the dominant isoform in the myometrium instead of PR-B. This change of ratio leads to the functional withdrawal of local progesterone, which leads to functional activation of estrogen and increased myometrial sensitivity to estrogen.[29,30] Interruption of progesterone production at the

early stage of pregnancy would lead to spontaneous abortion; however, administration of exogenous progesterone did not prolong pregnancy, nor does estrogen administration initiate labour.[23]

Because of the important roles of progesterone in each stage of pregnancy, even though progesterone withdrawal does no initiate labour in human, it is possible that an unknown progesterone analog is involved in the maintenance of human pregnancy and withdrawal of this analog initiate human parturition. Preliminary results from our lab suggest that ED is structurally similar to synthetic progestin and that it binds to the progesterone receptor with relatively high affinity. These data suggest that ED may function like a progestational agent in human pregnancy. Moreover, since the level of ED remains high during pregnancy and falls dramatically in association with parturition, it is possible that the withdrawal of ED is the trigger of human parturition.

1.2.3. Estrogen

Another key characteristic of human pregnancy is massive production of estrogen with a large portion of it as estriol and the rest of it are estradiol and estrone.[31 32] At the early stage of pregnancy, estradiol is the major estrogen produced from the ovaries.[33] After the sixth week of pregnancy, the placental begins to produce estradiol using the maternal and fetal DHEAS as substrates.[34,35] The placenta becomes the major site of estradiol synthesis after the first trimester. Estrone is also of placental origin and its production parallels that of estradiol.[36] Estriol is produced only by the placenta and is first detected in maternal plasma at the ninth week of pregnancy. At term, the fetal adrenal cortex produces 90 percent of the estriol precursor.[37,38] All the placental estrogens increase throughout gestation.[39] The level of these estrogens cannot be truly reflected by their levels in the maternal plasma since they are bound to sex hormone-binding-globulin (SHBG) with varying affinity. Their levels are better reflected by maternal urinary excretion levels of their conjugated forms (sulfate/glucuronide).[40,41]

In most species, systemic progesterone withdrawal followed by estrogen activation triggers parturition and the estrogen activation is mediated by increasing the circulating estrogen levels. In human, however, the estrogen activation is mediated at the functional level, by increasing in myometrial estrogen responsiveness. As noted above,

estrogen activity is mediated by ER-α. It has been shown that ER-α mRNA levels are correlated to the PR-A /PR-B mRNA ratio.[30] During parturition, elevated myometrial PR-A level induces the local ER-α expression thereby transforming the myometrium to a contractile state. Moreover, placental estrogens are essential for the preparation of the cervix for labor and for the initiation of contractions. Placental estrogens are involved in inducing the sensitivity of myometrium to oxytocin by up-regulating the oxytocin receptors and increasing the myometrial gap junction formation required for the coordinated muscle contraction at term. Placental estrogen deficiency can lead to failure of cervical ripening and spontaneous labor.[42]

1.2.4. Prostaglandin

Uterotonins are the agents that modulate the myometrial tone and contractility during pregnancy and parturition. The major uterotonins involved in human labour are prostaglandin (PG) and oxytocin. Prostaglandin is a 20-carbon chain fatty acid formed from arachidonic acid and functions as local hormone. Arachidonic acid first undergoes oxygenation and reduction to form an intermediate product PGH_2, which is catalyzed by PGH synthase or cyclooxygenase (PGHS or COX). There are two isoforms of PGHS: PGHS-1(COX-1) and PGHS-2(COX-2). Inhibition of PGHS enzymes decrease synthesis of all PGs. PGH_2 is then converted to one of the biologically active PGs (D_2, E_2, $F_{2\alpha}$, I_2, or TXA_2) by specific PG synthases.[43] The transfer and metabolism of PG is facilitated by the PG transport protein (PGT), which expression level is increased in late gestation. PG functions via interacting with the receptor and each type of PGs has its own corresponding receptor.

A large body of evidence has demonstrated the important roles of PG in membrane rupture, cervical dilatation, myometrial contractility, placental separation and uterine involution.[44-46] *Bennett et al* has proposed that human parturition was associated with up-regulation of PGs synthesized by COX-2 within the uterus.[47] The increased level of PGs led to the fetal membrane and cervix remodelling with stimulation of myometrial contraction. A recent study done by *Fischer et al* has demonstrated that PG E_2, $PGF_{2\alpha}$ and PGTX mediate uterine contractility through interaction with their corresponding receptors.[48] Elevated levels of both PG bioavailability and the expression of PG receptors are reported before the onset of labour.[49] Since PGHS and PG receptors are the two key

regulators of the PG functions related to parturition, many strategies for inhibiting PG synthesis or actions delay or prevent preterm labour have been explored, such as various PGHS inhibitors and antagonists for the different PG receptors. However, in most cases, there are fetal or maternal side effects involved.

1.2.5. Oxytocin

Another major uterotonin in human parturition is oxytocin (OT), which is the most potent stimulus for myometrial contractions and responsiveness to OT at the time of uterine activation in late gestation. It has been demonstrated that clinical administration of OT initiates labour which is indistinguishable from spontaneous labour. And also pharmacological inhibition through the use of OT or its receptor antagonists can delay delivery of the fetus.[50] There is no clear evidence to support the idea that the level of OT directly correlates to the initiation of human parturition. However, an increasing number of studies have revealed that the OT receptor, as the OT regulator, may play important roles in human parturition.

OT is a nine amino acid peptide that is principally synthesized in the hypothalamus and transported down the axons of the posterior pituitary for secretion into blood. It is also synthesized in the ovary and the placenta during pregnancy.[51,52] The biological function of OT is mediated by the OT receptor (OTR), which is a G-protein coupled receptor. Upon binding of OT to OTR, the G-protein becomes activated and stimulates the phospholipase Cβ (PLCβ) enzyme.[53,54] PLC catalyzes the hydrolysis of membrane phosphatidylinositol-4,5-bisphophate (PIP_2) to produce inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG).[54] IP_3 binds to its receptor on the sarcoplasmic reticulum to cause the release of Ca2+ into the cytoplasm. It leads to the increased intracellular Ca2+ concentration which triggers the contractile mechanism of the myometrial cells.[55] The DAG stimulates protein kinase C (PKC). PKC then activates the mitogen-activated protein (MAP) kinase pathway which causes the up-regulation of COX gene expression and phospholipase A activity.[56] Both enzymes are involved in prostaglandin synthesis.[57] Phospholipase A is responsible for releasing the prostaglandin precursor arachidonic acid from membrane phospholipids while COX is involved in catalyzing the rate limiting step in prostaglandin synthesis. Even though there is no significant increase of the OT level during human parturition, a progressive increase in

the response to oxytocin in myometrium as pregnancy advances has been observed. The OTR concentration is 150times greater in labour than in the non-pregnant uterus, which corresponds to a 100-fold increase in sensitivity of myometrial muscle to OT.[58] Hence, OT is a key player in the timing of birth.

Estrogen is known as the key regulator of OT synthesis in women. The level of plasma OT is correlated with the plasma level of estrogen. Estrogen also stimulates the synthesis of OTR in human and other animals. *Soloff et al* first demonstrated that estrogen induced the increase in OT in the non-pregnant oophorectomized rat and this was later confirmed in vitro in human and rabbit tissues.[59] Moreover, it has been shown that administration of anti-estrogens and estrogen receptor antagonists cause the reduction in both OT and OTR levels as well as delay in parturition in rats.[60] Up-regulation of OT and OTR concentration is one of the essential mechanisms in estrogen-activated myometrial contraction.

In contrast to its effect on OT, progesterone has a negative effect on OTR synthesis. Administration of progesterone to rats can trigger the decreased uterine OTR synthesis at the translational level and also inhibit the estrogen-induced increase in OT levels.[61] Moreover, PR antagonists have been shown to up-regulate the OT levels and trigger parturition in rats.[62] Hence, suppression of OTR expression may be one mechanism whereby progesterone maintains the uterine quiescence in pregnancy.

1.3. Discovery of Estradienolone (ED) and Its Significant Properties

The novel endogenous steroid, Estradienolone (ED) was first reported by Philip and Murphy in a study of low polarity steroids that bind to sex hormone binding globulin (SHBG).[3,4] As the total androgen levels measured by competitive binding to SHBG in pregnancy serum have been shown to be 10-15 fold higher than those in non-pregnant serum, they noticed that approximately half of the SHBG-bound steroids in pregnancy serum were unidentified.[63-65] They then investigated the unidentified steroids of low polarity in maternal serum using Sephadex LH-20 chromatography and four major peaks were defined (designated 1a, 1b, 2 and 3) (Fig. 2). They demonstrated that all four peaks were of placental origin by comparing the change of their concentration at various gestational ages and at premature and term labour in maternal serum, cord serum, placenta and maternal urine. Based on the chromatographic elution patterns, binding

characteristics and GC-MS data, Peak 1a, 1b and 3 were later identified as 5α-prenane-3,20-dione, progesterone and 2-methoxyestrone, respectively. Peak 2, which had a different chromatographic elution pattern and chemical characteristics compared with any known SHBG-bound steroids, was identified as ED. There was no significant difference in the mean level of ED for 12-24 weeks to 30-38 weeks gestation; however, there was a significant decrease in the serum level of ED in association with parturition. The maternal serum level of ED at 30-38 weeks gestation was found to be 3.6±1.5 ng/mL (in testosterone equivalents), while ED level of women during spontaneous labour at the same gestation age was found to be 0.8±0.5 ng/mL (in testosterone equivalents) (Fig. 3). This suggests that ED may be involved in the maintenance of pregnancy and its decrease in the initiation of labour.

1.3.1. Structural Properties of ED

One significant structural property of ED is its ability to rearrange to estradiol in alkali condition which suggests that ED is an isomer of estradiol. Philip and Murphy have demonstrated that ED in maternal plasma, placenta or maternal urine is soluble in alkali (0.1N NaOH) when the biological sample was partitioned between organic solvent and alkali. ED extracted from the alkali phase was then chromatographed and co-eluted with ^{3}H-estradiol and the collected fractions were assayed in a radioimmunoassay using a specific anti-estradiol body. The immunoreactivity coincided with ^{3}H-estradiol peak, suggesting that ED was converted to estradiol under alkali condition (Fig. 4B). On the other hand, the pure ED not exposed to alkali condition eluted in the non-polar region as SHBG bindable material (Fig. 4A). The fact that ED can rearrange to estradiol and that the level of ED decreases towards term suggests that the decrease of ED during labour is due to a shift in the biosynthetic pathway to produce estradiol, which further contributes to the initiation of myometrial contraction.

1.3.2. Biological Properties of ED

(i) Binding of ED to Sex Hormone Binding Globulin (SHBG)

One remarkable biological property of ED is its high binding affinity for SHBG. SHBG is produced mainly in the liver; however, other tissues, such as placenta, endometrium, brain and prostate, have also been shown to express it. SHBG is responsible for the sequestering and blood transport of many principle steroids such as testosterone, dihydrotesterone and estradiol, as well as regulation of their bioavailability to target cells.[63] It is a glycoprotein homodimer containing a single binding site for androgens and estradiol and a binding site for its cell membrane G-protein coupled receptor. It has been shown that steroid target cells display a high affinity membrane receptor for SHBG that is an initiating component of a signalling system at the cell membrane for the sex steroids.[66] Circulating sex steroids bind mostly to SHBG and only a small portion are bound to serum albumin (10% to 30%). Sex steroids that are bound to SHBG are considered to be biologically unavailable and only the "free" steroids can enter the target cells and activate the corresponding receptors. The level of SHBG is increased significantly during pregnancy. Even though the reason behind it remains unknown, SHBG may regulate both the bioavailability and signal transduction of sex steroids during pregnancy.

As a potent ligand, ED has a high affinity for SHBG which is almost the same (95%) as testosterone under physiological conditions.[3] In general, the higher the affinity of a steroid for SHBG, the greater its biological potency. Hence, ED may play a role in human pregnancy. Moreover, since the detailed structure of ED is uncertain and there is no antibody available, the determination of ED level is based on the competition of ED with ^{3}H-testosterone for SHBG binding sites and the concentration of ED is expressed as testosterone equivalents.

(ii) Binding of ED to Methyltrienolone-Binding Protein (MTBP)

The Methyltrienolone (R1881) Binding Protein (MTBP) is an androgen binding protein of placental origin first identified by Macaulay and his colleagues.[67,68]. MTBP, also known as an orphan androgen binding protein, has unique physical and binding characteristics

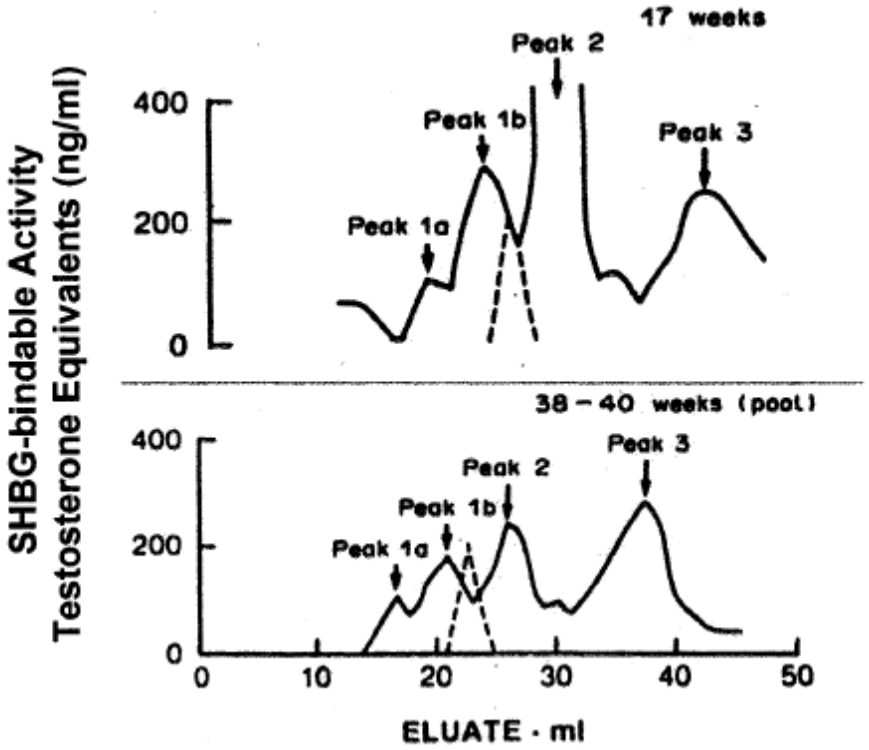


Figure 2: Elution patterns of unknown SHBG-bound material in the region of low polarity using detailed chromatography of pregnancy serum. Samples were chromatographed in a Sephadex LH-20 column (60 x 0.9cm). 45 fractions of 1mL each were collected using a mobile phase of dichloromethane, heptane and methanol (50:50:1), and an aliquot from each was assayed for binding to SHBG. The graphs are representative of numerous serum samples from women at 12-40 weeks gestation, without labour. Dashed peak arrow indicates the position (elution volume) of the ^{3}H-androstenendione tracer added to the sample prior to extraction and chromatography. Values are expressed in testosterone equivalents. Peak 2 represent the ED peak.

Philip and Murphy 1989[3]

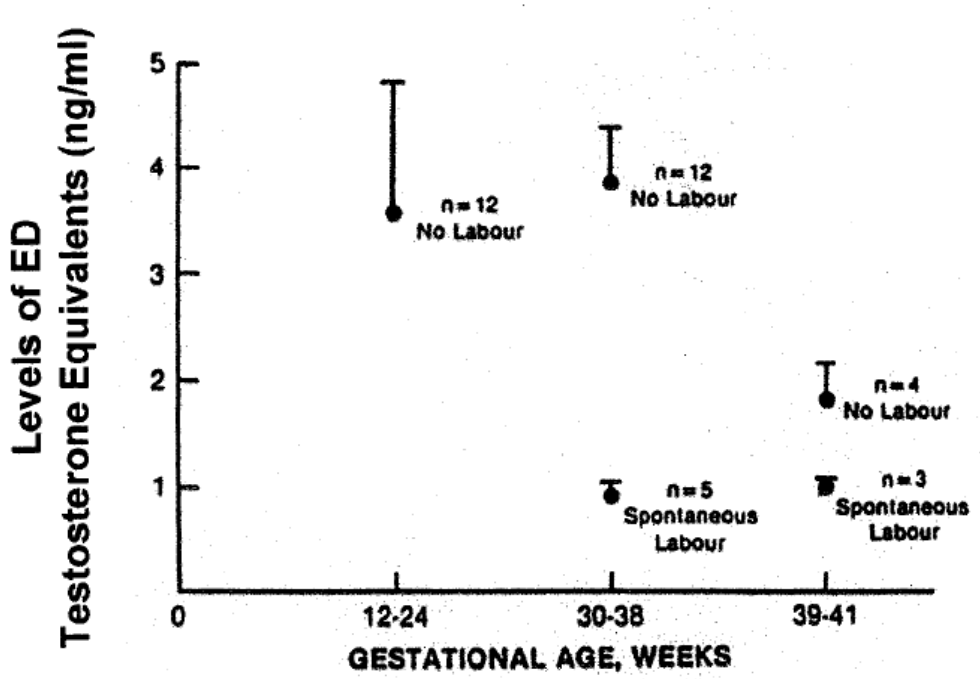


Figure 3: Concentration of ED in maternal serum declines in association with impending term or preterm labour. ED was purified from maternal serum at gestational ages of 12-24 weeks, 30-38 weeks and 39-41 weeks during spontaneous onset labour. ED levels were measured by SHBG binding assay following extraction and fractionation on Sephadex LH-20 column.

Philip and Murphy 1989[4]

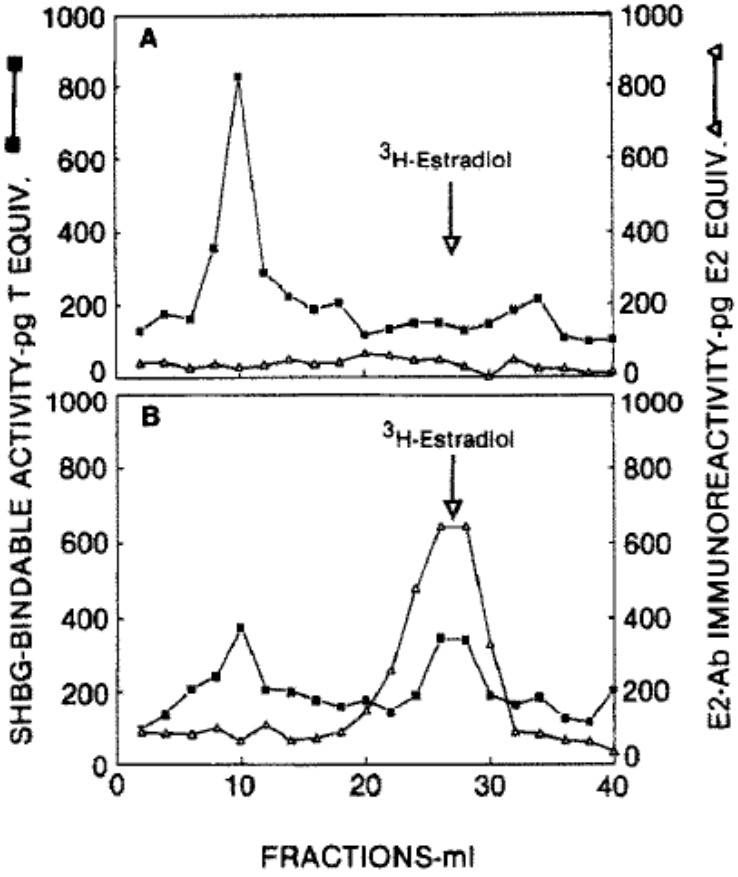


Figure 4: Elution patterns of ED before (A) and after (B) exposure to 0.1N NaOH. The purified ED treated with and without 0.1N NaOH were chromatographed on a Sephadex LH-20 column (10 x 1cm) and 40 fractions were collected. The fractions were then split and assayed by competitive binding to SHBG and antibody specific for estradiol. The arrow indicates the elution volume of ^{3}H-estradiol added to the sample before chromatography.

Philip and Murphy 1989[4]

that clearly distinguish it from classical androgen receptors. One of the unique steroid binding characteristics of MTBP is its high affinity to R1881 but lower affinity for dihydrotestosterone and mibolerone.[67,68] It has been demonstrated that MTBP is present in the choriocarcinoma cell line JEG-3 although the biological significance of it is unknown. A previous study in our lab has demonstrated that ED binds to MTBP with a higher affinity than any other natural steroids tested, including R1881 itself (Philip, unpublished data). This interesting result suggests that ED is the natural ligand of MTBP and the high concentration of ED in placenta may be explained by the binding of ED to MTBP. Nevertheless, the physiological significance of binding of ED to MTBP remains to be determined, which requires the detail structure of ED.

(iii) Binding of ED to Orphan Nuclear Receptors: Estrogen-Receptor-Related-Receptors (ERRs)

Steroid hormones exert their genomic effect by binding to specific nuclear receptors which belong to a superfamily of ligand-activated transcription factors that regulate the expression of target genes.[69] The nuclear receptors of this superfamily have 6 structural domains which are essential for their regulatory functions, including the ligand binding domain and DNA binding domain.[70] Upon the steroid hormone binding, the ligand binding domain undergoes a conformational change which induces the receptor dimerization. This leads to exposure of DNA binding domain of the nuclear receptor and which can now bind to the hormone response elements (HRE) in the promoter region of the target genes to activate the gene transcription.

Within this nuclear receptor superfamily there are a large number of cloned receptors for which no natural ligands have been identified. These receptors are known as orphan receptors. Identification of natural ligands for these orphan receptors may lead to the discovery of new hormone signalling pathways. The estrogen-related-receptors (ERRs) are part of these orphan receptors which consist of three isoforms: ERRα, ERRβ, ERRγ. The ERR isoforms display a high level of homology within their ligand binding domain. The ERRα gene is widely expressed both in the developing embryo and in adult tissue.[71] It has also been suggested as an unfavourable marker for breast cancer progession and hormonal insensitivity.[72,73] ERRβ is expressed early in the developing placenta, in a subset of cells in extra-embryonic ectoderm predestined to make up the

chorion.[74] ERRγ is expressed at a very high level in the fetal brain but at lower level in kidney, lung and liver.[71] It is a favourable biomarker for clinical breast cancer outcome and hormone insensitivity due to the fact that it is over expressed in 75% of the tumours.[72] ERRs share structural similarities with estrogen receptors (ERs) but have unique characteristics. They not only bind to the estrogen response elements as homodimers, but also bind to the ERR-response elements (ERRE) as monomers. However, estradiol and other natural estrogens do not bind to ERRs.[71]

Study of the crystal structure of the ligand binding domain of ERRs suggests that the transcriptional activity of the ERRs may be regulated by a natural inverse agonist instead of natural agonist ligand. Previous studies from our lab have demonstrated that ED does not display an affinity for the ERs (ERα and ERβ), but functions as an inverse agonist of ERRα and ERRγ by inhibiting their transcriptional activity. Significantly, ED is several-fold more potent than diethylstilbestrol (DES), which is a synthetic ligand for ERRα and ERRγ. Since both ERRα and ERRγ are important biomarker for breast cancer, ED may also play a key role in inhibiting breast cancer progression.

Taken together, confirmation of ED structure is fundamental for the study of all the significant biological functions of ED implicated above. Hence, structural characterization of ED is the primary goal of this project.

CHAPTER 2:

MATERIALS AND METHODS

Materials and Methods

2.1. Sample Collection

After obtaining informed written consent, blood samples were collected from pregnant women between 8-24 weeks gestation undergoing routine blood tests at the Royal Victoria Hospital (Montreal, Quebec). Samples were centrifuged at 3000 rpm for 5 minutes and the supernatant collected was frozen at -20°C until analyzed. 24-hour urine samples were obtained from pregnant women at 22-36 weeks gestation and pooled together. The pregnancy urine samples were then immediately stored at -80°C until analyzed. Non-pregnant (NP) blood samples and 24-hour urine samples were collected from the women in their twenties who were either on the birth control pill or not. All the NP samples were processed the same way as the pregnancy samples.

2.2. Urine hydrolysis and Extraction

2.2.1. Urine Sample Hydrolysis for Free ED

1 L of the pooled pregnancy urine was thawed at 4°C and the pH of the urine sample was adjusted to 5.2 with acetic acid. 45mL of 2M sodium acetate buffer (pH 5.2) and 25mL of Glusulase (helix pomatia intestinal preparation containing 10,000 units of sulfatase and 90,000 units of β-D-glucuronidase activity/mL [Perkin Elmer]) were added. The sample was then thoroughly mixed and incubated at 37°C for 36 hours. Once the incubation was completed, 20,000 cpm of ^{3}H-androstenedione tracer was added to the hydrolyzed urine sample. The tracer served as a radioactive marker for the identification of ED peak's elution pattern and allowed calculation of the sample recovery.

The same volume (1 L) of the pooled NP urine samples, one from the NP women on birth control pills and the other one from the NP women who were not on birth control pills, were hydrolyzed the same way as the pregnancy urine. These NP urine samples served as experimental controls.

2.2.2. Large-Scare Urine Extraction for Isolation of ED

The hydrolyzed urine sample was extracted twice with 3 times volume of ethyl acetate (Fisher Scientific Co.). The combined organic extract was evaporated to dryness under nitrogen (N_2) gas flow (VitalAire Canada Inc.).

2.3. Procedure for Low Pressure Liquid Chromatography/Fractionation of Extracted Urine Sample (10cm and 60cm column Fractionation)

The first purification was performed on a short glass column (10cm x 1cm) packed with Sephadex^{TM} LH-20 (GE Healthcare). The mobile phase consisted of a mixture of dichloromethane (Fisher Scientific Co.), heptane (Sigma) and methanol (Fisher Scientific Co.) in the ratio of 50:50:1. The previously dried sample extract was re-dissolved in 0.5 mL of mobile phase, applied to the column and 18 fractions of 1mL each were collected at the gravity-flow rate of approximately 1 mL/min. An aliquot of 60 µL from each fraction was added in 3mL of Scintisafe (Fisher Scientific Co.) and counted in a Beckman LS5801 liquid scintillation counter to locate the ^{3}H-androstenedione tracer peak. Since ED co-eluted with ^{3}H-androstenedione on this column, the fractions containing the highest tracer counts were pooled, evaporated to dryness with N2 gas and re-dissolved in 0.5 mL of the mobile phase for subsequent fractionation on the 60 cm column.

Samples were further chromatographed on a 60 cm x 0.9 cm Sephadex^{TM} LH-20 (GE Healthcare) column using the same mobile phase as described for the 10 cm x 1 cm column. At an upward flow rate of 1 mL/min (peristaltic pump, Fisher Scientific Co.), 48 fractions of 1 mL each were collected. An aliquot of 60 µL was taken for counting to locate the ^{3}H-androstenedione tracer peak. The ^{3}H-androstenedione was typically eluted 5 to 7 fractions prior to the ED peak. A suitable aliquot was also assayed for SHBG binding activity to further confirm that the fractions contained ED.

10 cm and 60 cm fractionations were also performed on the two NP urine samples as controls at the same condition.

2.4. Procedure for SHBG Assay

Since the detailed structure of ED is not available, ED levels were determined by assaying aliquots from column fractions using a competitive protein binding assay as described by Philip and Murphy. This assay measures ED bound to the SHBG from pregnancy serum on the basis of its ability to display ^{3}H-testosterone from SHBG binding sites

2.4.1. Buffers and Solutions Used for the SHBG Assay

Phosphate Buffer (pH 6.5)

0.2M sodium phosphate monobasic, monohydrate (Sigma)

0.2M sodium phosphate dibasic, heptahydrate (Sigma)

Both solution were prepared separately at room temperature. The dibasic solution was added to the monobasic solution with stirring until a pH of 6.5 was reached. The buffer was stored at 4°C until used.

Gelatin Water

A fresh mixture of 25 mg Gelatin (Sigma) and 50mL phosphate buffer (pH6.5) was prepared and stored at 4°C for up to one week. The solution was brought to room temperature prior to use in the SHBG assay.

Dextran Coated Charcoal (DCC) solution

A mixture consisting of 0.125 g charcoal (Sigma), 0.125 g dextran (Sigma) and 100 mL phosphate buffer (pH 6.5) was prepared and kept at 4°C. Constant stirring with a magnetic stir bar during use ensured that the charcoal remained in suspension.

Charcoal Stripped Late Pregnancy Serum (LPS) for SHBG

Late pregnancy serum from several blood samples of pregnant women (22 to 34 weeks) were pooled, aliquoted and stored at -20°C until use. For the assay, LPS was thawed and 120 µL aliquot was diluted with 0.5 mL nano-pure water and mixed with approximately 15 mg charcoal. This diluted LPS-charcoal mixture was vortexed for 1 minute and centrifuged at 3000 rpm for 10 minutes to remove endogenous steroids. The supernatant was isolated

and used as SHBG source in the assay.

Protein-Tracer Solution (PTS)

An ethanol solution containing 12,000 cpm of ^{3}H-testosterone (Perkin Elmer, Boston, MA) was evaporated to dryness in a test tube under low airflow. The stripped LPS was then added to the test tube with 10 mL of the gelatin water and the mixture was vortexed thoroughly.

2.4.2. Procedure of SHBG Assay

After locating the ^{3}H-androstenedione tracer peak, a suitable aliquot (normally 1-5 μL) of the fractions thought to contain ED plus one set of testosterone standards were assayed. The testosterone (Sigma) standard consisted of a series of test tubes in duplicate containing increasing amount of non-radioactive testosterone as follows: 0, 50, 100, 200, 400, 800, 1600 picograms. 100 μL of fresh PTS solution was added to all the assay tubes in which the sample aliquots and standards were dried down. The assay tubes were incubated at 37°C for 5 minutes and then incubated at 4°C for 30 minutes after gentle shaking. The unbound steroids were then separated from the SHBG-bound steroids by treatment with 1mL of DCC solution for 5 minutes. The assay tubes were then centrifuged at 3000 rpm for 10 minutes at 4°C. 0.5 mL of the supernatant of each assay tube was added to 3 mL Scintisafe for counts per minute (cpm). Counts from the testosterone standards were averaged and used to generate the standard curve. The SHBG-bound material (ED) in the column fractions was expressed in testosterone equivalents.

2.5. High Performance Liquid Chromatography (HPLC) of Sephadex LH-20 Purified ED Samples

Further purification of ED obtained from 60cm fractionation was performed by HPLC. Fractions that contain ED (normally the 5th to 7th fractions after the ^{3}H-androstenedione tracer peak) were pooled together and dried down to approximately 80 μL. A mobile phase consisting of a mixture of dichloromethane, heptane and methanol at a ratio of 30:70:0.1 was used on a Nova Pak normal phase silica-diol capped column (3.9

X 150 mm, Waters). A Waters HPLC system consisting of a 600S model controller and 626 model pump was employed. After injecting the 80 µL sample into the HPLC system, 1 mL fractions were collected at a flow rate of 1 mL/min with 100% mobile phase for 48 minutes. The column was then washed using 100% mobile phase for 1 hour at the rate of 1ml/min. Suitable aliquots were removed for counting to locate the radioactive tracer peak and for locating ED by SHBG assay. Once assayed, the fractions were dried down by N_2 gas and re-suspended in 1mL of anhydrous ethanol (Fisher Scientific Co).

2.6. Serum Extraction

Serum samples were extracted twice with 3 volumes of ethyl acetate (Sigma). The combined organic phase extract was evaporated to dryness under N_2 flow and re-suspended in the 100% mobile phase for the 10cm and 60cm Sephadex^{TM} LH-20 fractionation. The same 10 cm, 60 cm and HPLC fractionation procedures described in section 2.3 and 2.5 were applied to the serum extract. The fractions containing ED was located using the SHBG assay procedure described in section 2.4.

2.7. Gravity-Flow-Based Extraction Procedures for Isolating the Sulfate-Conjugated Steroids from Pregnancy Urine

2.7.1. Sephadex LH-20 Extraction of Sulfate-Conjugated Steroids

1 g of Sephadex^{TM} LH-20 was pre-soaked in nano-pure H_2O overnight and then packed into an empty disposable PD-10 column (Sigma). Each mini-column was pre-conditioned with 0.1N HCl before saturating with urine sample. 10 mL of pregnancy urine was extracted with equal volume of chloroform in methanol (2:1, v/v) (Sigma). The aqueous layer containing all the conjugated steroids was acidified with equal volume of 0.1N HCl (total volume of approximately 20mL) and then applied to the mini-column. The column was washed with 5 mL of 0.1N HCl followed by 7 mL of 0.1M sodium acetate buffer (pH 4.0) (Sigma) to elute the glucuronide conjugates. The sulfated conjugates were then eluted with 22 mL of nano-pure H_2O. The mini-column was then stripped with 0.1N NH_4OH in ethanol (1:1,v/v) (Sigma) and re-generated with 0.1N HCl. In order to obtain a more concentrated sulfate conjugates, the H_2O (sulfate) eluants were pooled and re-acidified with equal volume of 0.1N HCl. The acidified sulfate conjugates

were passed through the mini-column again and washed with 5 mL 0.1N HCl. All the sulfate conjugates that were bound to the column were eluted with 0.1N NH_4OH in ethanol (1:1, v/v) (Sigma) and stored at - 20°C until further analysis. Large-scale extraction of the sulfate conjugates which contain ED was carried out on 50 Sephadex™ LH-20 mini-columns simultaneously using the same procedure noted above.

2.7.2. Oasis WAX Weak Anion-Exchange Solid Phase Extraction of Sulfate-Conjugated Steroids

12 mL of pregnancy urine was acidified with 2.4 mL of 2M sodium acetate buffer (pH 5.2) and passed through an Oasis Wax cartridge (Waters) that was pre-conditioned with 1mL of 2M sodium acetate buffer (pH 5.2). The cartridge was first washed with 1 mL of nano-pure H_2O and the glucuronide conjugates bound to the anion exchange solid phase were eluted with 4 mL of 10% formic acid in methanol (95:5, v/v) (Sigma). After washing the cartridge with 1mL of 5% ammonium hydroxide in methanol (20:80, v/v) (sigma), the sulfated conjugates were eluted with 4mL of 5% ammonium hydroxide in methanol (90:10, v/v) (Sigma) and stored at -20°C untill further analysis. Since the capacity of each cartridge was 12 ml, large-scale sulfate conjugates extraction from pregnancy urine was carried out on multiple cartridges simultaneously using the same procedure noted above.

2.7.3. Hydrolysis and Extraction of Sulfate Conjugate Preparation

The sulfate conjugate extracts from Sephadex™ LH-20 mini-column extraction and Oasis WAX weak anion-exchange solid phase extraction were hydrolyzed by Glusulase (Perkin Elmer) to generate the unconjugated (free) ED and fractionated on 10 cm and 60 cm Sephadex™ LH-20 (GE Healthcare) columns following the same procedures described in section 2.2 to 2.5. The fractions containing ED was located using the SHBG assay procedure described in section 2.4.

2.8. Gravity-Flow-Based Procedures for Separating Sulfate-Conjugated Steroids

In order to develop an optimal separation system for sulfate-conjugated steroids, two well known radiolabeled sulfate-conjugated steroids: ^{3}H-pregnenolone sulfate (^{3}H-PS) and ^{3}H-estrone sulfate (^{3}H-ES) were used to demonstrate the efficiency of gravity-flow-based systems.

2.8.1. Sephadex LH-20 Solid Phase Separation of Sulfate-Conjugated Steroids

Ethanol aliquot containing 50,000 cpm of ^{3}H-PS and ^{3}H-ES was evaporated under airflow and re-suspended in an equal volume of 0.1N HCl in separate test tubes. The two radioactive samples were passed through two different mini-columns and eluted with 5 mL of 0.1N HCl, followed by 70 mL of 0.1M sodium acetate buffer (pH 4.0) (Sigma). 75 fractions of 1 mL each were collected and a suitable aliquot of each fraction was taken to count. The two mini-columns were stripped with 5ml of 0.1N NH_4OH in ethanol (1:1,v/v) (Sigma) and re-generated with 5 mL of 0.1N HCl.

2.8.2. DEAE Sephadex A-25 Weak Anion Exchange Solid Phase Separation of Sulfate-Conjugates Steroids

An alternative system was also examined. DEAE Sephadex A-25 (GE Healthcare) was pre-soaked in nano-pure H_2O overnight and then packed in 10 cm x 1 cm, 15 cm x 1 cm and 35 cm x 0.9 cm columns. Ethanol aliquot containing 40,000 cpm of ^{3}H-PS and ^{3}H-ES were dried down by air flow and re-suspended in a suitable volume of 0.3M NaCl (Sigma). The radioactive samples were then passed through the DEAE Sephadex A-25 (GE Healthcare) columns and eluted with suitable 0.3M NaCl. 1 mL fractions were collected and aliquots taken from each fraction to count for locating the radioactivity.

2.9. HPLC Separation of Sulfate-conjugated Steroids

Radiolabeled sulfate-conjugated steroids: ^{3}H-PS and ^{3}H-ES, were also used to demonstrate the efficiency of the HPLC systems. A Waters HPLC system consisting of a 600S model controller, 626 model pump and 2487 model pump UV detector was employed.

Nova Pak normal phase silica-diol capped column (3.9 X 150mm, Waters) was the HPLC system used in this experimental approach. 3 gradient mobile phases were

tested with this HPLC system: (1) 16% ammonium acetate and 84% acetonitrile (pH 4.0) to 45% ammonium acetate and 55% acetonitrile over 35 minutes; (2) 16% ammonium acetate and 84% acetonitrile (pH 4.0) to 24% ammonium acetate and 76% acetonitrile over 35 minutes; (3) 35% triethyl ammonium acetate and 65% acetonitrile (pH 6.8) to 65% triethyl ammonium acetate and 35% acetonitrile over 15 minutes, further to 90% triethyl ammonium acetate and 10% acetonitrile over 25 minutes. An isocratic mobile phase consisting of 45% methanol and 55% nano-pure H_2O was also tested.

Ethanol aliquot consisting 2000 cpm of ^{3}H-PS and ^{3}H-ES was dried down by airflow and re-suspended in 80 μL of the mobile phase. The sample was then injected into the HPLC system and 1mL fractions were collected at a flow rate of 1mL/min. Aliquots were taken from each fraction to locate the radioactivity. The column was washed and regenerated with an optimal mobile phase.

CHAPTER 3:

RESULTS

Results

3.1. Sephadex LH-20 purification and HPLC purification of extracted urine and blood samples

The first stage of the study involved the use of an established method to isolate and purify large quantities of ED in order to pursue biological studies and structure analysis.[4] Even though maternal serum is the ideal source for pure free ED, it is ethically difficult to collect a sufficient amount of maternal blood sample. Pregnancy urine, on the other hand, is shown to be a suitable source and much less difficult to collect. ED, along with other steroids, is excreted in the urine as sulfate or, to a less extent, glucuronide conjugates which are water soluble. In order to obtain a unconjugated form of ED, the pregnancy urine was hydrolyzed by treatment (36-hour incubation) with the enzyme Glusulase which has both sulfatase and β-D-glucuronidase activity. Free ED was then extracted from the urine samples by two-phase extraction.

In order to further confirm that ED exists only during pregnancy and it is not similar to any known metabolites, urine and serum samples from NP women either taking birth control pill or not also underwent the same extraction procedure as the pregnancy samples and serve as a negative control in biological studies. A blank H_2O control was also used.

Following the extraction from the pregnancy urine sample, free ED was purified on a series of columns packed with Sephadex^{TM} LH-20 (GE Healthcare) (Fig. 5). Sephadex LH-20 is a dextran gel which is widely used for steroid separation and purification. It contains both hydrophilic and lipophilic properties and gives excellent separation of closely related steroids.[75] In the present study, the steroids in the urine samples were separated on different lengths of Sephadex^{TM} LH-20 columns. Non-polar steroids including ED and androstenedione were eluted in the region of low polarity. As an initial step in the separation of non-polar steroids from the polar steroids, the extract was fractionated on a 10cm Sephadex LH-20 column. Since ED was co-eluted with ^{3}H-androstenedione on the 10cm columns, the fractions containing the tracer (and ED) were pooled and further separated on a 60 cm Sephadex LH-20 column. On the 60cm column, ED was eluted at approximately the 5th to 7th fractions after the tracer peak and the ED-containing fractions were identified by SHBG assay.

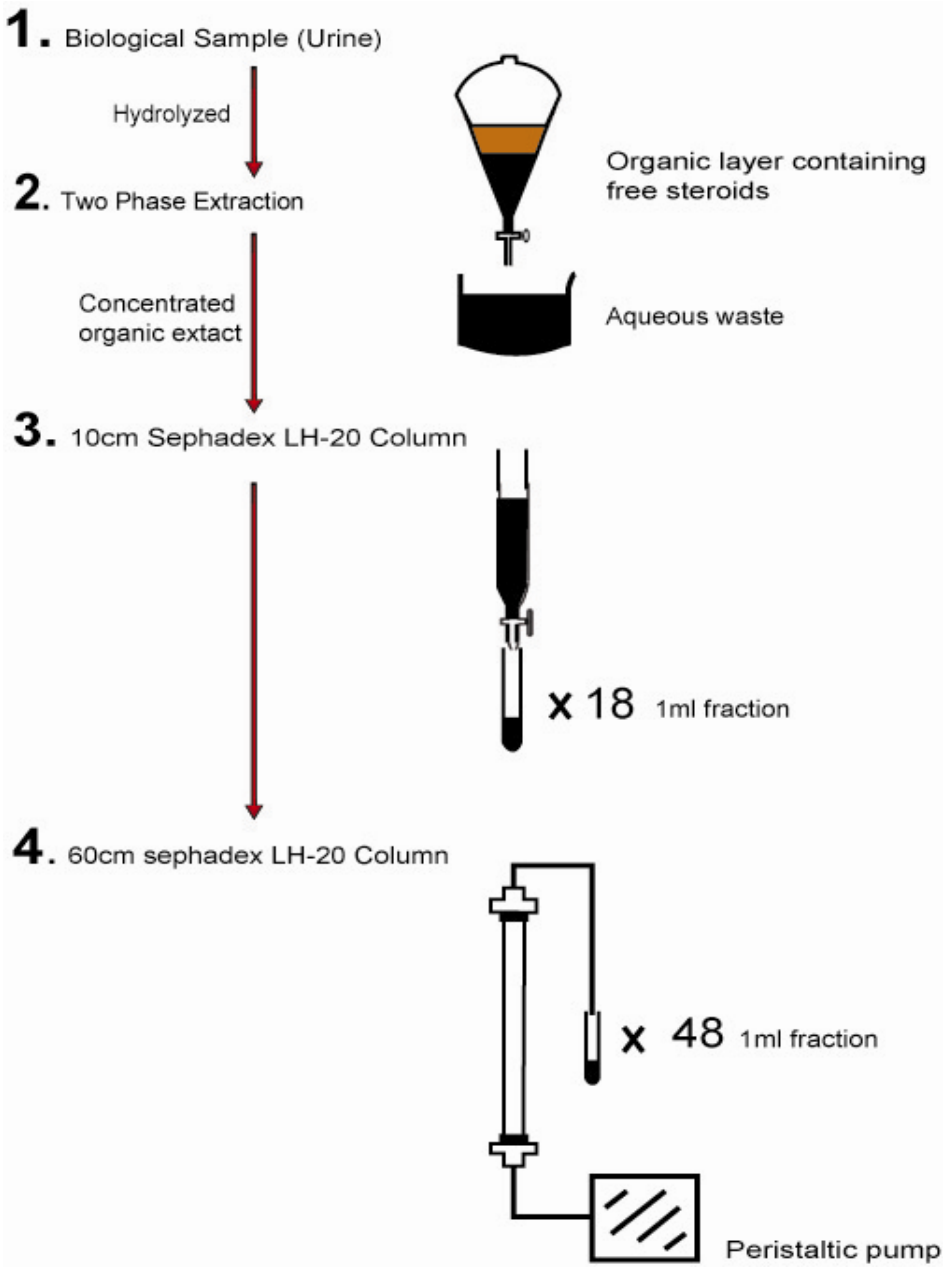


Figure 5: Sephadex LH-20 column extraction and purification scheme: 1) Biological sample hydrolysis by Glusulase. 2) Hydrolyzed sample extraction with 2 x 3 volumes of ethyl acetate. 3) Initial separation on Sephadex LH-20 column (10 cm x 1 cm) and 18 fractions of 1mL each was collected. 4) Detailed separation on Sephadex LH-20 column (60 cm x 1 cm) and 48 fractions of 1mL each was collected at a flow rate of 1 mL/min. a 60 µL aliquot of each fraction was taken to locate the tracer peak by liquid scintillation counting and to determine the ED concentration by SHBG assay. The ED obtained from the 60cm fractionation was further purified on HPLC.

In order to obtain a more pure ED sample, the pooled 60cm column fractionated ED fractions were fractionated by the HPLC to further separate other non-polar or compounds that may co-elute with ED on the 60 cm column. A chromatogram of SHBG-bound material obtained by HPLC separation is shown in Figure 6A which clearly shows that there are two distinct peaks. The first peak is small and eluted 2 fractions prior to the ^{3}H-androstenedione tracer peak. The second peak is the major peak which is eluted one fraction after the tracer peak. This HPLC elution pattern of pregnancy urine sample is consistent with the results obtained by the previous researchers from our lab. It has also been previously demonstrated that the second major peak contains bioactive ED.[4] The HPLC chromatogram of NP urine samples from women either taking birth control pills or not is presented in Figure 6B. Similar to the result from the Sephadex LH-20 60cm column fractionation, there is no clear peak or pattern shown in either HPLC chromatogram in Figure 6B. There is also no trace of SHBG-bound material in the chromatogram of water control (Fig. 6C) as expected.

For comparison, blood serum samples from pregnant and NP women were also extracted and purified by Sephadex LH-20 columns and the elution profile of the 60 cm column fractionation is represented in Figure 7. In contrast to the elution profiles of the pregnancy serum samples (Fig. 7A), there is no trace of SHBG-bound materials shown in the elution profiles of the NP serum (Fig. 7B) indicating the low concentration of SHBG-bound material in NP serum.

3.2. Gravity-Flow-Based Extraction of Sulfate-conjugated ED from pregnancy urine

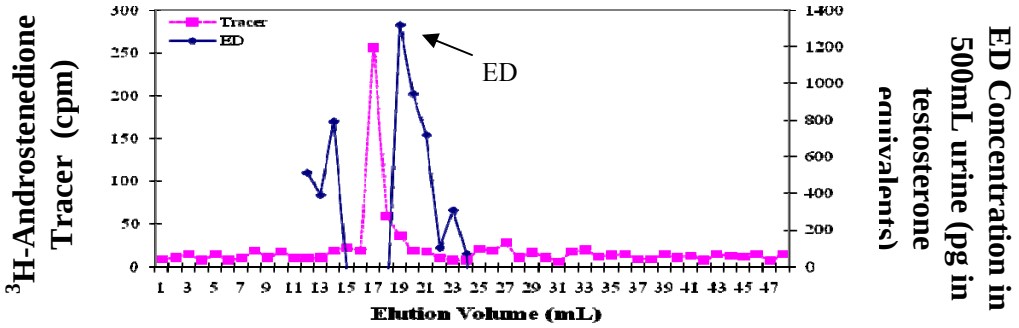
Due to the inconsistencies in yields, the loss of ED during extraction probably due to its relatively unstable nature and the expense of the hydrolysis enzyme, attempts at extracting its conjugates form, ED-sulfate, were made. Since ED is excreted in urine mostly as sulfate conjugates and the conjugate form of it is much more stable, extraction and purification of ED-sulfate was attempted.

3.2.1. Sephadex LH-20 Extraction of Sulfate-Conjugated Steroids

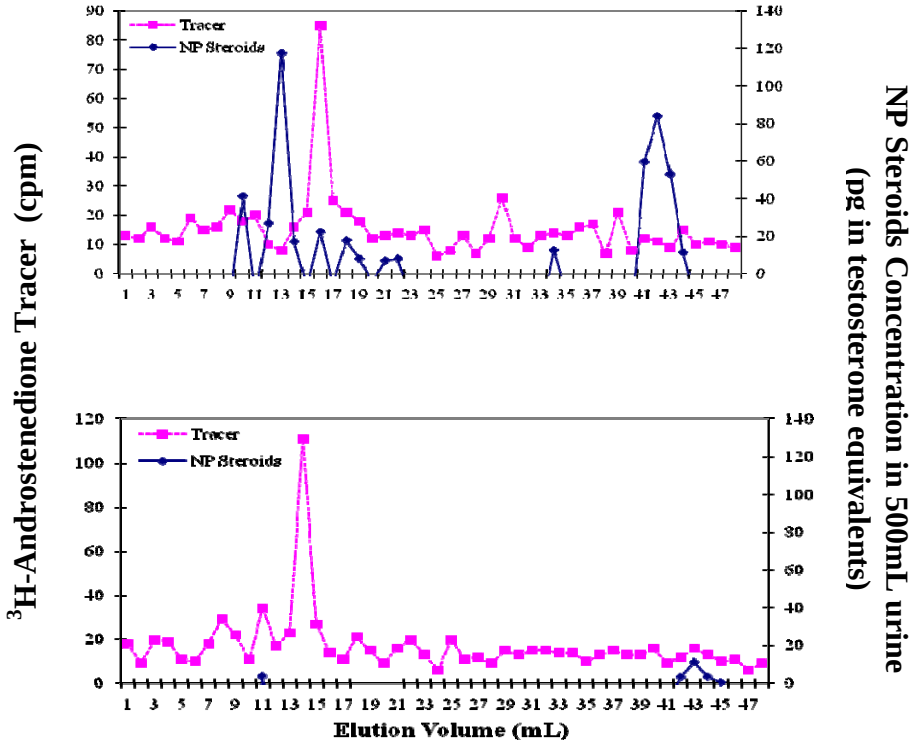
This Sephadex LH-20 mini-column methodology was first developed by Finnson for separating the thyroid hormone glucuronide conjugates and sulfate

Figure 6: HPLC chromatogram of ED peak obtained from pregnancy urine sample NP urine samples and water control. Equal volume (1L) of pregnancy urine from women at 12-36 weeks gestation *(A)*, NP urine from women taking birth control pills *(B, top)*, NP urine from women that were not taking birth control pills *(B, bottom)* and pure water *(C)* were treated with Glusulase and extracted using the same procedure. Fractions containing ED or corresponding to the location of ED from the Sephadex LH-20 60cm column fractionation were pooled and further separated by HPLC. 48 fractions (30 fractions in figure 6C) of 1mL each were collected using a mobile phase consisting of dichloromethane, heptane and methanol (30:70:0.01) at a flow rate of 1 mL/min (Nova Pak normal phase diol-capped silica column, 3.9 x 150mm). 60 µL aliquot from each fraction were taken for radioactivity counting and SHBG assay. The ED peak is indicated by an arrow.

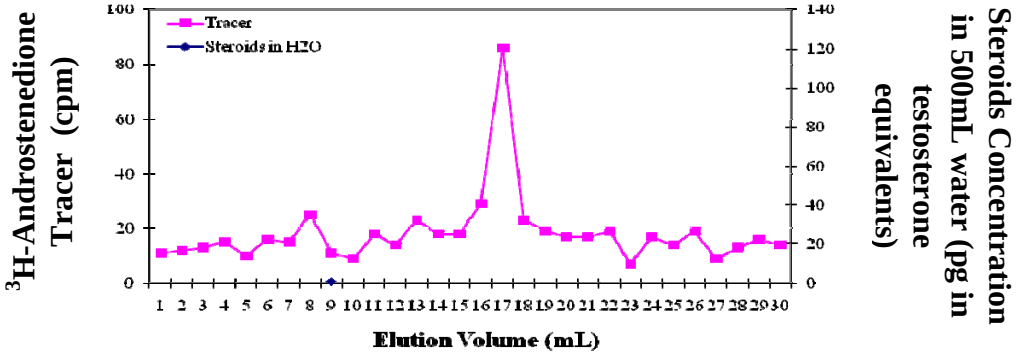
(A)


Tracer
ED
ED
^{3}H-Androstenedione Tracer (cpm)
ED Concentration in 500mL urine (pg in testosterone equivalents)
Elution Volume (mL)

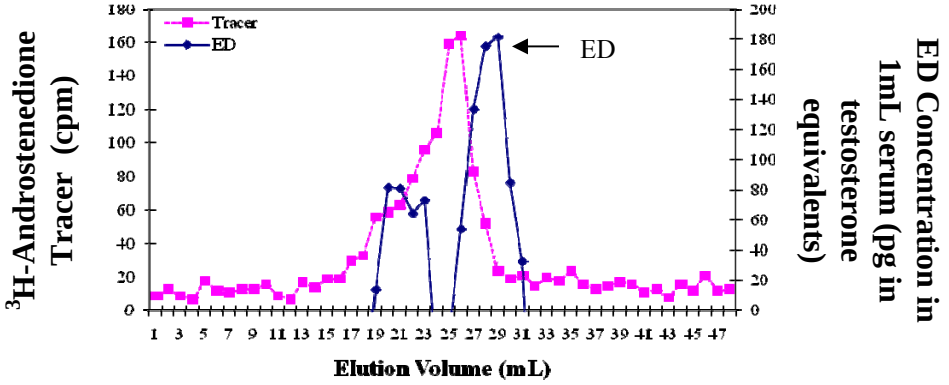
(B)


Tracer
NP Steroids
Tracer
NP Steroids
^{3}H-Androstenedione Tracer (cpm)
NP Steroids Concentration in 500mL urine (pg in testosterone equivalents)
Elution Volume (mL)

(C)


Tracer
Steroids in H2O
^{3}H-Androstenedione Tracer (cpm)
Steroids Concentration in 500mL water (pg in testosterone equivalents)
Elution Volume (mL)

(A)



(B)

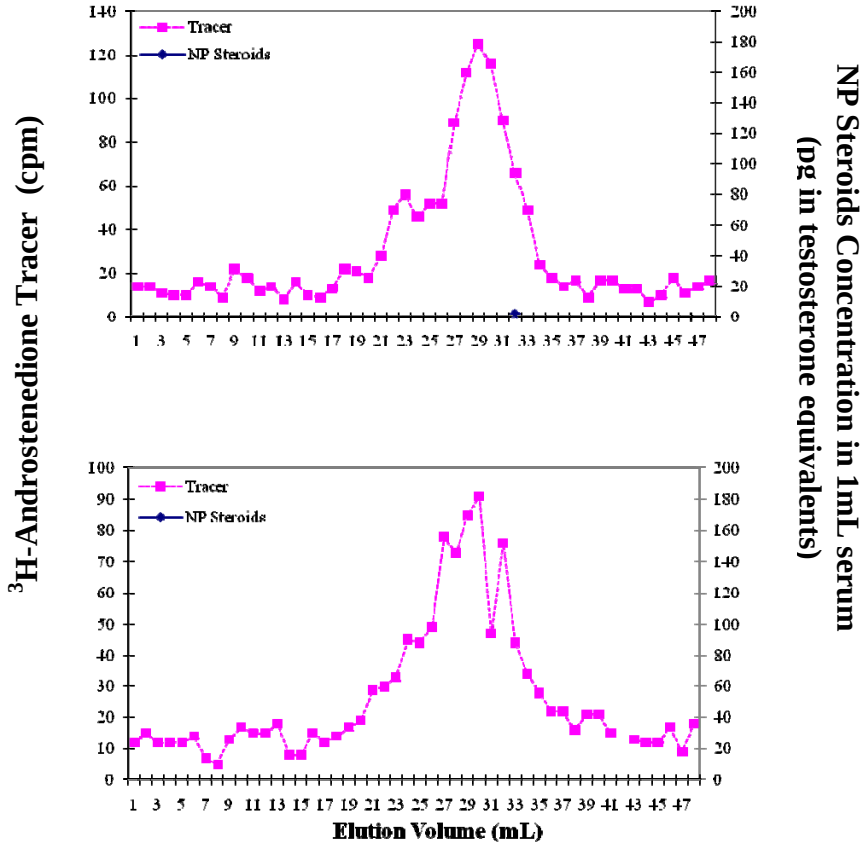


Figure 7: Elution patterns of SHBG-bound material in the region of low polarity from the pregnancy serum (A) and the NP serum samples (B) on the Sephadex LH-20 60cm column. Blood sample were collected from the women at 12-36 weeks gestation *(A)*, NP women taking birth control pills *(B, top)* and NP women that were not taking birth control pills *(B, bottom)*. Equal volume of serum samples were manipulated. Samples were chromatographed on a Sephadex LH-20 column (60 x 0.9cm) with a mobile phase consisting of dichloromethane, heptane and methanol in the ratio of 50:50:1. 48fractions of 1mL each were collected and an aliquot was assayed by the SHBG assay. The ED peak is indicated by an arrow.

conjugates and proven to be efficient.[76] Even though the same type of resin was employed (Sephadex LH-20), the solvent system was completely different from the one used for the free ED purification. In the present study, the pregnancy urine sample was first extracted with an equal volume of chloroform in methanol (2:1, v/v) and all the conjugated steroids remained in the aqueous layer while the free steroids were present in the organic phase. After acidification the aqueous phase with equal volume of 0.1N HCl, the sample was then applied to the mini-columns. After washing with 5 mL 0.1N HCl, the glucuronide conjugates were first eluted with 7 mL 0.1M sodium acetate buffer (pH 4.0). The sulfate conjugates including ED-sulfate were eluted by 22 mL H_2O. Since ED is excreted in urine mostly as a sulfate conjugate, the H_2O fractions were pooled, acidified and re-chromatographed using the same procedure mentioned above. The mini-columns containing ED-sulfates were eluted with 5 mL 0.1N NH_4OH in ethanol (1:1,v/v).

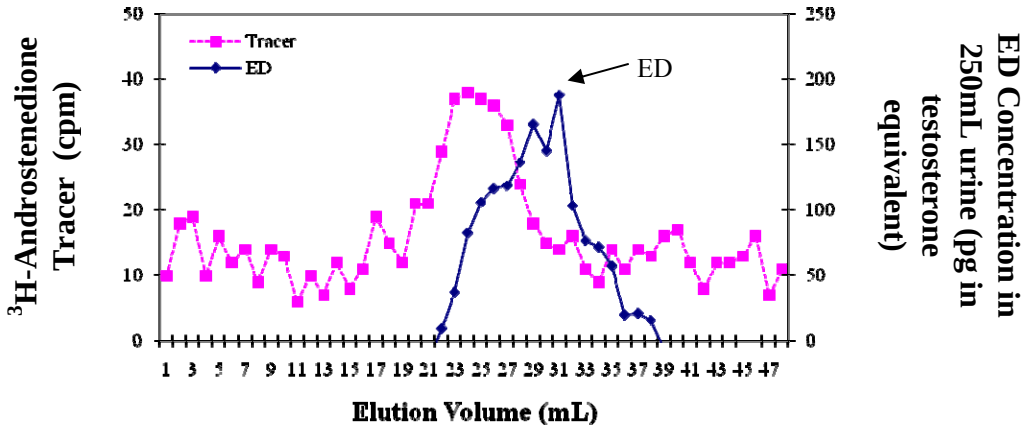
In order to further confirm that this new system is suitable for extracting ED-sulfate, the NH_4OH/methanol extracts were then evaporated and the sulfate conjugates were re-suspended in the $NaC_2H_3O_2$ buffer for the Glusulase hydrolysis. The extraction and purification of hydrolyzed ED were then carried out the same manner as the regular urine extraction described earlier, followed by Sephadex LH-20 60 cm column fractionation. Figure 8A represents the ED peak from the extract of hydrolyzed urine but was not subjected to a sulfate conjugate extraction procedure and figure 8B represents the ED peak from the sulfate conjugates extract. Even though the ED concentration from the sulfate conjugates extract was lower (~120 pg/µL compared to ~210 pg/µL), ED peak shown in the diagram is sharper compared to the ED peak in figure 8A.

3.2.2. Oasis WAX Weak Anion-Exchange Solid Phase Extraction of Sulfate-Conjugated Steroids

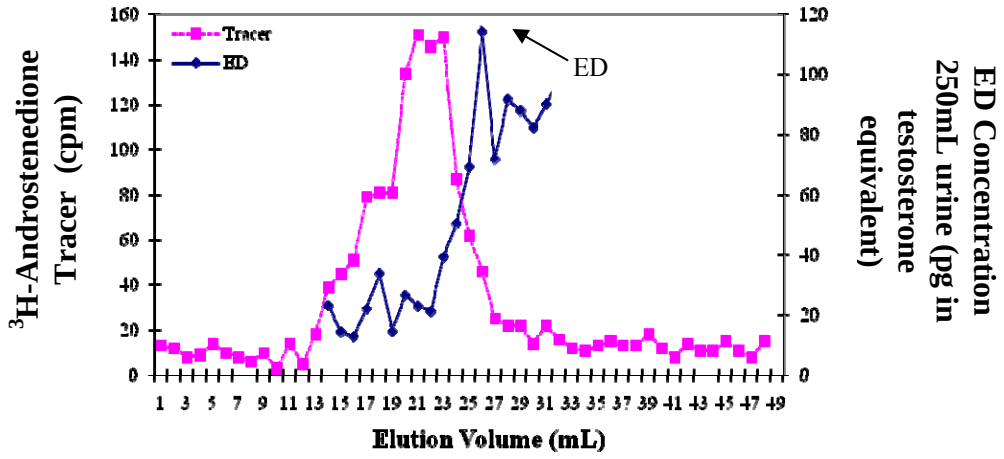
This extraction protocol was first developed by *Strahm et a*l to extract 19norsteroid sulfate conjugates for further Liquid Chromatography/Mass Spectrometry/ Mass Spectrometry (LC/MS/MS) analysis.[77] It has been demonstrated in their studies that this protocol is sufficient for separation of glucuronide conjugates and sulfate conjugates of two structurally related steroids and less than 1% of the glucuronide conjugates was found in the separated sulfate conjugates. In the present study, the pregnancy urine sample was first acidified with acetate buffer and applied to the cartridges. Glucuronide

Figure 8: Elution patterns of SHBG-bound material in the region of low polarity from the pregnancy urine sample (A) and sulfate extracts (B), (C) on the Sephadex LH-20 60cm column. 24-hour urine sample were collected from the women at 22-36 weeks gestation. ***(A)*** 250mL of the pooled urine sample was hydrolyzed by Glusulase for 36 hours at 37°C prior to extraction. ***(B)*** Another 250mL urine sample was first extracted with equal volume of chloroform in methanol (2:1, v/v). The aqueous layer was then acidified with equal volume of 0.1N HCl before applying to 10 Sephadex LH-20 mini-columns (20mL each). The eluted H_2O fractions were pooled together, acidified and re-chromatographed on the same mini-columns. The columns were stripped with 0.1N NH_4OH in ethanol (1:1,v/v) and the eluants were pooled, evaporated and re-suspended in the acetate buffer for Glusulase hydrolysis. The sulfate conjugates were then hydrolyzed by Glusulase for 36 hours at 37°C prior to extraction. ***(C)*** The third portion of 250mL of pregnancy urine was first acidified with an equal volume of 2M acetate buffer and then applied to the Oasis Wax cartridges. The eluted 5% NH_4OH in methanol (90:10, v/v) fractions were pooled, evaporated and re-suspended in the acetate buffer for Glusulase hydrolysis. The sulfate conjugates were then hydrolyzed by Glusulase for 36 hours at 37°C prior to extraction. Steroid extracts were chromatographed on Sephadex LH-20 column (60 x 0.9cm) with a mobile phase consisting of dichloromethane, heptane and methanol (50:50:1). 48 fractions of 1mL each were collected at a flow rate of 1 mL/min and assayed by SHBG assay. The ED peaks are indicated by arrows.

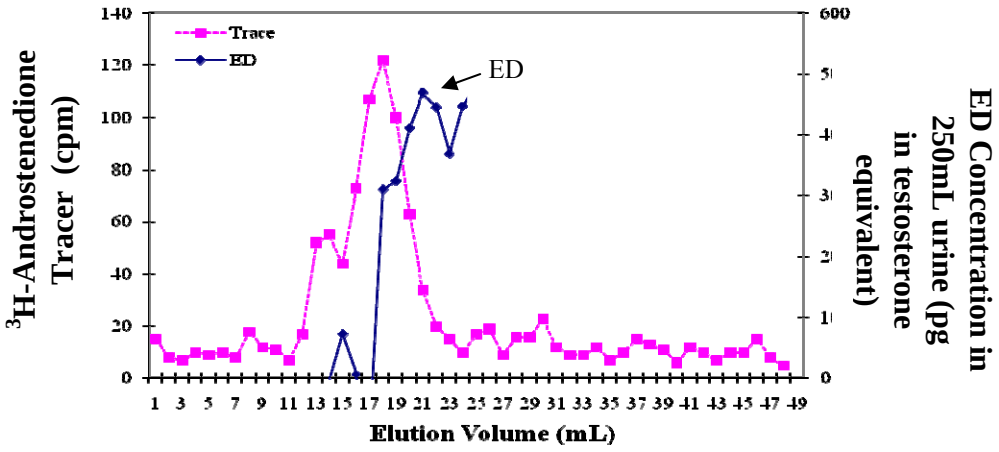
(A)


Tracer
ED
ED
^{3}H-Androstenedione Tracer (cpm)
ED Concentration in 250mL urine (pg in testosterone equivalent)
Elution Volume (mL)

(B)


Tracer
ED
ED
^{3}H-Androstenedione Tracer (cpm)
ED Concentration 250mL urine (pg in testosterone equivalent)
Elution Volume (mL)

(C)


Trace
ED
ED
^{3}H-Androstenedione Tracer (cpm)
ED Concentration in 250mL urine (pg in testosterone equivalent)
Elution Volume (mL)

conjugates were neutralized and eluted at low pH with a large proportion of organic phase in the mobile phase (10% formic acid in methanol, 95:5, v/v) and sulfate conjugates remained charged and bound to the column. The ionic interactions between sulfate conjugates and the solid phase were interrupted as the concentration of NH_4OH increased. By using 5% NH_4OH in methanol (90:10, v/v), the sulfate conjugates were completely eluted.

The sulfate extracts were evaporated and re-suspended in acetate buffer and subjected to Glusulase hydrolysis, as described before. The hydrolyzed sulfate conjugates were further extracted and purified using the regular free ED extraction procedure and the obtained elution profile of the Sephadex LH-20 60 cm column fractionation is represented in Figure 8C. There is also a distinct peak shown in the diagram which suggests that the system is working properly except that the free ED concentration is much lower than the one obtained from the same amount of urine sample which was not extracted by the cartridges (Fig. 8A).

3.3. Gravity-Flow-Based Procedures for Separating Sulfate-Conjugated Steroids

A large portion of steroids including ED is excreted in urine conjugated to a sulfate group. Hence, the sulfate extracts obtained from the pregnancy urine sample contain various sulfate-conjugated steroids besides ED-sulfate. In order to obtain pure ED sulfate from the steroid sulfate preparation, it is necessary to develop an optimal separation system for sulfate-conjugated steroids.

3.3.1. Sephadex LH-20 Solid Phase Separation of Sulfate-Conjugated Steroids

^{3}H-Pregnenolone Sulfate (^{3}H-PS) and ^{3}H-Estrone Sulfate (^{3}H-ES) were used to demonstrate the efficiency of the employed system. Approximately 10,000 cpm of each sulfate were applied to two separate mini-columns after acidified with 0.1N HCl. The elution was carried out using isocratic elution with 0.1M sodium acetate buffer (pH 4.0) as the mobile phase. The elution profiles of both radioactive sulfates are represented in Figure 9. As shown in the diagram, there are two peaks in the elution profile of ^{3}H-ES which is probably due to a contaminant or break-down products in the ^{3}H-ES sample. The peak representing ^{3}H-PS is much broader compared to the ones from the ^{3}H-ES

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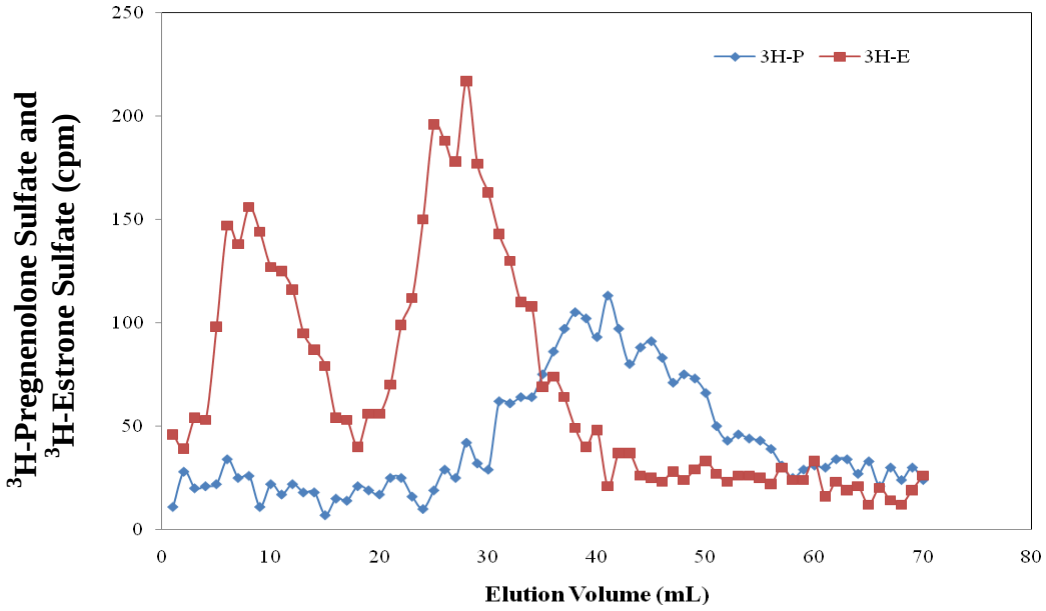

Figure 9: Elution profiles of ^{3}H-Pregnenolone Sulfate and ^{3}H-Estrone Sulfate on Sephadex LH-20 mini-columns. Approximately 10,000 cpm of each steroid sulfate were first acidified with 0.1N HCl and applied to separate Sephadex LH-20 mini-columns. The sulfates were chromatographed on the mini-columns by isocratic elution with 0.1M sodium acetate buffer (pH 4.0). 70 fractions of 1mL each were collected and 500 µL of each fraction was taken for radioactivity counting.

elution profile. The fact that there is an overlap between the major peak from the ^{3}H-ES elution profile and the ^{3}H-PS peak which suggests that the separation of the two steroid sulfates was not complete. Complete separation of the two steroid sulfates by Sephadex LH-20 column can probably be achieved by increasing the column length; however, the flow rate was too low as the column length increased, making it impractical.

3.3.2. DEAE Sephadex A-25 Weak Anion Exchange Solid Phase Separation of Sulfate-Conjugated Steroids

Diethylaminoethyl (DEAD) Sephadex is a weak anion exchange resin that can separate the conjugated steroids based on their different charge and different polarity. isocratic separation of estrogen conjugates on DEAE Sephadex was first demonstrated by *Musey et al*.[78] In their study, the estradiol sulfate and estrone sulfate were clearly separated on a 60 x 0.5 cm DEAE Sephadex column by isocratic elution with 0.3M NaCl buffer. In the present study, ^{3}H-PS and ^{3}H-ES were applied on the DEAE Sephadex A-25 columns and the separation efficiency of the columns with different lengths were tested. All the separations were carried out by isocratic elution with 0.3M NaCl solution and the elution profiles obtained from different length of columns are shown in Figure 10. The NaCl solution neutralizes the polar effect of the sulfate group and allows conjugated steroids to separate out according to their own properties. The two sulfates were chromatographed on two separate 10 x 1 cm DEAE Sephadex A-25 columns in order to identify the location of each peak. On DEAE Sephadex A-25 columns, ^{3}H-PS was eluted out before ^{3}H-ES and the ^{3}H-PS was sharper than the ^{3}H-ES peak as shown. On the 10 cm x 1 cm DEAE Sephadex A-25 column, the two sulfates were not completely separated and had a minor overlap which suggests that 10cm as the column length is not sufficient for separating the two sulfates. The 15cm and the 35 cm DEAE Sephadex A-25 columns, on the other hand, gave clean separations of the two sulfates. The two sulfates had longer retention time on the 35cm column which suggests that the column length is essential for achieving better resolution. Nevertheless, same as in the Sephadex LH-20 separation system, the flow rates of the DEAE Sephadex A-25 columns were too low for aqueous mobile phase as the column length increased. The flow rate for the 35cm DEAE Sephadex A-25 column was approximately 83 µL/min. When the column height reached 60cm, the aqueous mobile phase could barely flow through the column. Hence this

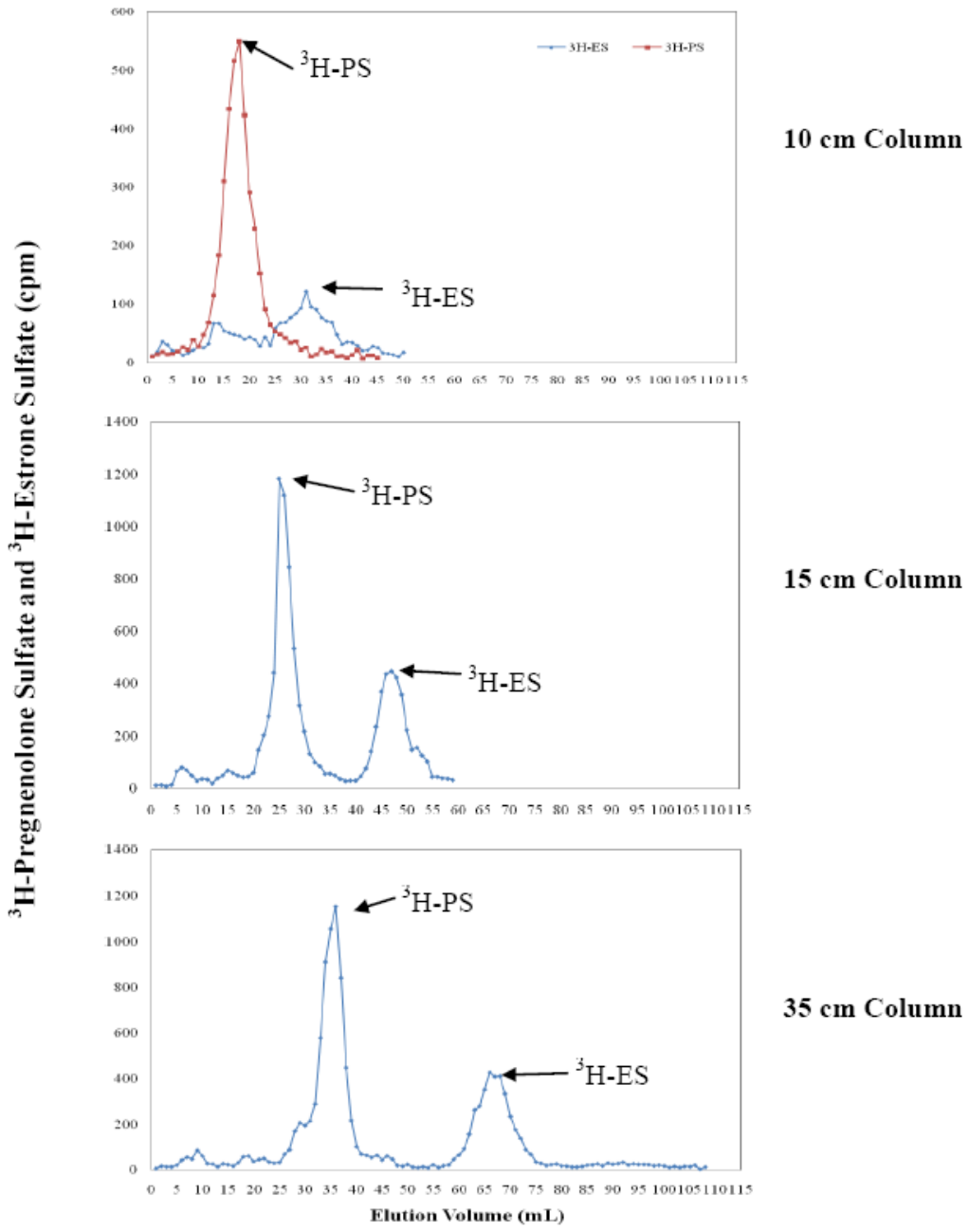

Figure 10: Elution profiles of ^{3}H-Pregnenolone Sulfate and 3H-Estrone Sulfate on DEAE Sephadex A-25 columns. Same amount of radioactivity of ^{3}H-PS and ^{3}H-ES were applied to separate 10 x 1cm, one 15 x 1cm and one 35 x 0.9cm DEAE Sephadex A-25 columns. Each column was eluted with 0.3M NaCl in isocratic mode. 1mL fractions were collected and 500 µL of each fraction were taken for radioactivity counting.

separation methodology is not considered to be the optimal due to the fact that it is not practical for large-scale manipulation.

3.4. HPLC Separation of Sulfate-conjugated Steroids

Since the flow rate was the major issue encountered during the gravity-flow-based separation mentioned above, HPLC may represent a better approach. Normal phase HPLC has been used extensively for separating steroids according to their polarities. In the present study, attempts at separating the sulfate conjugates by Normal Phase HPLC in the gradient mode were taken. 3 gradient systems were tested (refer to section 2.9.1) and each gradient system consisted of an aqeous phase (eg. ammonium acetate) and an organic phase (eg. acetonitrile). The proportion of the two phases were changed over a period of time, thereby disrupting the interactions between the sulfate conjugates and the stationary phase. The sulfate conjugates were separated out according to their structural properties. However, the ^{3}H-PS and ^{3}H-ES were eluted at the same time in all three gradient systems which indicates that the polar effect of the sulfate group was still dominant over the core steroid's structural properties. Hence, an alternate HPLC system should be explored.

CHAPTER 4:

DISCUSSION

Discussion

The economic, social and health costs associated with preterm birth is enormous. Researchers and clinicians have been working for decades to find the cause of it but with little success. In animal models, parturition is initiated by a decrease in the circulating progesterone to estrogen ratio leading to increased uterine contractility. In the human, however, there is no change in the circulating progesterone levels observed during parturition and the trigger of labour remains elusive.[8,9] Since progesterone is essential in maintaining pregnancy in mammals, it is possible that there is an endogenous progesterone agonist involved in maintaining human pregnancy and the decrease in the level of this agonist triggers the parturition in humans.

ED is a little-known endogenous steroid that was first reported by Philip and Murphy in 1989.[3,4] It has been demonstrated that the level of ED in both the blood and the placenta remains high during pregnancy, but decreases in association with term and preterm labour. ED is an isomer of estradiol and can rearrange to estradiol in alkali. The decrease in ED levels in association with labour is possibly due to its increased conversion to estradiol, which would further increase uterine contractility. Despite its structural similarity to estradiol, ED displays little affinity to the estrogen receptor but a high affinity for the progesterone receptor, which suggests that ED may act as a progestational agent. Preliminary results from our lab have demonstrated that ED is the natural ligand of MTBP and also serves as an inverse agonist of ERRα and ERRγ by inhibiting their transcriptional activity. The unique structural and biological properties of ED provide compelling reasons for the further study its physiological functions and therapeutic potential.

In order to fully investigate this endogenous novel steroid, large quantities of chemically synthesized ED are necessary for preparing radiolabeled-ED and generating an anti-ED antibody for further study of its biological functions. All of these need the final confirmation of the detailed structure of ED.

In the present project, ED was purified from pregnancy urine and serum samples using the methods described previously in section 2. NP urine and blood serum samples were also processed in the same way to serve as experimental controls. Purification of ED relies on Sephadex™ LH-20. It is a dextran gel which is widely used on steroid separation and purification. It contains both hydrophilic and lipophilic properties and

gives excellent separation of closely related steroids. Separation by Sephadex LH-20 can be achieved by molecular size, absorption and ion exclusion.[75] Since Sephadex LH-20 preferentially absorbs the more polar compounds, non-polar molecules, such as ED, are eluted out first in the non-polar region using the described method. Androstenedione, which is also a non-polar steroid, is used as a tracer in this method and ED is known to elute at approximately the 5^{th} to 7^{th} fractions after the ^{3}H-androstenedione peak on the 60 cm Sephadex LH-20 column. The level of ED was measured by SHBG assay which is a competitive protein binding assay based on the fact that ED has high binding affinity to SHBG. In this assay, the ability of ED to displace the testosterone from the SHBG binding site was measured and the ED concentration was presented as the testosterone equivalents. The Sephadex LH-20 60cm column elution profiles of the pregnancy urine samples showed a distinctive ED peak right after the tracer peak. The elution profiles of the NP urine samples from women either on birth control or no taking birth control pills also showed some SHBG-bound materials which were probably the steroid metabolites from the birth control pills. The ED peak, however, was not observed in any elution profiles of the NP samples. This further indicates that ED is exclusively produced in pregnant women.

The level of ED obtained in the present study is lower than the value reported by Philip and Murphy. This may be due to the fact that the individual ED concentration varied from one patient to another. Moreover, ED is known to be relatively unstable and is converted to estradiol under alkali and acidic condition. ED may also be sensitive to the direct airflow and strong light. The relatively unstable nature of ED also makes it difficulty for direct NMR or LC/MS/MS analysis. In order to minimize the conversion of ED during extraction, samples were evaporated under N_2 gas; all the samples were always covered with tin foil; fractions were immediately evaporated and stored at -20°C since ED is stable in ethanol.

Due to the inconsistencies in yields, the considerable loss of ED during extraction because of its relatively unstable nature and the expensive cost of the Glusulase enzyme, extraction of ED-sulfate is considered to be a better approach since ED is excreted in urine mostly as sulfate conjugate which is more stable. In the present study, two gravity-flow-based extraction procedures were attempted for obtaining steroid conjugates from pregnancy urine: the Sephadex LH-20 mini-column extraction and the

Oasis WAX weak anion exchange solid phase extraction. Since the sulfate conjugates were extracted and concentrated by evaporation prior to the hydrolysis, the amount of Glusulase used for the hydrolysis of the sulfate fractions extracted was much lower than the amount used for hydrolysis of equal volume of urine sample. Plus the ED purified from the sulfate extract was more pure. Nevertheless, the concentration of ED obtained from the sulfate extract was lower than the one purified directly from urine (~120 pg/μL compared to ~210 pg/μL). One possible cause of this is that the NH_4OH concentration was too high in the sulfate extract and it directly affected the Glusulase enzyme activity. Developing an optimal de-salting system should be the next step for this study. An alternate solution is to perform a direct analysis of the sulfate extract by LC/MS/MS. The LC/MS/MS identification and quantification of sulfate conjugates extracted from the urine sample via Oasis WAX weak anion exchange solid phase extraction can be achieved at a low concentration.[77]

The sulfate extracts obtained from the pregnancy urine sample contain various sulfate-conjugated steroids besides ED-sulfates. Hence, it is crucial to develop an optimal separation system for sulfate-conjugated steroids. The Sephadex LH-20 mini-column separation of the two radiolabeled steroid sulfate conjugates was not complete because the column was not long enough to give each sulfate conjugate a proper retention time. However, increasing the column length was not a practical solution since the flow rate decreased dramatically. The DEAE Sephadex A-25 columns, on the other hand, provided a very clean separation of the two sulfate conjugates. Yet the same problem was encountered while increasing the column length. It is not practical for large-scale manipulation.

An alternative solution for this is HPLC separation. Since the effect of the sulfate group was dominant over the core steroid's own structural properties, the sulfate conjugates could not be separated by normal phase HPLC which separated the compound according to their polarity. As the weak anion exchanger (DEAE Sephadex) can separate the two sulfate conjugates, the ion exchange HPLC may be able to separate the sulfate conjugates without any pressure issue. It has been demonstrated that a strong anion exchanger HPLC column (μ Partisil 10 SAX, 25 cm x 4.6 mm) was able to separate the estrogen sulfate conjugates.[79] The next step of this study should be testing out this HPLC system.

To date, ED is the only known steroid which plasma and placental levels remain high during pregnancy and decrease in association with term and preterm labour in the human. In order to fully investigate its unique biological properties and its therapeutic potential in diagnosis and treatment of preterm labour, availability of sufficient quantities of ED for further structural analysis is essential in the project. However, due to its relatively unstable nature, the yield of unconjugated ED was low and not sufficient for further structural analysis using NMR or LC/MS/MS. Therefore, several methodologies for purification of its conjugated form (ED sulphate) were attempted and optimized in the present study. The obtained ED sulfate preparation is ready for further NMR or LC/MS/MS studies. Once the detailed structure of ED are confirmed, it can be chemically synthesized which facilitates the future biological studies of ED in human parturition.

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