Methodology Development for the Study of Estrogen Carcinogenesis

BY

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THESIS

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This thesis is dedicated to my parents, my brother, and my cousin Cherry for their support and encouragement during my MS study. Without them, it would never have been accomplished

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AD	Alzheimer's disease
ALP	Alkaline phosphatase
CYP	Cytochrome P450
dA	2'-Deoxyadenosine
dC	2'-Deoxycytidine
dG	2'-Deoxyguanosine
CNL	Constant neutral loss
DMSO	Dimethyl sulfoxide
E_2	17β-Estradiol
EN	Equilenin
EQ	Equilin
ER	Estrogen receptor
ERE	Estrogen response element
ESI	Electrospray ionization
GC	Gas chromatography
HPLC	High performance liquid chromatography
HRT	Hormone replacement therapy
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LOD	Limit of detection
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NADPH	β-Nicotinamide adenine dinucleotide phosphate, reduced
NP1	Nuclease P1
4-OHEN	4-Hydroxyequilenin
4-OHEQ	4-Hydroxyequilin
P450	Cytochrome P450
PPB	Potassium phosphate buffer
ROS	Reactive oxygen species
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
VPH	Venom phosphodiesterase I

SUMMARY

I.

Evidence from clinical epidemiology and *in vivo* and *in vitro* experiments have a strong correlation between estrogen exposure and breast cancer. However, the mechanisms of estrogen carcinogenesis are highly complex. One of the proposed pathways is the hormonal pathway, by which estrogen stimulates cell proliferation which enhances the chance of genetic error during DNA replication. Another pathway, which is the focus of this project, is the chemical pathway in which estrogens are metabolized to form reactive *o*-quinones that covalently modify DNA resulting in DNA adduct formation.

Hormone replacement therapy (HRT) has been used for the relief of postmenopausal symptoms since its introduction in the 1930s. Clinical results from the two landmark studies, the Women's Health Initiative in 2002 and the Million Women Study in 2003, have raised questions about the use of HRT. Premarin (Wyeth-Ayerst), the most prescribed drug for HRT, is mostly made of conjugated equine estrogen. When taken into body, equine estrogen conjugates are hydrolyzed to their parent estrogens. These equine estrogens can be oxidatively metabolized by cytochrome P450s to form 2- and 4-hydroxyl metabolites. Previous studies have shown that compared to endogenous estrogens predominantly metabolized to 2-hydroxyl metabolites, increasing unsaturation in the B-ring shifts the metabolism of equine estrogens to mainly 4-hydroxylation. Both 4-hydroxyequilenin (4-OHEN) and 4-hydroxyequilin

SUMMARY (continued)

 (4-OHEQ) will rapidly autoxidize to *o*-quinone and 4-OHEQ has been found to oxidize to 4-OHEN-*o*-quinone. Studies suggest that 4-OHEN-*o*-quinone is more reactive than endogenous estrogen quinones, and potentially more toxic and carcinogenic.

4-OHEN can react with DNA to form stable adducts, which could be potential biomarkers for cancer risk assessment. LC-MS/MS, with high sensitivity, and ability for structural identification and accurate quantification, is having an increasing role in the detection of DNA adducts. The adducts standards were chemically synthesized and then characterized with tandem MS. TSQ Quantum triple quadrupole was found to be 4 to 5 times more sensitive than the API 3000. For 4-OHEN-dA and –dC, the limit of detection was estimated to be 50 fmol on column, equivalent to 3.8 adducts/10^8. The detection limit for 4-OHEN-dG was a little higher (100 fmol; 7.7 adducts/10^8 nucleotides). The DNA extraction and hydrolysis efficiency of method used was determined to be around 98% and 60% respectively. In combination of these factors, a table of correlation between adducts frequency and DNA amounts required is listed. In vitro experiments of formation of 4-OHEN-DNA stable adducts in the MCF-7 cells were conducted. However, no positive results were shown. Based on the optimization work and calculation table, it can be concluded that the levels of adducts formed are below limit of detection. Therefore, a more sensitive instrument will be required.

II.

Alzheimer's disease (AD), the most common form of dementia, is a neurodegenerative disorder affecting memory, cognition, and behavior. Advancing age is considered one of the greatest risk factors to develop AD. In addition, women have been found to be at higher risk of developing AD compared to men. Postmenopausal depletion of endogenous estrogens may also contribute to increased risk. Clinical results have shown that in women, previous history of using HRT is associated with reduced risk of AD. Anastrozole, an aromatase inhibitor which has been studied for the treatment of early breast cancer in postmenopausal women, was found to cause poorer hippocampal-dependent tasks such as verbal and visual learning and memory.

The goal of the study was to evaluate the effect from depleting circulating and/or local brain estrogens through gonadectomy and/or treatment with anastrozole. Blood brain barrier penetration of anastrozole was also analyzed in a mouse model. Analysis of hippocampal amyloid-beta deposition by Dr. Cassia Overk demonstrated alteration of AD pathology in female but not male 3xTgAD mice with orally administered anastrozole. Hypothesis that AD pathology may be affected by sex is supported by the results. To investigate the blood brain barrier penetration of anastrozole, a method for the determination of brain anastrozole level was developed Brain anastrozole extraction was carried out with methanol. The reproducibility was evaluated by spiking the same amount of anastrozole into

SUMMARY (continued)

different amounts of brain tissue. Qualification and quantification of the levels of

anastrozole was performed with the aid of LC-MS/MS. The concentration in the

brain was detected at 4.7 pg/mL. Compared to serum level of 10.19 ng/mL, brain

anastrozole concentration is 0.05% of that in serum. A significantly positive

correlation between serum and brain levels of anastrozole was observed. Based

on the experiment results, it can be concluded that anastrozole can penetrate

blood brain barrier and alter AD pathology in female 3xTgAD mice.

CHAPTER 1 INTRODUCTION

1.1 <u>**Hormone Replacement Therapy and Risk of Breast Cancer**</u>

Premarin (Wyeth-Ayerst), which consists of conjugated equine estrogens

is the most widely prescribed hormone replacement therapy (HRT) drug used to

relieve menopausal symptoms (1). Vasomotor symptoms such as hot flashes and

night sweats, CNS symptoms such as insomnia and memory loss, and urogenital

symptoms such as viginal dryness and urinary tract infections are the most

common (2). In addition, HRT has been reported to have beneficial effects on

decreasing the risks of osteoporosis, reducing cardiovascular mortality, and

maintaining good serum lipid profiles (3,4). Also, estrogen has been shown to be

effective in improving memory in patients with Alzheimer's disease (5). However,

evidence suggests that, despite of the benefits HRT, long-term estrogen therapy

increases the risks of breast and endometrial cancer (6).

The link between HRT and breast cancer has been documented in the

epidemiological literature since the 1980s (7). The Nurses' Health Study was the

first large study indicating the risk of breast cancer was significantly increased

among women currently using estrogen alone (relative risk, 1.32) or estrogen

plus progestin (relative risk, 1.41), compared with nonusers (8). It was not until

the results published from the Women's Health Initiative (WHI) study in 2002 and

the Million Women Study in 2003, that women and doctors re-evaluated the use

of HRT (9,10). An analysis in 2009 on the WHI trial comparing the group

receiving 0.625 mg of conjugated equine estrogens (CEE) plus 2.5 mg of

medroxyprogesterone acetate daily, with the group receiving placebos, showed an increased risk of breast cancer (11). This trail was stopped early in 2002, after a mean of 5.2 years of follow-up, because risks exceeded benefits (10). The estrogen-only trial was stopped in 2004, since it showed no net health benefit. The use of CEE increased the risks of stroke, had no effects on coronary heart disease (CHD) incidences and showed little indication of breast cancer risks reduction, even it did decrease the risk of hip fracture (12,13).

Clinical prescription data demonstrated that since the publication of the trial results in 2002, the use of HRT declined. Current users were advised to reduce the therapy dosage or even discontinuation (1). Data analysis from the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) registries showed that, the age-adjusted incidence rate of breast cancer fell by 6.7% in 2003, which corresponded to the fact that the use of HRT had decreased by 38% by the end of 2002 (14).

1.2   Mechanism of Estrogen Carcinogenesis

Breast cancer has been related to long-term exposure to estrogens and its metabolites, but the mechanisms of estrogen carcinogenesis are still not totally understood (15). Malignant phenotypes could arise from the accumulation of random mutations during DNA replication errors, which may include mutation in DNA repair genes, tumor suppressor genes and oncogenes (16).
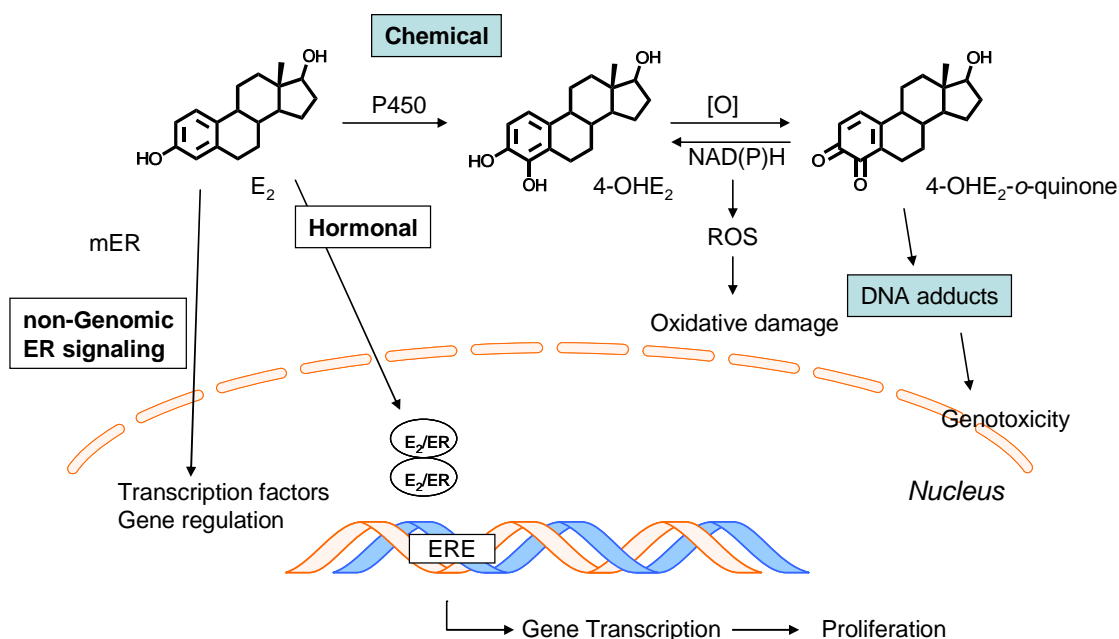
Figure 1. The proposed mechanisms for estrogen carcinogenesis.
Hormonal pathway includes the estrogen-driven cell proliferation. Chemical pathway includes formation of *o*-quinones.

One of the major pathways of estrogen carcinogenesis is the extensively studied hormonal pathway, by which estrogens stimulate cell proliferation through the nuclear estrogen receptor-mediated signaling pathway. In this classical pathway, estrogen-bound ERs dimerize and function as a transcription factor which binds to estrogen response element (ERE) in the promoter region of target genes (17). Estrogen-driven cell proliferation increases the chance of random genetic errors which will lead to a malignant phenotype if not corrected (16,18-20).

The nongenomic pathway is believed to be mediated by extranuclear 7-transmembrane G protein-coupled receptor, GPR30. In contrast to transcriptional

regulation, which occurs on a time frame of hours, the rapid nongenomic estrogen signaling, including events such as generation of the second messenger and activation of receptor tyrosine kinases, occurs within seconds to minutes (21-23).

The third pathway is the chemical pathway in which estrogens are oxidatively activated by cytochrome P450 to form *o*-quinones and, reactive oxygen species (ROS) are generated from redox cycling of *o*-quinones. These reactive oxidative intermediates can directly or indirectly damage DNA, resulting in genotoxicity. It has been reported that both hormonal and chemical pathways contribute to estrogen carcinogenesis (24).

1.3   Oxidative Metabolism of Estrogens

Estrogens are eliminated from body by conversion to hormonally inactive or less active water-soluble metabolites excreted in the urine and/or feces (25). The metabolic disposition of estrogens includes oxidative metabolism and conjugative metabolism.

Several cytochrome P450 enzymes are involved in the oxidative metabolism of estrone and estradiol to 2-hydroxycatechol (cytochrome P450 1A, and 3A) and 4-hydroxycatechol (cytochrome P450 1B) estrogens (26-28). Cytochrome P450 enzymes are reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enzymes catalyzing estrogen oxidative metabolism. In human liver, 2-hydroxylation is the major pathway and CYP1A2

and 3A4 are main contributors to hepatic hydroxylation reaction (29,30). The 4-hydroxylation of estradiol catalyzed by P450 3A family is a minor pathway in human liver (31,32).

In contrast, studies showed that 4-hydroxylation is the major pathway in extrahepatic tissues. The predominant extrahepatic hydroxylation members are CYP 1A1 and 1B1 (33,34). It has been known that CYP1A1 catalyzes the hydroxylation at C-2, -6α, and -15α positions of estradiol. Hydroxylation activity at C-4 position is only 4.1% of that at C-2 position (35). CYP1B1 mainly catalyzes the hydroxylation at C-4 position, while the activity catalyzing at C-2 position is only 1.9% of C-4 (28). For example, in human breast cancer MCF-7 cells treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the 2-hydroxylation is predominantly mediated by CYP450 1A1/1A2 (35-37). In human breast and uterus, P450 1B1 is considered to be the major enzyme for 4-hydroxylation (28,34,38).

1.3.1 <u>Endogenous Estrogens</u>

The endogenous catechol estrogens, such as 2- and 4-hydroxyestradiol, can be further oxidized to *o*-quinones by oxidative enzymes or metal ions (24,39). The estrogen *o*-quinones are unstable under physiological conditions, and are easily isomerized to quinone methides (40,41). The half-life of 2-hydroxyestrone-*o*-quinone (2-OHE-*o*-quinone) is 47 sec, while the 4-hydroxyestrone-*o*-quinone (4-OHE-*o*-quinone) is 12.2 min (40). The *o*-quinones are Michael acceptors,

which directly conjugate with nucleophilic DNA bases and lead to genotoxic effects (39,42-44).
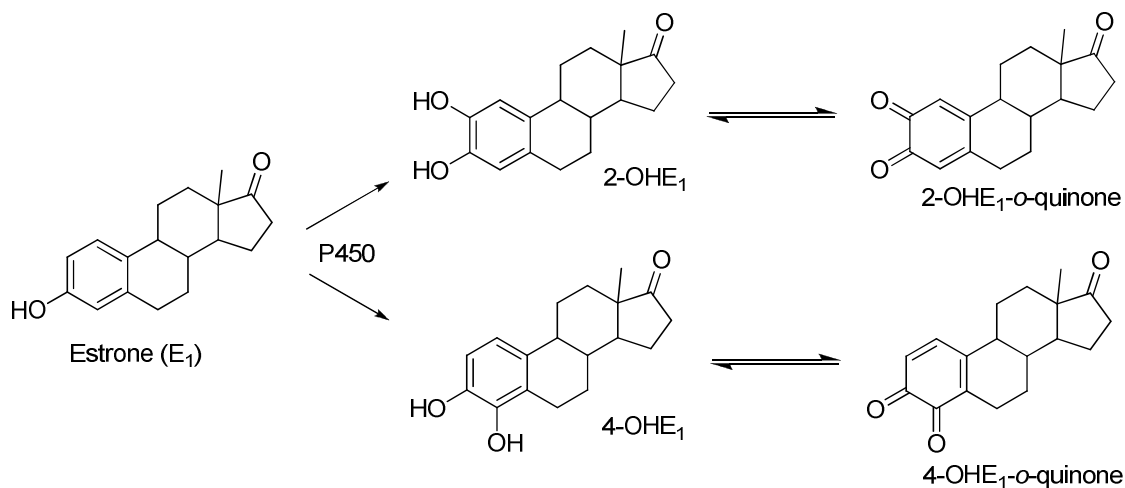
Figure 2. Metabolism of estrone to 2-/4-hydroxyestrone and o-quinones (45).

Studies suggested that high levels of 4-hydroxylation estrogen metabolites in estrogen targeted tissues played an important role in estrogen carcinogenesis (15). The ratio of 4-/2-hydroxyestradiol in neoplastic mammary tissue were elevated (46) and CYP1B1 was overexpressed in 92% of human breast cancer tissue specimens compared with normal tissue (47). Overexpression of CYP1B1 results in elevated level of 4-hydroxylated estrogens (37). Although 2- and 4-hydroxylated estrogens are mutagenic in human breast epithelial cells (48), 4-hydroxyestradiol is more carcinogenic than 2-hydroxyestradiol (49,50).

1.3.2 <u>Equine Estrogens</u>

Premarin is composed of at least 10 different estrogens, including estrone, estradiol, equilin (EQ), equilenin (EN) and their 17-beta-hydroxylated analogues, most of which are present as sulfate esters (51). It has been reported that hamsters treated for 9 months with estrone, equilin plus d-equilenin, or hydrolyzed Premarin exhibited 100% renal tumor incidences and abundant tumor foci (52). In the Postmenopausal Estrogen/Progestin Interventions (PEPI) trial, endometrial hyperplasia occurred at a daily dose of 0.625 mg of Premarin (53).

The major equine estrogens components in Premarin are EQ and EN (52). Similar to endogenous estrogens, both EQ and EN are metabolized by P450 aromatic hydroxylation to their phase I catechol metabolites (54,55). It has been shown that the ratio of 2-hydroxylation to 4-hydroxylation varies dramatically with estrogen substrates. In reference to estrone and 17β-estradiol, increased unsaturation in ring B shifts the metabolism of EQ and EN from the predominant 2-hydroxylation to 4-hydroxylation (55-57). EN is metabolized to 4-hydroxyequilenin (4-OHEN) and EQ is metabolized to 4-hydroxyequilin (4-OHEQ). Similar to 4-OHEN, 4-OHEQ also autoxidizes to an *o*-quinone which oxidizes to 4-OHEN-*o*-quinone (Figure 3). (55). This is significant because in Syrian hamster model, 4-hydroxyestrone is carcinogenic but not 2-hydroxyestrone (49,50).
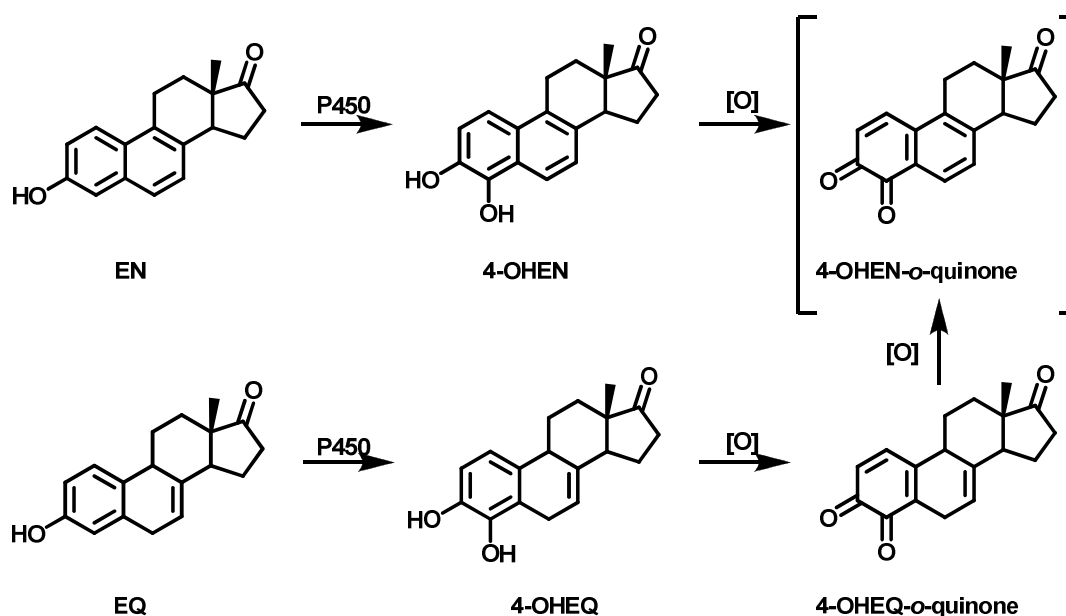
Figure 3. Phase I metabolism pathway of equine estrogens. EN and EQ are oxidized by P450 1B1 to genotoxic 4-OHEN-*o*-quinone.

Unlike endogenous catechol estrogens, 4-OHEN rapidly autoxidizes to the *o*-quinone without the aid of enzymes. In comparison with the stability of endogenous estrogen *o*-quinones, 4-OHEN-*o*-quinone is much more stable with a half-life of 2.3 h at pH 7.4 and 37 °C (45). Also, 4-OHEN-*o*-quinone is a potent redox active compound. Redox cycling of 4-OHEN-*o*-quinone resulted in formation of hydroxyl radicals, a potent oxidant which may induce damage in cells (58). In addition, 4-OHEN-*o*-quinone is a Michael receptor which will react with nucleophiles such as DNA bases and cause cytotoxic/genotoxic effects (55). It has been reported that 4-OHEN induces different kinds of DNA damage (59) and causes cellular transformation (60), and apoptosis (61).

1.4   <u>DNA Damage by Quinone Alkylation</u>

In cells, DNA carries the genetic information for all of the proteins that the organism will ever synthesize. DNA damage, the first step in carcinogenesis, can be caused by environmental alkylating carcinogens, ultraviolet (UV)-light, and ionizing radiation (IR) (62). Chemical carcinogens can cause the formation of carcinogen-DNA adduct, or induce oxidative damage, alterations to DNA ultrastructure which include DNA-strand crosslinking, DNA-strand breakage, or chromosomal rearrangements and deletions (63). Different repair mechanisms prevent DNA from procuring permanent damage. Impaired DNA repair may cause the accumulation of mutation in target genes and accelerate cell malignant transformation. The ability of cells to repair DNA determines the cellular susceptibility to endo- and exogenous substances, including carcinogens. Therefore, the cell's response to DNA damage and its ability to maintain genomic stability through DNA repair influences cancer initiation and progression (64).

For quinone-containing antitumor agents, such as doxorubicin and daunorubicin, there has been considerable evidence of their activity from free radicals generated by redox reactions of the quinone ring (65,66). Free radicals can produce cytotoxic effects by lipid peroxidation or induction of DNA strands break; however, the quinone moiety with alkylating activity also contribute to the cytotoxic effects (67). DNA-alkylating agents are either intrinsically active or, more typically, activated by enzymes to produce reactive intermediates. Cellular interaction between DNA and electrophilic small molecules can result in DNA structural damage due to covalent DNA adduct formation (68). These adducts

may cause mutation during DNA replication if not correctly repaired. DNA

adducts are potential biomarkers of cancer risks (69).

1.4.1 Endogenous Estrogen DNA Adducts

DNA adducts can be classified as stable or depurinating adducts.

Depurinating adducts are spontaneously released from DNA by breakage of the

glycosidic bond (70). Reaction of endogenous catechol estrogen quinones with

the nucleoside 2'-deoxyguanosine (dG) and adenine (Ade) resulted in formation

of 4-OHE$_1$(E$_2$)-1(α,β)-N7Gua and 4-OHE$_1$(E$_2$)-1(α,β)-N3Ade, respectively (41,71).

Both *in vitro* and *in vivo*, the main adducts, depurinating N7-guanine and N3-

adenine produced from 4-OHE$_2$-*o*-quinone, are products by 1,4-Michael addition

(39,43,72,73).

4-OHE$_1$(E$_2$)-1-N3Ade   4-OHE$_1$(E$_2$)-1-N7Gua

E$_1$: R, =O
E$_2$: R, -OH
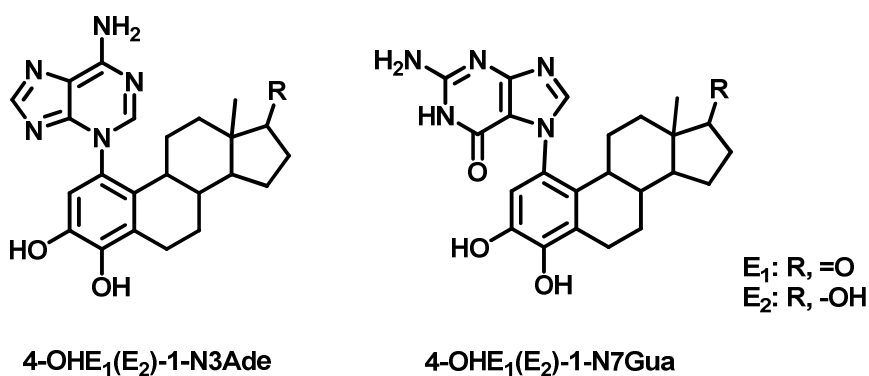
Figure 4. Structures of depurinating adducts of 4-OHE$_1$(E$_2$).

Stable adducts were also reported when $4\text{-OHE}_2\text{-}o$-quinone reacted with DNA; however, the amount is only 0.02% of the depurinating adducts (70). In contrast, $2\text{-OHE}_1(E_2)\text{-}o$-quinones act as their tautomers, $2\text{-OHE}_1(E_2)\text{-}o$-quinone methides, to form stable adducts via 1,6-Michael addition (73).

1.4.2 <u>Equine Estrogen DNA Adducts</u>

Unlike endogenous estrogen catechols which primarily form depurinating adducts, the 4-OHEN-o-quinone can react with DNA to form unusual cyclic adducts via semiquinone radical or o-quinone mechanism (45,74). The stable adducts classified as those which are covalently bonded to DNA without being excised or repaired.

The adducts were synthesized in aqueous solution by reacting 4-OHEN with four 2'-deoxynucleosides except reaction with 2'-deoxythymidine (74). There are actually three chiral centers for each adduct, which means eight stereoisomers, only four stereoisomer were detected in each of the adducts because of the high strain in a trans configuration (75). The structural characterization and stereochemistry properties of the adducts have been studied with LC-MS, NMR experiments and theoretically computational studies (75-78).
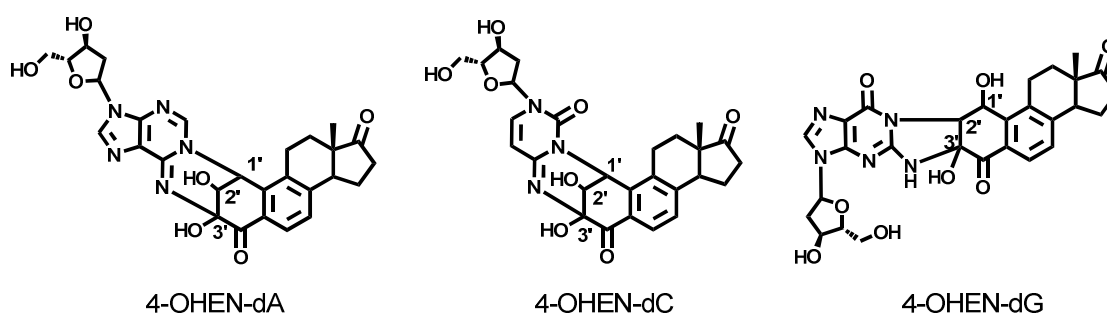
Figure 5. Structures of cyclic stable 4-OHEN-DNA adducts. The chiral centers are indicated in the structures.

In an animal study, stereoisomers of 4-OHEN-dA and 4-OHEN-dG were detected in the mammary tissues following direct injection of 4-OHEN (79). Mutagenic events were observed in the *sup*F gene carried on vector plasmids transfected into human fibroblast (80). Also, the cyclic 4-OHEN-DNA adducts have been detected from breast tumor tissues by the highly sensitive nano LC-MS/MS analysis in patients with a history of using of Premarin. Although the sample size was small, in four out of ten samples, stable 4-OHEN-dA, -dC and – dG adducts were detected for the first time (81). Therefore, these studies suggested that 4-OHEN was potentially carcinogenic through the mechanism of DNA adducts formation.

1.5   Review of Methods Developed for the Detection of 4-OHEN-DNA Adducts

The formation of DNA adducts by genotoxic carcinogens is one of the essential steps for the development of cancer (82). Some genotoxic carcinogens directly react with DNA but the majority requires metabolic activation to form electrophiles which covalently bind to nucleophilic sites of DNA (83). If not timely repaired, DNA sequence alteration may occur upon DNA replication. These adducts can be used as a biomarker for the risk assessment of human exposure to genotoxic carcinogens. Various analytical methods have been developed for the detection of DNA adducts and they vary in sensitivity, selectivity, and applicability for human studies (84)

1.5.1 ^{32}P-Postlabeling Assay

The ^{32}P-postlabeling assay has been in existence since 1980s for the detection of carcinogen-DNA adducts and other forms of DNA modification. It has been a widely applied technique with high sensitivity (0.1-1 adduct/10^9-10^{10} nucleotides) (85,86). The standard method includes the enzymatic digestion of DNA to nucleosides 3'-monophosphates, 5'-labelling of these nucleotides with an isotopically labeled phosphate group, and the resolution and detection of the labeled products (87). The enrichment procedures applied after digestion of DNA to nucleoside 3'-monophosphate normally include *n*-butanol extraction (88), nuclease P1digestion (89), immunoaffinity chromatography (90), or HPLC.

Even the sensitivity of ^{32}P-postlabeling assay is high, it depends on the specific activity of the isotope used, and the efficiency of adduct labeling. Since in many cases adduct labeling efficiency is not quantitative, one of the disadvantages is that the procedure is not a highly accurate quantitative technique (91). In the initial developed method, thin-layer chromatography (TLC) on polyethylenimine (PEI)-cellulose is generally used for the separation of ^{32}P-labeled adducts using several different buffers. This technique is simple, sensitive, and does not require costly equipment. Also, it provides the advantage of using multi-direction chromatography and avoids cross-contamination.

However, the disadvantages are that it is a slow and labor-intensive procedure, and has limited resolving power. Only one labeled sample can be analyzed on one plate, and several buffer conditions are needed for separation. Also, the difference between home-made TLC plates and those commercially prepared will affect results (85).

Terashima and coworkers developed a ^{32}P-postlabeling analysis coupled with nondenaturing 30% polyacrylamide gel electrophoresis (^{32}P-postlabeling/PAGE) based on their observation that oligonucleotides containing a single DNA adduct migrated slower than the unmodified oligonucleotides (92). The detection limit of this method for 5 µg of DNA is about 7 adducts/10^9 nucleotides. For example, in a reaction of calf thymus DNA and 4-OHEN, dC and dA adducts were detected with minor unknown products. However, although this technique is more convenient and less time-consuming than ^{32}P-

postlabeling/TLC method, the major weakness of ^{32}P-postlabeling is the lack of specific qualitative information regarding identity of the adducts (93).

1.5.2 <u>Immunoassay</u>

The fact that antibodies can be generated against the normal nucleosides suggested that antibodies against modified nucleosides could be employed for DNA adducts detection (94). It has been reported that the usage of specific antibodies in immunoassay can improve DNA adducts detection. Since the immunoassays do not require DNA hydrolysis, one of the advantages is that immunoassays are rapid (95,96). Additional advantages which make immunoassays ideal for adducts detection are the ease of use, low cost, and high throughput capability (97).

Both polyclonal and monoclonal antibodies have been developed against carcinogen-DNA adducts. Development of polyclonal antibodies is quicker since only sera collection is needed after immunization of animals several times over the 2-3 months. For monoclonal antibodies, it may take an additional 3-4 months to process, but the major advantage is the unlimited supply available by growing *in vivo* or *in vitro* (97). However, the disadvantage is cross-reactivity to other structurally similar adducts since cross-reactivity has been observed with both polyclonal and monoclonal antibodies (98,99).

Competitive ELISAs has been a widely used immunological method for DNA damage quantification and the sensitivity is relatively high (ca. 1 adduct/10^8-

10^9 nucleotides). Also, a standard curve can be generated for quantification. Okahashi and coworkers developed specific monoclonal antibodies for 4-OHEN-DNA adducts, with the detection limit close to 5 adducts/10^8 nucleotides in 1 µg DNA (100). This method is advantageous because expensive equipment and radiolabeled compounds are not needed for the immunoassays.

Furthermore, immunohistochemical detection of DNA adducts can be performed on either fixed cells or tissue sections providing qualitative and semiquantitative data. The major advantage of immunohistochemical method is the ability to detect DNA adducts in specific cell types within a tissue and the applicability to small amounts of sample. Less than 1 ml of blood is required for analysis of lymphocytes compared with the 30 ml required to isolate sufficient DNA for ELISA. The disadvantages of immunohistochemical methods are like those of immunoassays. Cross-reactivity of antibodies may result in errors in quantification and the sensitivity depends on the characteristic of antibodies used (97).

1.5.3 Liquid Chromatography-Mass Spectrometry

The earlier role of mass spectrometry was limited to providing analysis of newly identified DNA adducts, or elucidating structures of DNA adduct standards used by other detection methods (93). With the development of liquid chromatography (LC) coupled to mass spectrometry (MS) and advances in

ionization methods, the advantages of MS has increased and LC-MS is now commonly used for the quantification of DNA adducts (101,102).

Before LC-MS became the main method for DNA adducts detection, gas chromatography-mass spectrometry (GC-MS) with electron ionization or chemical ionization is the predominantly used method (103). GC-MS has been extensively applied for determination of protein adducts and has the advantage of greater peak resolution when compared with LC-MS (104). However, the limitation of GC-MS is that only non-polar and volatile compounds can be analyzed, yet the majority of DNA adducts are polar and/or non-volatile molecules which require derivatization before GC-MS analysis. Therefore, with the advent of electrospray ionization (ESI) source, direct analysis of polar molecules without derivatization can be carried out with direct coupling of LC to MS (82).

Compared to other methods, the major advantage of MS is that it gives structural identification and allows extreme accurate quantification. The stable isotope internal standards which have an identical chromatographical profile as the DNA adducts of interest but different mass which can only be differentiated by MS. This allows quantification and structure confirmation of the product ions from unlabelled DNA adducts (105). Also, unlike ^{32}P-postlabeling sensitivity improvement which has been limited over recent years, the selectivity and sensitivity continued to increase in light of technological advances that improved efficiency of ionization, transmission, and ion detection.

The triple quadrupole is the most common instrument design for adducts analysis and this configuration allows for tandem MS with multiple reaction monitoring (MRM) or constant neutral loss (CNL), leading to greater sensitivity. Therefore, the amount of DNA needed for analysis decreased to the level applicable to human sample studies (91). The typical detection limits of LC-ESI-MS for various DNA adducts range from 0.5-5 adducts/10^8 nucleotides, which is less sensitive compared with 0.1-1 adduct/10^9 nucleotides with ^{32}P-postlabeling for some adducts (91,105).

In the sample preparation procedure for LC-MS, the problem that high levels of unmodified nucleoside interfere with analysis was also encountered as in ^{32}P-postlabelling method. Fortunately, with the innovation of column switching, direct analysis of digested DNA sample without prior purification is permissible (106,107). Comparison has been made between the results obtained from LC-MS and ^{32}P-postlabeling. Soglia and coworkers demonstrated that there exists a good correlation between data acquired from LC-MS and ^{32}P-postlabeling, but the levels of adducts determined by ^{32}P-postlabeling was lower (108). LC-MS/MS has numerous advantages and its role in DNA adducts detection is increasingly more important.

1.6 **Alzheimer's Disease and Risk Factors**

Dementia is the disease occurring progressively with the increase in age, especially over 65 years old. Among the different forms of dementia, Alzheimer's disease (AD) is the most common (109). AD is a neurodegenerative disorder causing disruption in mental function such as memory loss and difficulty in visual and spatial recognition. In the prospective analysis from the Canadian Study of Health and Aging, about two thirds of the cases of dementia are AD (110). Extracellular deposition of amyloid plaques and presence of neurofibrillary tangles in the brain are the neuropathological hallmarks of AD (111). The characteristically morphologic change in patients is a reduction in cerebral cortex and hippocampal regions (112).

Among the factors increasing risks of AD such as advanced age, gender, and lower education level, age has been considered to be the main contributor (110,113). In addition, genetic factors are also involved (114). The apolipoprotein E (APOE) ε4 allele is reported to be a risk factor for Alzheimer's disease and associated with earlier age of sporadic AD onset, increased amyloid plaques count and neurofibrillary tangles (115,116), and cholinergic dysfunction in hippocampal and frontal cortex region (117,118). In familial Alzheimer's disease (FAD), mutations in genes including amyloid precursor protein gene (*APP*), *presenilin-1* gene (*PSEN1*) and *presenilin-2* gene (*PSEN2*), have been identified to cause early onset (119,120).

1.7   Relationship between Estrogen and Alzheimer's Disease

Estrogens are known to affect the hypothalamus and brain areas related to ovulation and reproduction. In clinical studies and animal models, estrogens also exert actions in brain areas controlling functions other than reproduction, such as midbrain and brain stem neurons producing serotonin and catecholamines, spinal cord, cerebral cortex, and hippocampus. The estrogens acting on these areas play an important role in memory, learning, emotion, motor coordination, and pain sensitivity (121). The intracellular mechanisms of action include the pathways via classical nuclear estrogen receptors and non-classical receptors at the plasma membrane, in mitochondria, and in the cytoplasm (122,123). Generally, estrogens directly promote cell survival and synaptic plasticity, prevent axonal and dendritic pruning, modulate neurotransmitter receptor function, and enhance neurogenesis. Through these mechanisms, estrogen exerts neuroprotective ability (124).

The evidence of estrogens in neuroprotection can be observed both *in vitro* and *in vivo*. For example, estradiol is the ingredient added to serum-free media for neuron cell cultures (125). Addition of estradiol increases the viability, survival, and differentiation of primary cultures from hypothalamic neurons and hippocampal neurons (126,127). In ovariectomized rat models, estrogen replacement therapy can reverse the estrogen deprivation-induced impairment of learning and memory performance (128,129). Estrogens improve two different types of memory processes depending on striatal (non-spatial navigation) and hippocampal (spatial) memory systems (130).

In clinical studies, in addition to the age-dependency of AD, it is reported from a meta-analysis that women are at higher risk for AD than men (131). Increasing evidence showed that in postmenopausal women who take hormone replacement therapy, improved cognition function is observed, and the likelihood of suffering from AD is significantly less than women without HRT (132-134).

1.8   **Aromatase Inhibitor**

In premenopausal women, the ovaries are the main source of estradiol circulating in the body to act on target tissues. However, in postmenopausal women, whose ovarian estrogen secretion ceases, and in men, aromatase is the enzyme converting testosterone to estradiol in peripheral tissues and throughout the brain (135,136). Aromatase, belonging to a cytochrome P450 superfamily, CYP19, catalyzes the local conversion of C_{19} testosterone to estradiol (137). This pathway becomes the main process of estrogen synthesis and can generate high levels of estradiol locally but not significantly affect circulating estrogen levels (138).
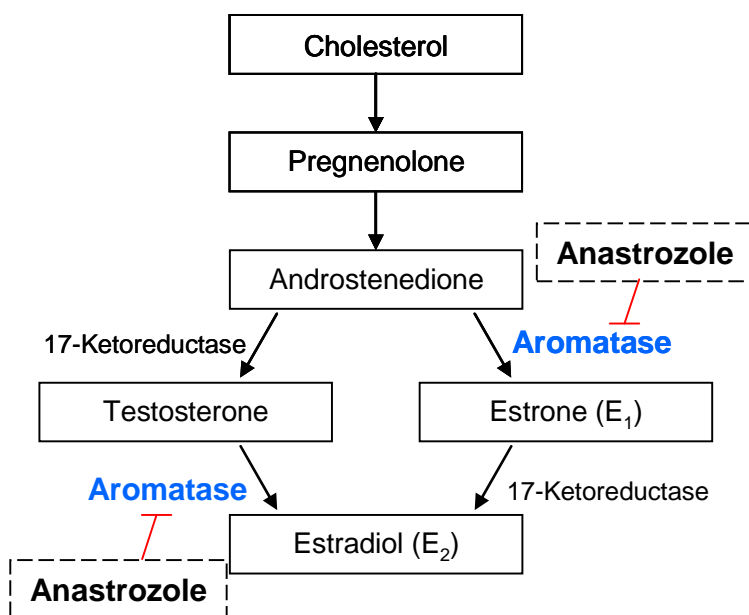
Figure 6. Biosynthesis of estradiol, including associated enzymes.

Aromatase inhibitors can be classified to four generations by the chronology of clinical trials, but in fact all the inhibitors are more easily subdivided chemically into steroidal and non-steroidal compounds. The structures of steroidal inhibitors are close to androstenedione and these substrate analogues, which are more hydrophobic than others, possess the best affinity for the enzyme (139). A series of steroidal inhibitors were evaluated systematically by Schwarzel and coworkers (140). 4-OHA, which acts as a suicide inhibitor binding irreversibly to enzyme active site, showed a high degree of selectivity for aromatase (141). In the study conducted by Brodie and coworkers, 4-OHA is the most potent suicide inhibitor they have evaluated, and the most effective and selective for treating breast cancer (142 ,143).
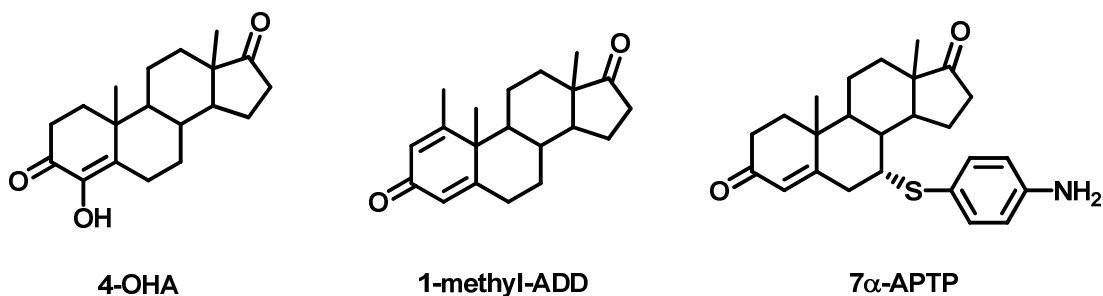
Figure 7. Structures of steroidal aromatase inhibitors. 4-OHA, 4-Hydroxyandrost-4-ene-3,17-dione; 1-methyl-ADD, 1-Methylandrosta-1,4-diene-3,17-dione; 7α-APTP, 7α-(4'-aminophenyl)thioandrost-4-ene-3,17-dione.

The other class is non-steroidal inhibitors, which mostly consists of imidazole and triazole derivatives. These compounds, working as competitive enzyme inhibitors, possess a common chemical feature of heteroatom coordinately binding to the heme iron of the cytochrome P450s, thus interfere with steroid hydroxylation (144). Aminoglutethimide is the first non-steroidal aromatase inhibitor developed. However, the more recently discovered letrozole and anastrozole have higher specificity for aromatase for the treatment of advanced breast cancer in postmenopausal women (145). Both letrozole and anastrozole are more potent with the IC_{50} of 11.5 and 15 nM respectively, in human placental microsomes (146,147).

The aromatase activities can be detected in breast tumor and normal breast tissues, and the expressions are higher in or near breast tumor sites (148,149). Therefore in breast cancer, inhibition of aromatase is an important approach for reducing estrogen growth-stimulatory effects in estrogen-dependent breast cancer (144).
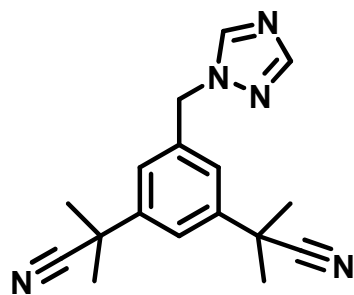
Anastrozole
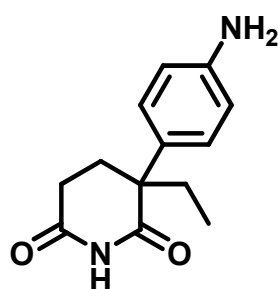
Aminoglutethimide
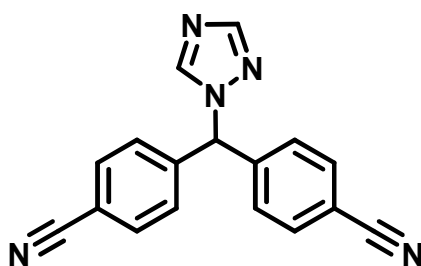
Letrozole
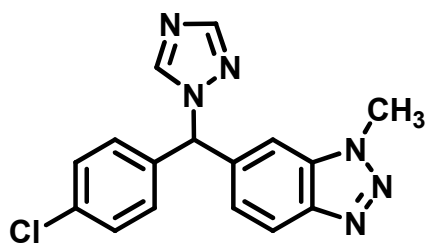
Vorozole

Figure 8. Structure of non-steroidal aromatase inhibitors.

1.9   <u>Specific Aims for this Study</u>

I.

Formation of estrogen-DNA adducts could be potential biomarkers for cancer risks assessment. Development and elaboration of sensitive LC-MS/MS was perform for the identification and quantification of adducts. Efficiency of sample preparation was also evaluated. In combination of sample preparation efficiency and analytical sensitivity, the correlation between adduct frequency and DNA required to achieve limit of detection can be summarized. The final goal of this study was to apply the analysis technique for the detection of 4-OHEN-DNA adducts formed in cells.

II.

Clinical observation found that use of HRT in postmenopausal women is associated with reduced risk of AD. In patients with early-stage breast cancer, reduced hippocampal-dependent tasks was observed in patients receiving anastrozole treatment. The pathology of AD could be changed by manipulating circulating and local brain estrogens. We hypothesized that the aromatase inhibitor anastrozole, an analogue of letrozole, can penetrate blood brain barrier after long-term oral administration. Inhibition of brain estrogen synthesis by aromatase inhibitor will result in alteration on AD pathology.

CHAPTER 2 MATERIALS AND METHODS

2.1   <u>Materials</u>

Caution: *The catechol estrogens are potentially hazardous carcinogen which should be handled with care in accordance with NIH guidelines for the laboratory use of chemical carcinogens.* All chemicals were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Itasca, IL) unless stated otherwise. 4-OHEN was synthesized by treating equlin with Fremy's salt as described previously with minor modification (51). 4-OHE was purchased from Steraloids (Newport, RI). Phosphodiesterase I (VPH) was from Worthington (Lakewood, NJ).

Stable adducts 4-OHEN-2'-deoxyadenosine, 4-OHEN-2'-deoxycytosine, and 4-OHEN-2'-deoxyguanosine were synthesized by incubating 4-OHEN with 2'-deoxyadenosine (dA), 2'-deoxycytosine (dC), and 2'-deoxyguanosine (dG), respectively (74). Four adducts were found with dG and two major and two minor adducts were found with dA and dC. Purification of adducts was carried out by Perkin Elmer (Shelton, CT) preparative HPLC system with series 200 autosampler. Liquid chromatography was carried out on a Kromasil 100-5C18 (20 x 250 mm) column (Technikrom, Evanston, IL) connected to Perkin Elmer 785A UV/VIS detector at 280 nm. The elution solvents were methanol with 0.1% formic acid as solvent C, and water with 10% methanol and 0.1% formic acid as solvent D. The gradient, with 6 mL/min flow rate, started from 10% solvent C for 5 min, increased to 60% over 15 min, and then increased to 90% over 20 min. After holding at 90% for 10 min, solvent C was back to 10 min over 5 min. The

compounds were dried and weighted to make known concentration stock solutions. Anastrozole was from AstraZeneca (London, UK) and verapamil was from US Pharmacopeia (Rockville, MD).

2.2   Cell Culture

All cell culture reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise stated. MCF-7 WS8 human breast cancer cells (a kind gift from Dr. V.C. Jordan, Fox Chase Cancer, Philadelphia, PA) were maintained in RPMI 1640 containing 10% (v/v) fetal bovine serum (Atlanta Laboratory, Atlanta, GA), 1% GlutaMAX^{TM}, 0.1 mM non-essential amino acids, and 6 µg/mL bovine insulin. Estrogen-free medium for MCF-7 WS8 cells were prepared by supplementing charchaol-dextran-treated fetal bovine serum to phenol read-free RPMI 1640, whereas other components remained the same. Cells were plated in 10-cm dishes for 24 h and changed to phenol red-free media 72 h prior to treatment for adduct formation.

2.3   Formation of 4-OHEN-DNA Adducts in Calf Thymus DNA

Calf-thymus DNA was dissolved in molecular biology grade water containing 10 mM Tris and 1 mM EDTA and subject to gently inversion overnight. Concentration of DNA solution was determined by measuring the absorbance at 260 nm and one absorbance unit corresponds to approximately 50 µg/mL of DNA.

4-OHEN powder was dissolved in DMSO freshly before experiment every time.

4-OHEN was added to DNA solution and incubated in 37 ^{o}C for 1 hour.

2.4 Extraction of DNA

Cellular DNA extraction was performed as previously described (154) with minor modification. Cells cultured in 10 cm dish were collected by cell scraper. The cell pellets were homogenized with lysis buffer (320 mM sucrose, 10 mM Tris, pH 7.4, 5mM MgCl2, 10 mM Triton X-100, and 50 mM mannitol). After centrifuge, the nuclei pellets were suspended in solution buffer (1% SDS, 1 mM EDTA, 10 mM Tris, pH 7.4, and 0.45 M NaCl) supplied with RNase T1 and RNasa A. After 30 min incubation at 37 ^{o}C, proteinase K was added and the solution was incubated further for 30 min at 37 ^{o}C. Then NaCl and Tris were added to adjust the concentrations to 0.62 M and 20mM, respectively. An equal volume of n-butanol was added and vortexed thoroughly before sending the mixture to centrifuge. After centrifugation (4,000 rpm, 15 min), the bottom aqueous layer was collected. Equal volume of isopropanol was added for DNA precipitation which is then washed twice with 70% ethanol. Calf thymus DNA extraction was performed with addition of an equal volume of n-butanol followed by the same procedure described above.

2.5   Hydrolysis of DNA for Adduct Detection

The DNA was then dissolved in 25 mM ammonium acetate buffer (pH 5.3) containing 0.1 mM $ZnCl_2$ and determined concentration at 260 nm. Per 100 µg of DNA was hydrolyzed with 10 units nuclease P1 (NP1, from *Penicillium citrinum*) for 4 hours at 55 ^{o}C and then further incubated with 15 units alkaline phosphates (ALP, from calf intestine) and 0.5 units phosphodiesterase I (VPH, from *Crotalus admanteus* Venom) for 4 hours at 37 ^{o}C in a reaction buffer containing 0.5 mM $MgCl_2$, with 0.1 M diethanolamine to achieve final pH ~ 9.8. After hydrolysis is completed, the mixture was centrifuged for 25 min at 13,000 g using Amicon Ultra 10K centrifugal filters (Millipore). An aliquot was sent to LC-MS/MS analysis.

2.6   Determination of Extraction Efficiency

In both sets of experiment, 4 dishes of MCF-7 cells were propagated for each set without 4-OHEN treatment. After the cells cultured in dishes were collected, in the first set 50µl of the calf thymus DNA already reacted with 4-OHEN was added. The same cellular DNA extraction procedure was performed and DNA was precipitated with isopropanol. In the second set, followed by the steps of DNA extraction of cultured cells and DNA precipitation with isopropanol, 50µl of the calf thymus DNA reacted with 4-OHEN was added. Then hydrolysis was performed on both samples and an aliquot from each sample was subject to LC-MS/MS analysis. Efficiency of the whole procedure is determined by comparing the amounts of adducts from both set, with the second set as control.

2.7   Determination of Hydrolysis Efficiency

For the measurement of hydrolysis procedure, dG was used as the marker. All the samples were analyzed on API3000 with electrospray positive mode, and an Xbridge Phenyl (3.5 µm; 2.1 mm × 150 mm) column was used. Multiple reaction monitoring (MRM) of 268 → 152 was set to detect dG. Stock solution of dG was dissolved in water:methanol (50:50, v/v). Calibration curve of dG was prepared at concentrations of 0.5, 1, 10, 100, 250, 500, 1000 nM. After 290 µg of calf thymus DNA were digested, actual amount of dG was calculated based on the dG calibration curve generated. The hydrolysis efficiency was determined as the ratio of dG experimental value to theoretical value.

2.8   Analysis of Stable 4-OHEN-DNA Adducts by LC-MS/MS

Measurement of 4-OHEN-2'-deoxynucleoside adducts was carried out by using API 3000 (Applied Biosystem, Foster City, CA) triple quadrupole mass spectrometer connected to Agilent 1200 HPLC (Agilent Technologies). The mass spectrometer ion source was electrospray ionization with positive mode. Liquid chromatography was performed on an Xbridge Pheny (3.5 µm; 2.1 mm × 150 mm) column (Waters Corporation, Milford, MA) at room temperature. The mobile phase consisted of water with 10% methanol and 0.1% formic acid as solvent A and methanol with 0.1% formic acid as solvent B. For the analysis of these adducts, a linear gradient starting from 30% solvent B to 90% over 23 min, and then returning to 30% over 3 min was employed. Followed was a reequilibration

held at 30% B for 11 min prior to the next sample injection. The flow rate was 0.2

mL/min. Tuning of each parameter was performed with synthesized standards.

The nebulizer gas was 12, the curtain gas was 7, the collision gas was 4, the

ionspray voltage was 4500 V, and the gas temperature was set at 400 ^{o}C. For 4-

OHEN-dC and 4-OHEN-dG adduct, the declustering potential was 51 V, and the

focousing potential was 220 V. For 4-OHEN-dA adduct, the declustering potential

was 71 V and the focousing potential was 310 V. Collision energy for 4-OHEN-

dC, 4-OHEN-dA, and 4-OHEN-dG was 23, 35, 21 V, respectively. Multiple

reaction monitoring (MRM) channels of 525 $\rightarrow$ 408, 548 $\rightarrow$ 432, and 564 $\rightarrow$ 448

were set to detect 4-OHEN-dC, 4-OHEN-dA, and 4-OHEN-dG, respectively.

2.9   Calibration Curve and Quality Control of Anastrozole

Stock solutions of anastrozole and verapamil were dissolved in

acetonitrile:water (50:50, v/v) at concentration of 1 mg/mL and stored in -20 ^{o}C.

The 50 ng/mL verapamil working standard and various concentrations of

anastrozole working standards were prepared by dilutions of the stocks using

acetonitrile:water (50:50, v/v). Calibration curves of anastrozole were prepared in

duplicate by spiking blank brain tissue with verapamil (10 µL; 50 ng/mL) and

anastrozole (10 µl) at concentrations of 0.625, 1.25, 2.5, 5, 10, 20 ng/mL. The

same brain extraction procedure as described above was performed. Quality

control samples were prepared using blank brain at concentrations of 1.8 and 3.6

ng/mL. Final concentrations were ten-fold more dilute.

2.10   <u>**Brain Tissue Anastrozole Extraction**</u>

Brain anastrozole level was quantified with the aid of liquid chromatography-tandem mass spectrometry (LC-MS/MS). LC-MS/MS was performed in duplicate runs per sample. Solvent-resistant pipette tips with charcoal filters (Molecular BioProducts, San Diego CA) were used for all procedures involving solvents. Briefly, ice cold HPLC-grade methanol was added to duplicate brain samples according to the ratio of 1 mL methanol per 100 mg brain tissue. Samples were spiked with 50 ng/mL internal standard (10 µl), ground twice, and centrifuged under 4 ^{o}C for 15 minutes at 14,000 rpm (Eppendorf 5810R; Hamburg, Germany). The supernatant fraction was transferred to a clean 1.5 mL eppendorf tube and evaporated to dryness under nitrogen stream. The dried samples were reconstituted with 100 µl of HPLC-grade acetonitrile:water (50:50, v/v) containing 0.1% formic acid, vortexed for 10s, and centrifuged (14,000 rpm). The clear supernatant was transferred to a sample vial (Waters; Milford, MA) and subjected to LC-MS/MS analysis. Brain anastrozole levels were corrected for the variation in brain mass. Brain weight was converted into mL brain tissue using the specific gravity for mouse brain (1.05) (Nelson et al., 1971) and the density of water (1 mg/mL).

2.11   <u>**Analysis of Anastrozole Concentration in Brain Tissue by LC-MS/M**</u>S

A Waters Alliance 2695 Separation Module was used to resolve each brain extract. Liquid chromatography was carried out on an Xbridge C18 (3.5 µm;

2.1 mm x 100 mm) column (Waters Corporation, Milford, MA) at room temperature. The mobile phase consisted of water with 0.1% formic acid as solvent A and acetonitrile with 0.1% formic acid as solvent B. The compounds were eluted with 70% isocratic solvent B, and the flow rate was 0.2 mL/min. An aliquot (10 µl) of each sample was injected onto the column. Total run time for each sample was 8 min.

Mass spectrometric analysis was performed using a Thermo-Finnigan TSQ Quantum triple quardrupole mass spectrometer (West Palm Beach, FL, USA) equipped with an electrospray (ESI) source operating in positive mode. Multiple reaction monitoring (MRM) was performed for transitions $294 \rightarrow 225$ and $455 \rightarrow 165$ for anastrozole and the internal standard, respectively. The source temperature was set at 300 ^{o}C and the ESI voltage set to 3.5 kV. Collision energy was set to 19 eV and 28 eV for internal standard and anastrozole, respectively. Scan width was 2.0 atomic mass units and scan time was 1.0 s for each compound.

CHAPTER 3 DNA Adducts Formed From Equine Estrogens: Method Development

Metabolic activation of estrogens to their hydroxylated metabolites by cytochrome P450 is one of the major pathways of estrogen carcinogenesis. Formation of *o*-quinone from the catechol estrogens can induce DNA damages through alkylation to DNA. Since unrepaired estrogen-DNA adducts can lead to cancer, 4-OHEN-DNA stable adducts could be used as a biomarker to evaluate the risk of breast cancer correlated with the use of HRT. Methods such as ^{32}P-postlabeling/polyacrylamide gel electrophoresis analysis (^{32}P-postlabeling/PAGE analysis) and immunoassay have been applied for the detection of adducts (80,100). However, despite the advantages of highly sensitive ^{32}P-postlabeling/PAGE analysis posses, it lacks the ability to give appropriate structural information. Immunoassay has advantages such as low cost, relatively high sensitivity and is easy to use, but it is unable to give structural information and its high sensitivity is dependent on the antibody characteristics (91). LC-MS/MS provides characters of high sensitivity as well as structural elucidation. For *in vivo* studies, amount of sample is limited and DNA adducts of interest are formed in low quantities. Therefore, development of sensitive technique with optimized procedure is important.

Results and Discussion

3.1 <u>Synthesis and Characterization of 4-OHEN-DNA Adducts Standards</u>

To use LC-MS/MS as an approach for the detection of 4-OHEN-DNA stable adducts, synthetic standards are required. The stable adducts standards were prepared by the reaction of 4-OHEN with dA, dC, and dG, respectively. The products were separated from unreacted 2'-deoxynucleosides via preparative HPLC as shown in Figure 9. Identities of the adducts were confirmed by tandem mass spectrometry with the *m/z* of $[M + H]^+$ ions (Figure 10.). For 4-OHEN-dA as the ion with m/z 548 $[M + H]^+$, loss of deoxyribose gives product ion at m/z 432, and further loss of one molecule of H_2O gives product ion at m/z 414. The product ion at m/z 136 represents adenine. For 4-OHEN-dC as the ion with m/z 524 $[M + H]^+$, loss of deoxyribose gives product ion at m/z 408, and further loss of one molecule of H_2O gives product ion at m/z 390. The product ion at m/z 112 represents cytosine. Similarly, 4-OHEN-dG with the ion at m/z 564 $[M + H]^+$, loss of deoxyribose gives product ion at m/z 448, and further loss of one molecule of H_2O gives product ion at m/z 430. For MRM analysis, the transition is determined with precursor ion to most intensive product ion. Therefore, 548 → 432, 525 → 408, and 564 → 448 were set to detect 4-OHEN-dA, 4-OHEN-dC, and 4-OHEN-dG, respectively. The stable isotope labeled standard 4-OHEN-$[^{15}N_5]$dG was used as the internal standard and the corresponding transition for MRM was 569 → 453.
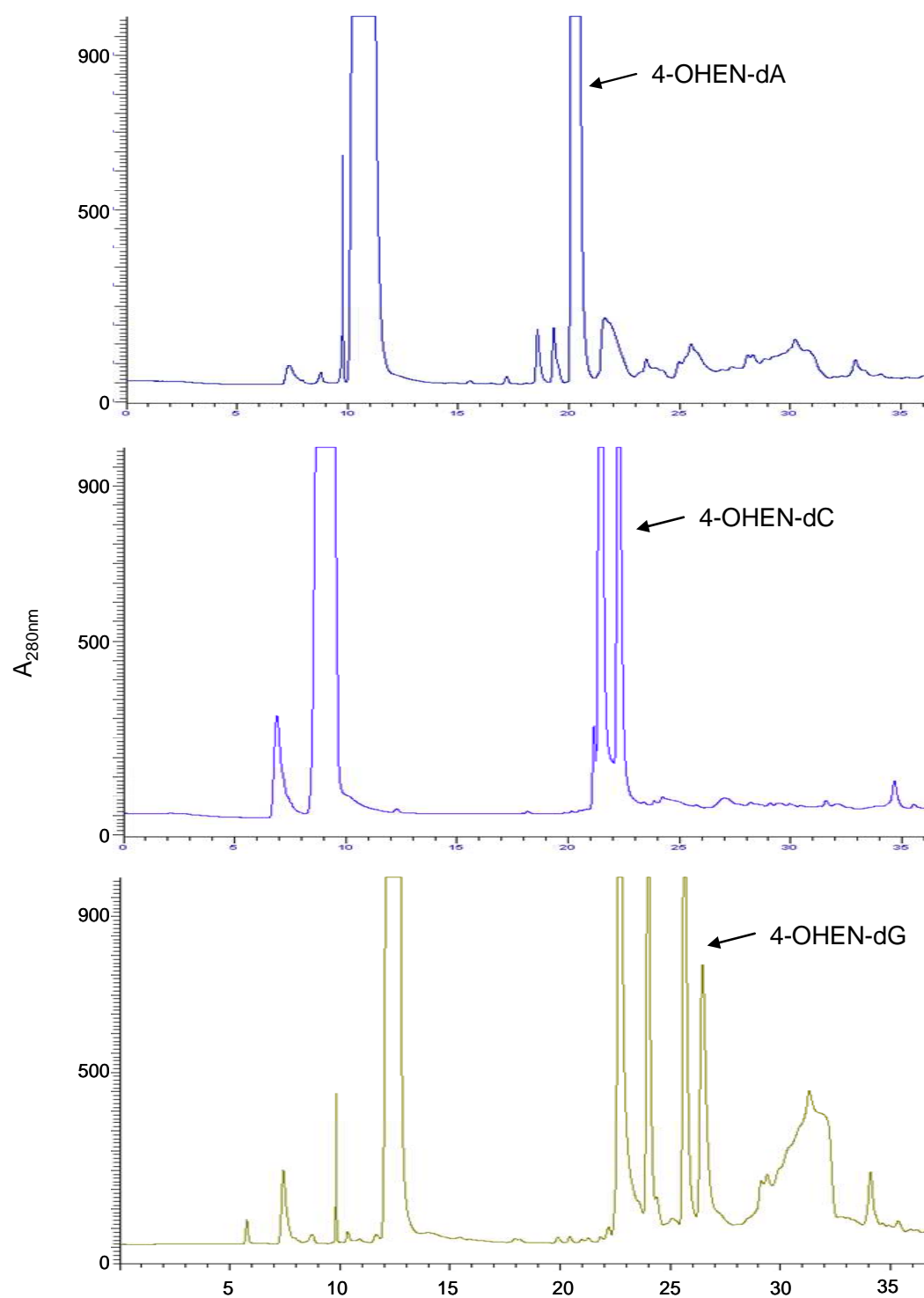
Figure 9. HPLC-UV chromatograms for separation of synthesized standards following the incubation of 4-OHEN and 2'-deoxynucleoside at 37 $^{\circ}$C overnight.

A) 4-OHEN-dA
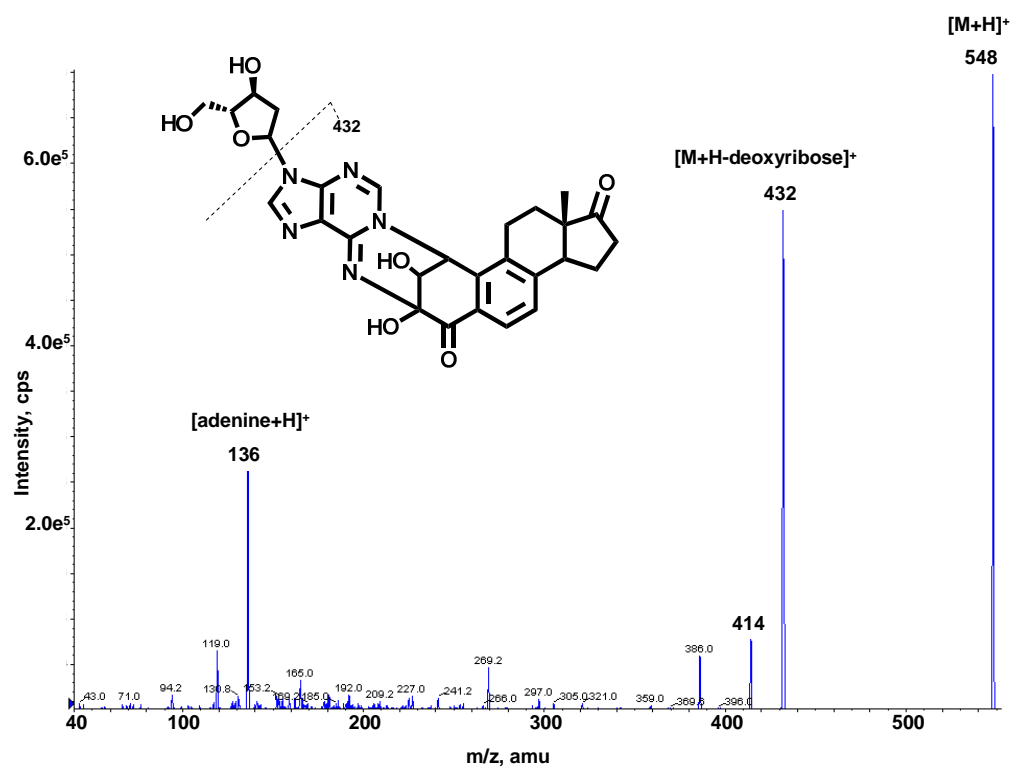
B) 4-OHEN-dC
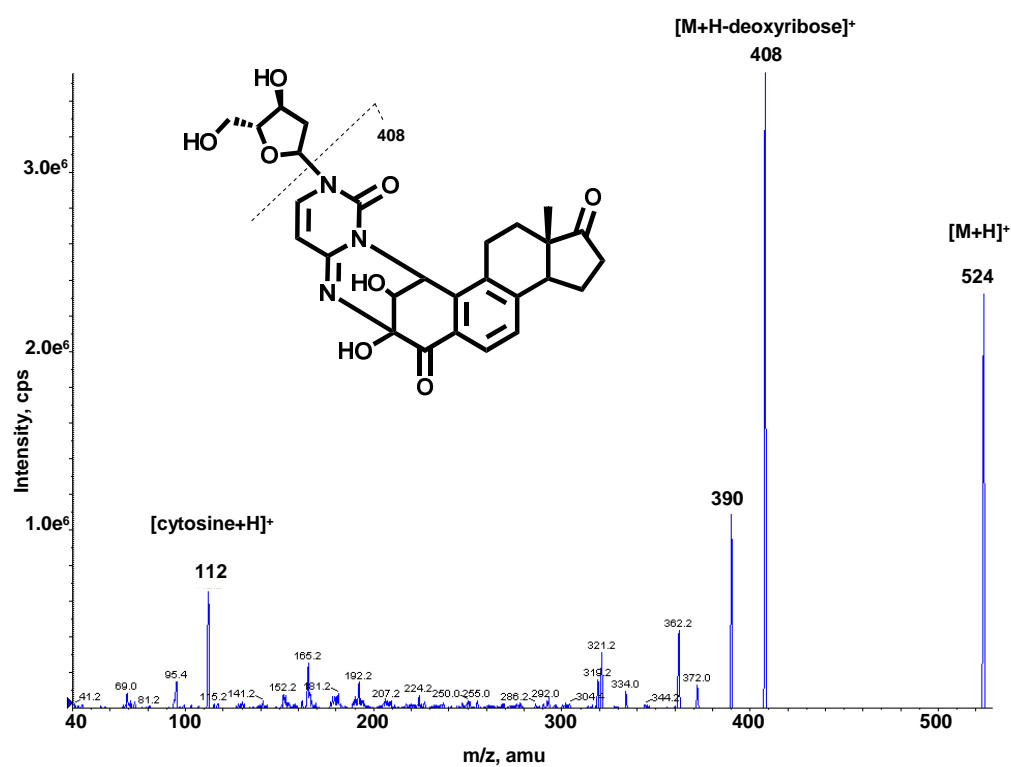
C) 4-OHEN-dG
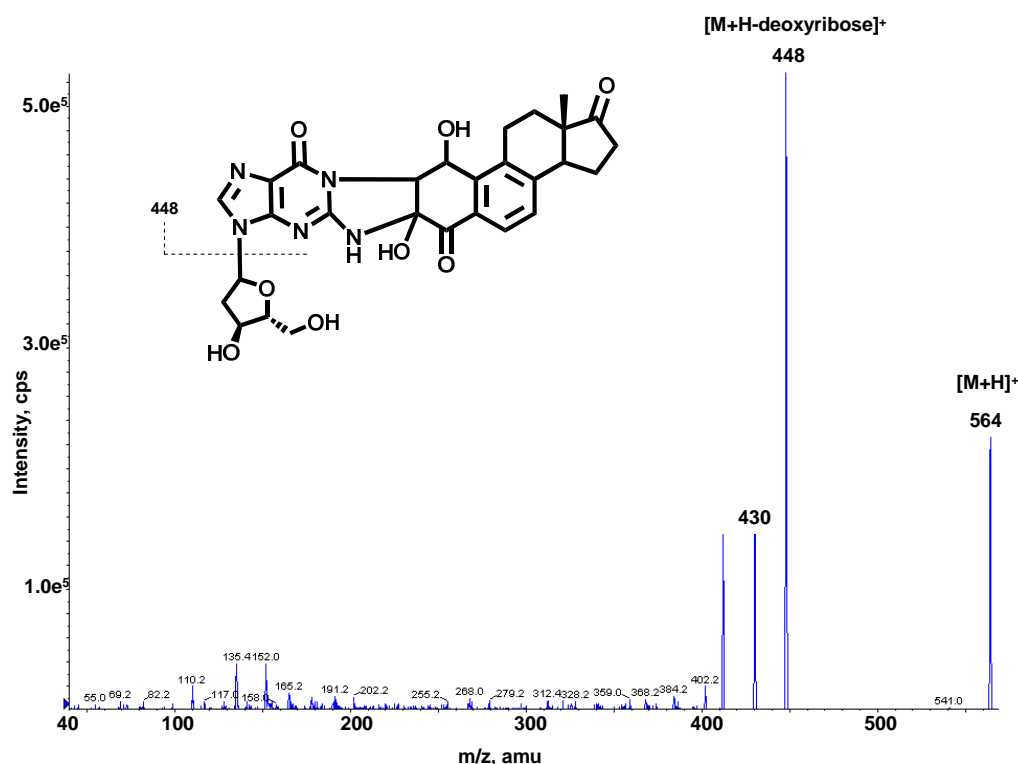
Figure 10. LC-MS/MS analysis of stable adducts.

3.2 Optimization of Liquid Chromatography System for Adducts Detection

Four combinations of mobile phases were evaluated for the best separation of adducts and least ion suppression for the detection of adducts by MS. First, water with 10% methanol and 0.1% formic acid as solvent A and methanol with 0.1% formic acid as solvent B; Second, water with 0.1% ammonium acetate (pH 6.8) as solvent A and methanol as solvent B; Third, water with 0.1% ammonium acetate (pH 4.8) as solvent A and methanol as solvent B.

The linear gradients were consistent across four solvent system combinations as described in methods.
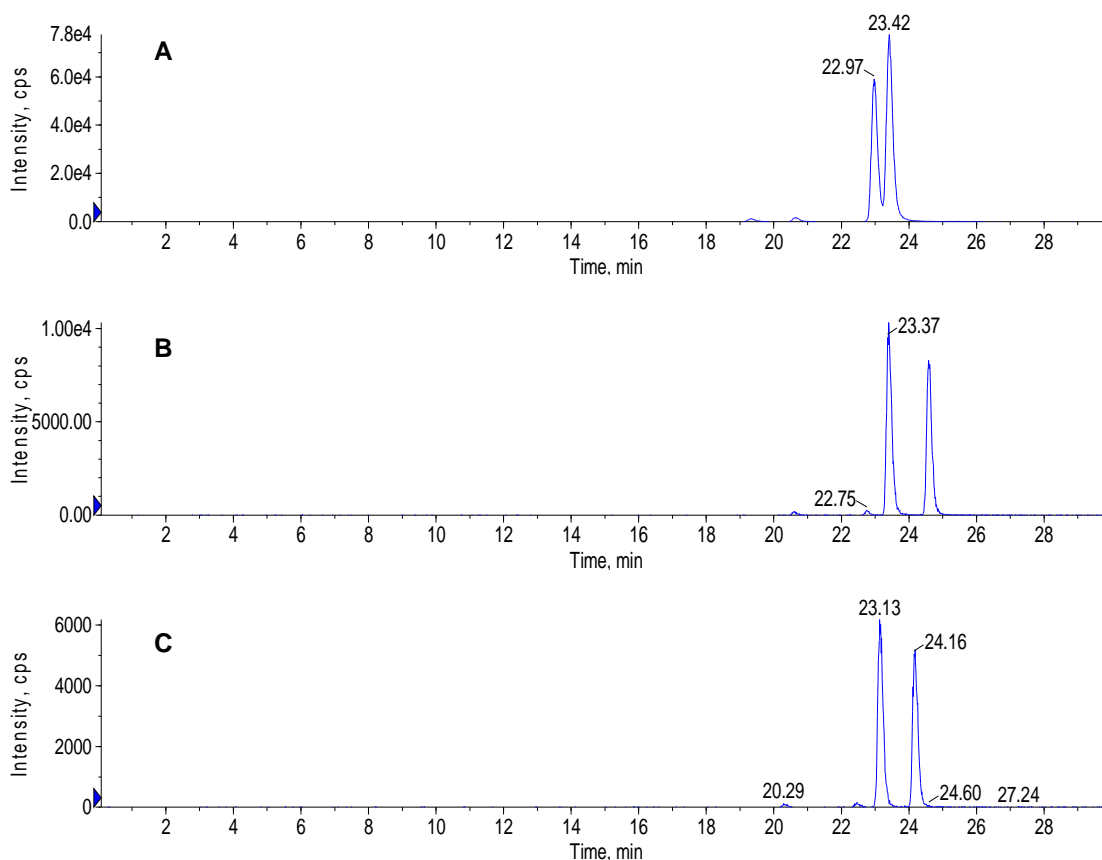
Figure 11. Chromatograms of 4-OHEN-dA in different mobile phases. A) water/methanol with 0.1% formic acid-methanol with 0.1% formic acid, B) water with 0.1% ammonium acetate (pH 6.8)-methanol, C) water with 0.1% ammonium acetate (pH 4.8)-methanol.
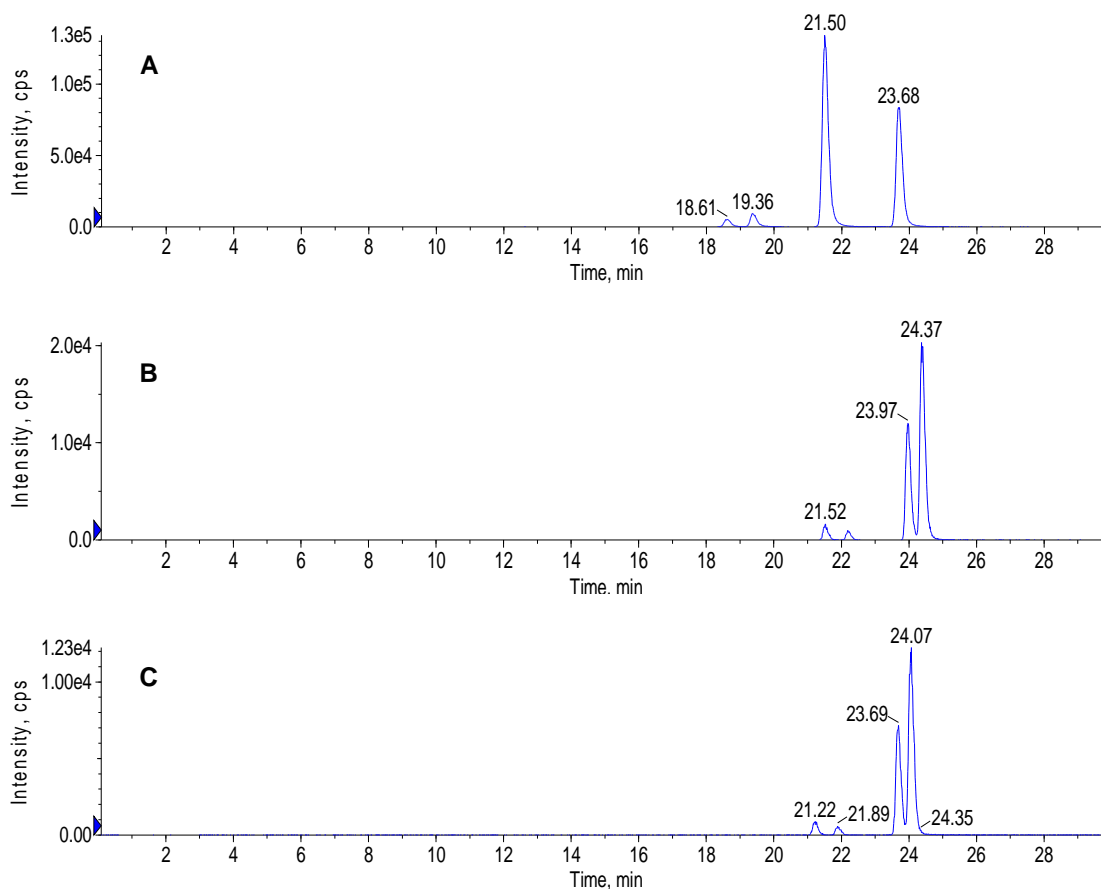
Figure 12. Chromatograms of 4-OHEN-dC in different mobile phases. A) water/methanol with 0.1% formic acid-methanol with 0.1% formic acid, B) water with 0.1% ammonium acetate (pH 6.8)-methanol, C) water with 0.1% ammonium acetate (pH 4.8)-methanol.
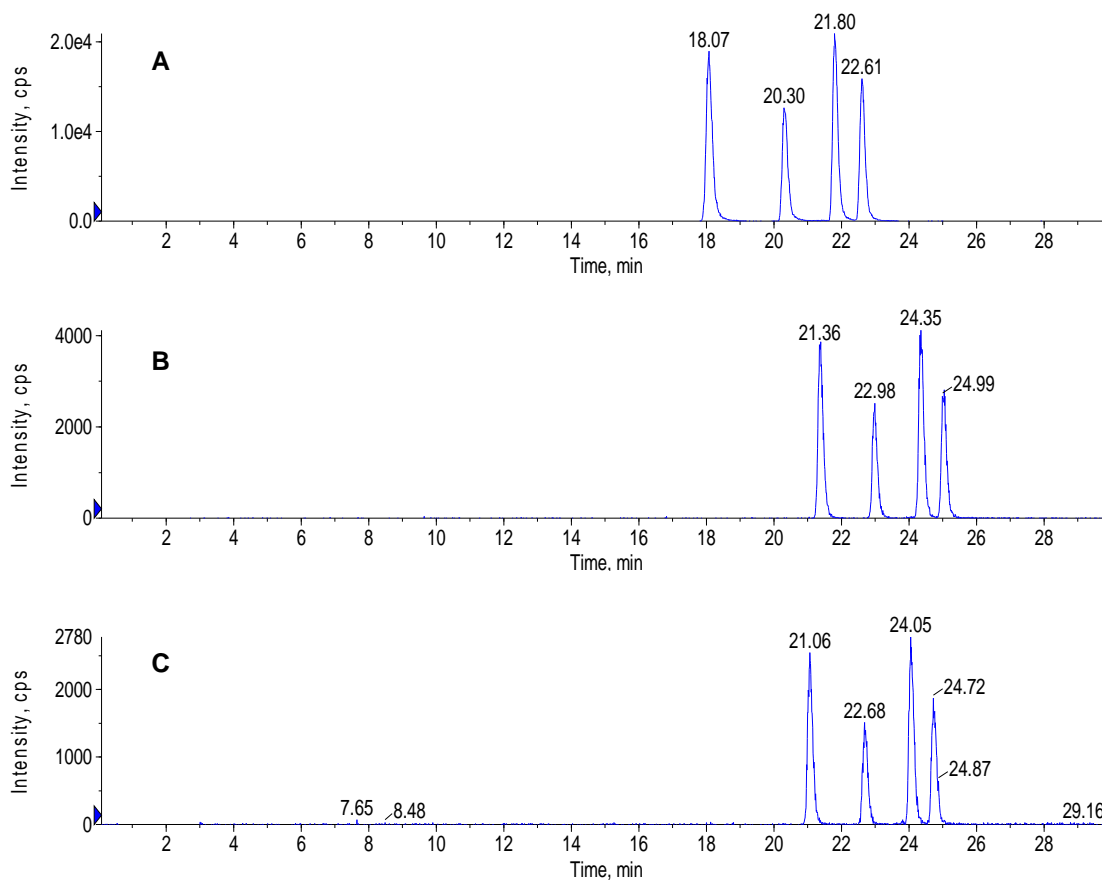
Figure 13. Chromatograms of 4-OHEN-dG in different mobile phases. A) water/methanol with 0.1% formic acid-methanol with 0.1% formic acid, B) water with 0.1% ammonium acetate (pH 6.8)-methanol, C) water with 0.1% ammonium acetate (pH 4.8)-methanol.

Results shown in Figure 11-13 revealed that water/methanol/formic acid-methanol/formic acid system provide the best baseline separation for 4-OHEN-dC adducts. Separation of 4-OHEN-dG adducts is moderate in all the four combinations. In the systems composed water with 0.1% ammonium acetate and methanol, separation of 4-OHEN-dA adducts is closer to baseline separation, compared to the first system. For the standards with the same concentration, all

the three types of adducts showed better ion intensities with

water/methanol/formic acid-methanol/formic acid system, especially for 4-OHEN-

dG adducts. Therefore, in observation of the overall results, water with 10%

methanol and 0.1% formic acid as solvent A and methanol with 0.1% formic acid

as solvent B will be the best choice for analysis.

3.3   Calibration Curve and Limit of Detection

A calibration curve was created for each individual adduct by analyzing a

series of standards containing various concentration of adduct standard and

constant amount of the stable isotope internal standard. All solutions were

prepared in methanol:water (70:30, v/v). The response was linear over a range of

0.5 to 500 nM (Figure 14). The calibration curve was used to determined the

levels of adducts in samples. The limit of detection on column for 4-OHEN-dA

and 4-OHEN-dC is 5 fmol, and for 4-OHEN-dG is 10 fmol.
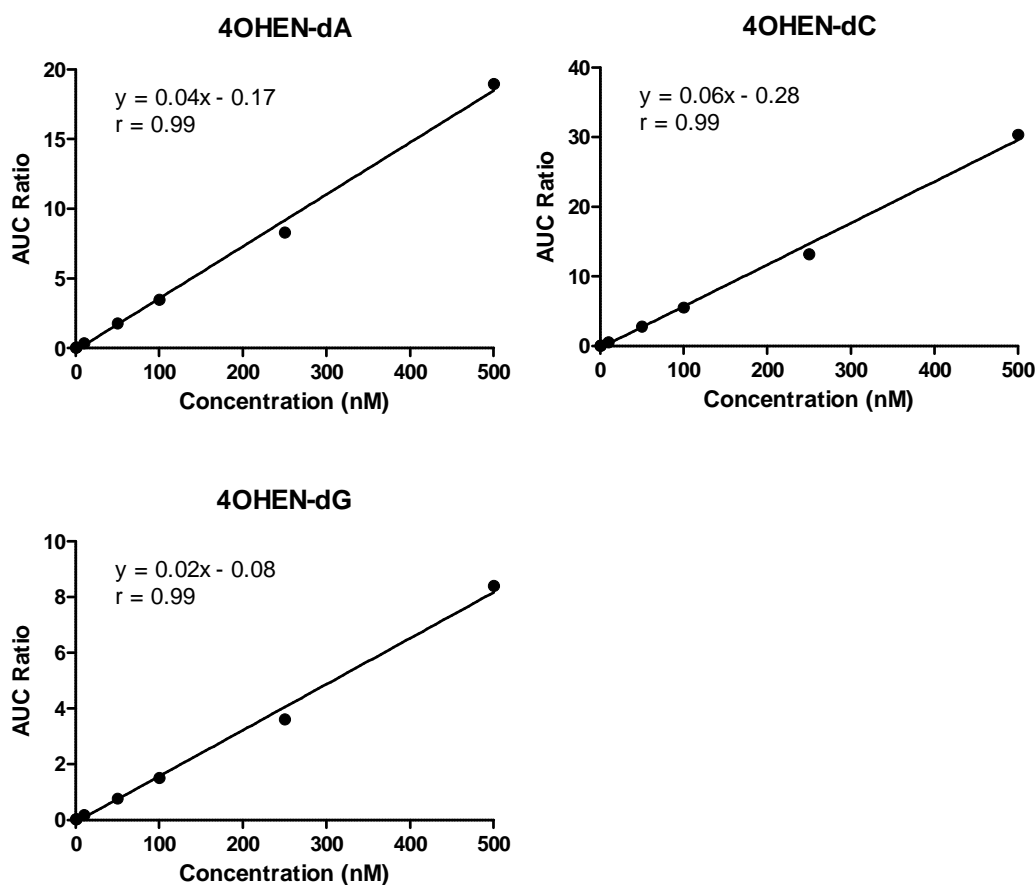
Figure 14. Calibration curves for 4-OHEN-DNA adducts. The ratio of peak area of adducts (4-OHEN-dA, 4-OHEN-dC, or 4-OHEN-dG) to 4-OHEN-[^{15}N$_5$]dG was plotted against the concentration of adducts (0.5, 1, 10, 50, 250, 500 nM).

To determine the limit of detection (LOD) of adducts after sample work-up, various amounts of adduct standards and constant amount of internal standards were mixed with 400 µg of calf thymus DNA in 1 mM potassium phosphate buffer (PPB). Each sample was subjected to the entire hydrolysis procedure and analyzed. The limit of detection on column was approximately 50 fmol for 4-OHEN-dA and 4-OHEN-dC, which is equivalent to 3.8 adducts/10^8 nucleotides for

400 µg of calf thymus DNA (assuming that 1 µg of DNA is equal to 3240 pmol of nucleotides (155)). For 4-OHEN-dG, detection limit is 100 fmol on column, equal to 7.7 adducts/10^8 nucleotides for 400 µg of DNA.

3.4   Comparison of Triple Quadrupole Detection Sensitivity

Triple quadrupole tandem mass spectrometer is the most commonly used instrument for DNA adduct quantitative analysis. Adducts standards were tested on different triple quadrupole mass spectrometer, such as API 3000, API 4000, TSQ Quantum, and Agilent QQQ. A standards mixture of all three adducts (5 nM) was used as a universal testing sample for instrument evalutaion. Chromatograms of 4-OHEN-dG were shown in Figure 15. In terms of ion intensity, TSQ Quantum showed the most intensive signal with the highest S/N ratio for all three adducts, followed by API 3000 and Agilent QQQ. Signals from API 4000 were unable to be distinguished from noise, suggesting the low signal to noise ratio.
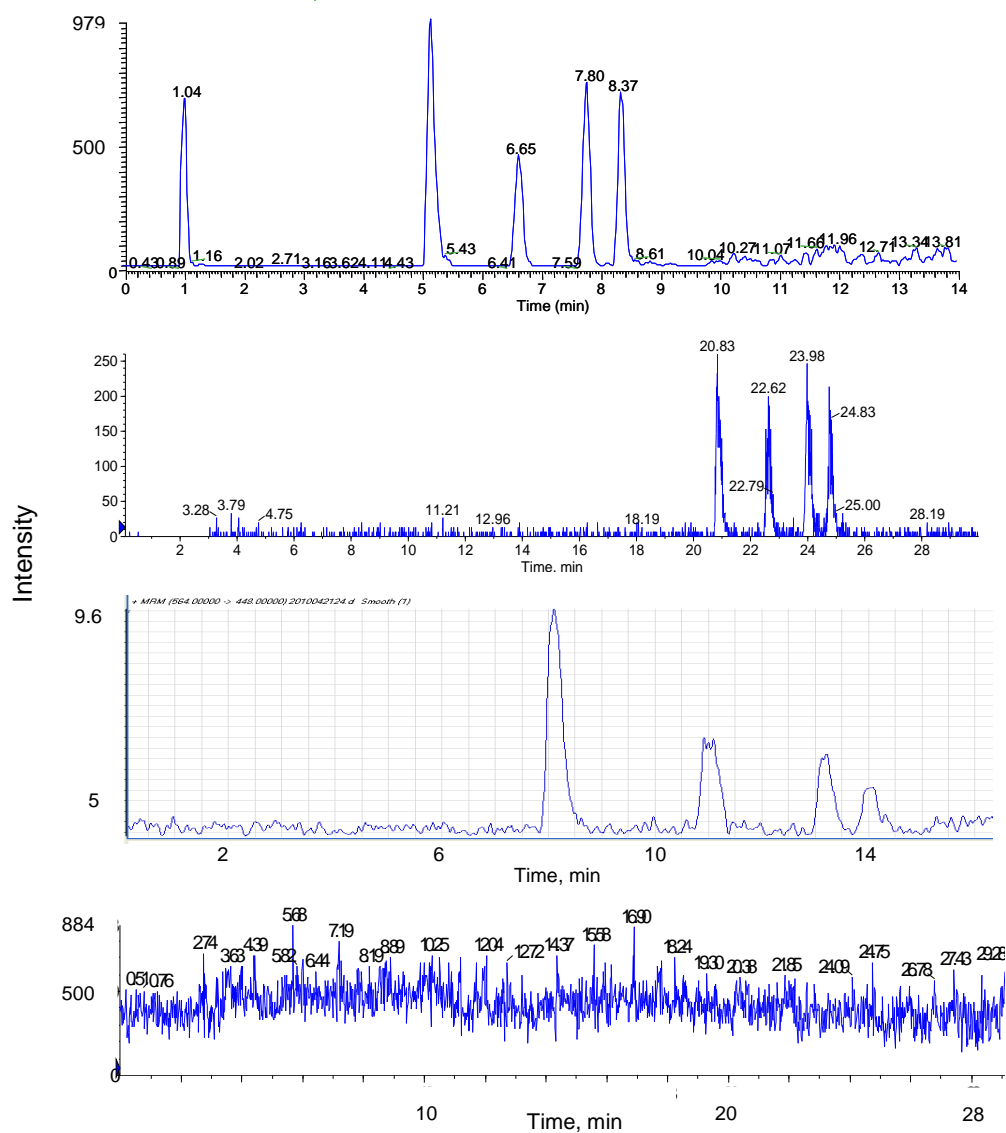
Figure 15. Chromatogram of 5 nM 4-OHEN-dG standard from four triple quadrupoles.

3.5   <u>Extraction Efficiency</u>

Collection of stable adducts formed from 4-OHEN and DNA requires
isolation and hydrolysis of DNA. For quantitative analysis of stable adducts,
unlike method such as ^{32}P-postlabeling which can analyze adducts using as little
as 10 µg samples, usually more than 200 µg is required for LC-MS/MS. In
examination of the procedure, extraction efficiency is one of the factors affecting
amount of DNA we can get. Calf thymus DNA incubated with 4-OHEN is used as
the marker. Adducted calf thymus DNA was added either before or after the
extraction procedure. After hydrolysis, adducts were measured with LC-MS/MS.
Chromatograms of 4-OHEN-dC with reacted calf thymus DNA added before and
after extraction were shown in Figure 17A and 17B respectively. The result
showed that the efficiency is 98%.

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                    ┌─────────────────────┐
                    │     Cell pellet     │
                    └─────────────────────┘
                              │
------------------ DNA extraction ------------------
                              ▼
            ┌─────────────────────────────┐
            │  Cell pellet homogenization │
            └─────────────────────────────┘
                              ▼
      ┌───────────────────────────────────────┐
      │ Centrifuge, nuclei pellet precipitation │
      └───────────────────────────────────────┘
                              ▼
          ┌─────────────────────────────┐
          │  Nuclei pellet homogenization │
          └─────────────────────────────┘
                              ▼
        ┌───────────────────────────────────┐
        │ DNA precipitation with isopropanol │
        └───────────────────────────────────┘
                              ▼
              ┌─────────────────────┐
              │    Dissolve DNA     │
              └─────────────────────┘
                              ▼
        ┌───────────────────────────────────┐
        │  DNA concentration measurement     │
        └───────────────────────────────────┘
                              │
------------------ DNA hydrolysis ------------------
                              ▼
            ┌─────────────────────────┐
            │     Adduct detection    │
            └─────────────────────────┘
```
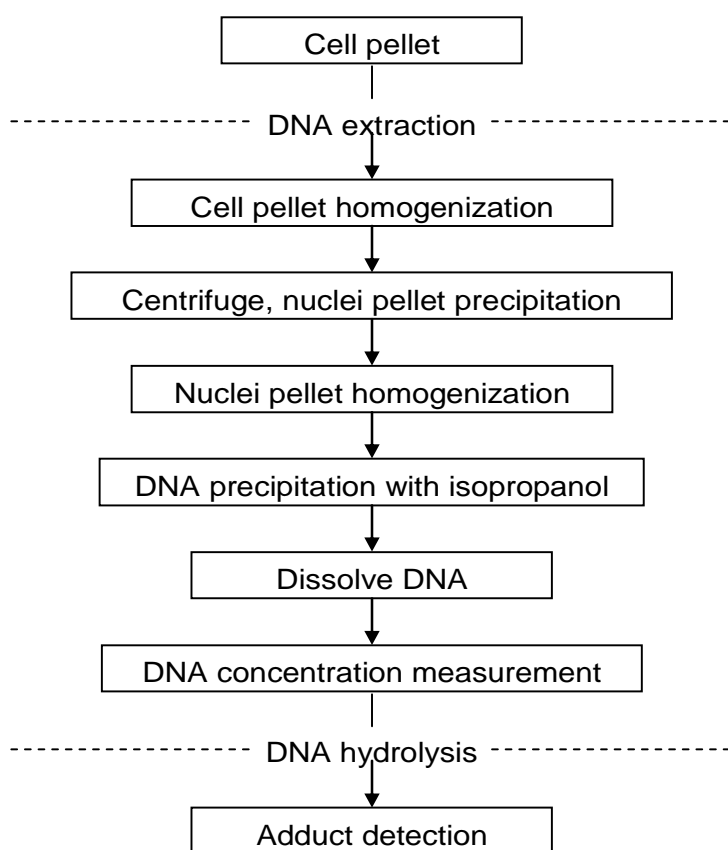
Figure 16. Steps of DNA extraction.
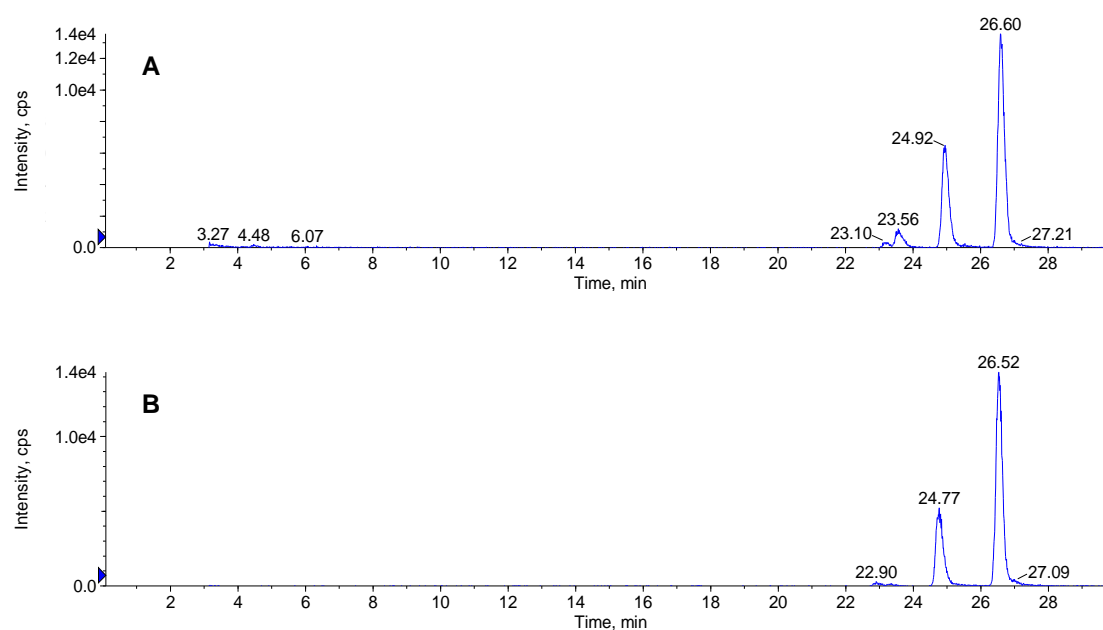
Figure 17. Representative chromatogram of 4-OHEN-dC adducts from experiment of DNA extraction efficiency determined by LC-MS/MS. A) Adducted calf thymus added before extraction, B) Adducted calf thymus added after extraction.

3.6   <u>Efficiency of DNA Hydrolysis</u>

Complete hydrolysis of DNA to single nucleoside is a critical step for adduct detection. Our group published a new hydrolysis method (156); however, the hydrolysis efficiency is not determined. Knowing the efficiency is critical since it affects the amounts of adducts required to achieve sufficient sensitivity. In the experiment, the efficiency for both calf thymus DNA and cellular DNA extracted from MCF-7 cells were determined. The measurement was made by quantifying levels of 2'-deoxyguanosine. The DNA from calf thymus and MCF-7 cells was hydrolyzed to nucleosides and the amount of dG was calculated by the calibration curve generated. Comparing the measured with theoretical levels of dG we got 62% and 59% hydrolysis efficiency for cellular and calf thymus DNA, respectively.
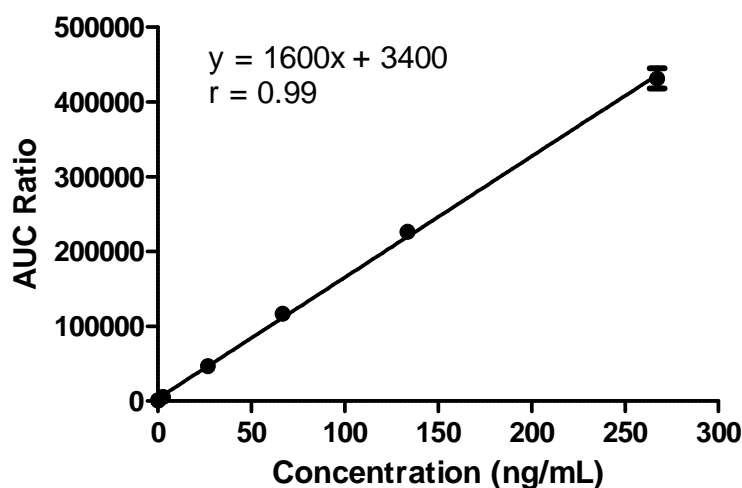
Figure 18. Calibration curve for the determination of 2'-deoxyguanosine (dG) by LC-MS/MS (MRM transition m/z 268 → 152). The peak area was plotted against the concentration of dG (ng/mL).
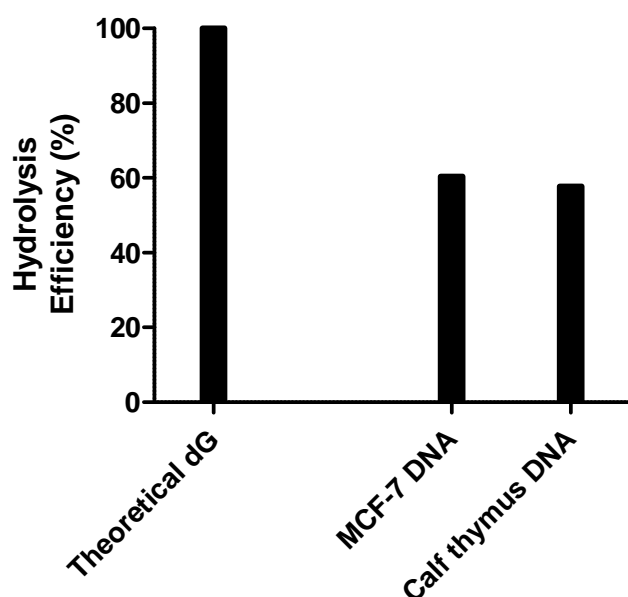
Figure 19. Hydrolysis efficiency of MCF-7 cell DNA and calf thymus DNA.

3.7   Formation of Stable Adducts in Calf Thymus DNA

Calf thymus DNA was incubated with 4-OHEN for 4 hours in PPB followed by isopropanol precipitation. Following hydrolysis, the resulting sample was analyzed by LC-MS/MS is shown in Figure 20. In addition to comparing to standards, we also ran 100x diluted samples and spiked diluted samples. The chromatogram (Figure 21) showed that after spiking, the increased intensity and overlap of calf thymus adducts and standards peaks indicated the formation of the unusual cyclic adducts.
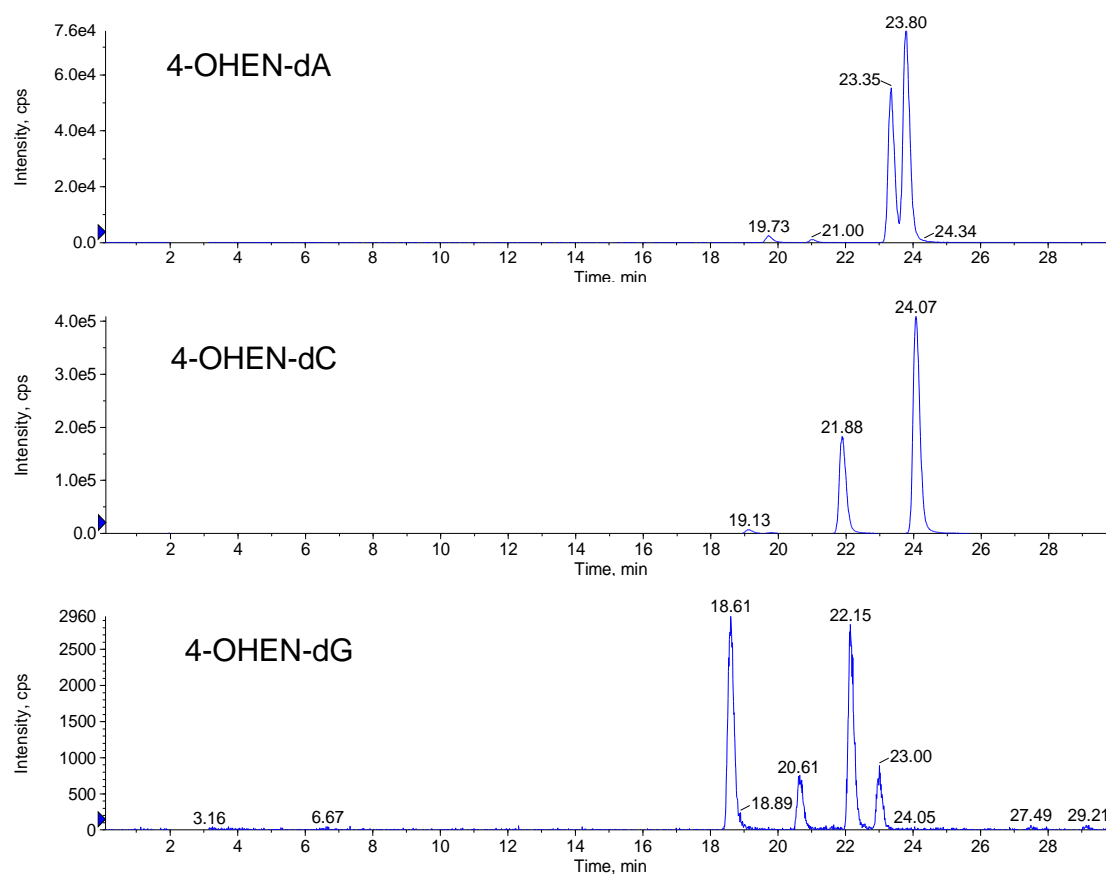
Figure 20. Chromatogram of calf thymus DNA reacting with 1 mM 4-OHEN at room temperature for 4 hours.
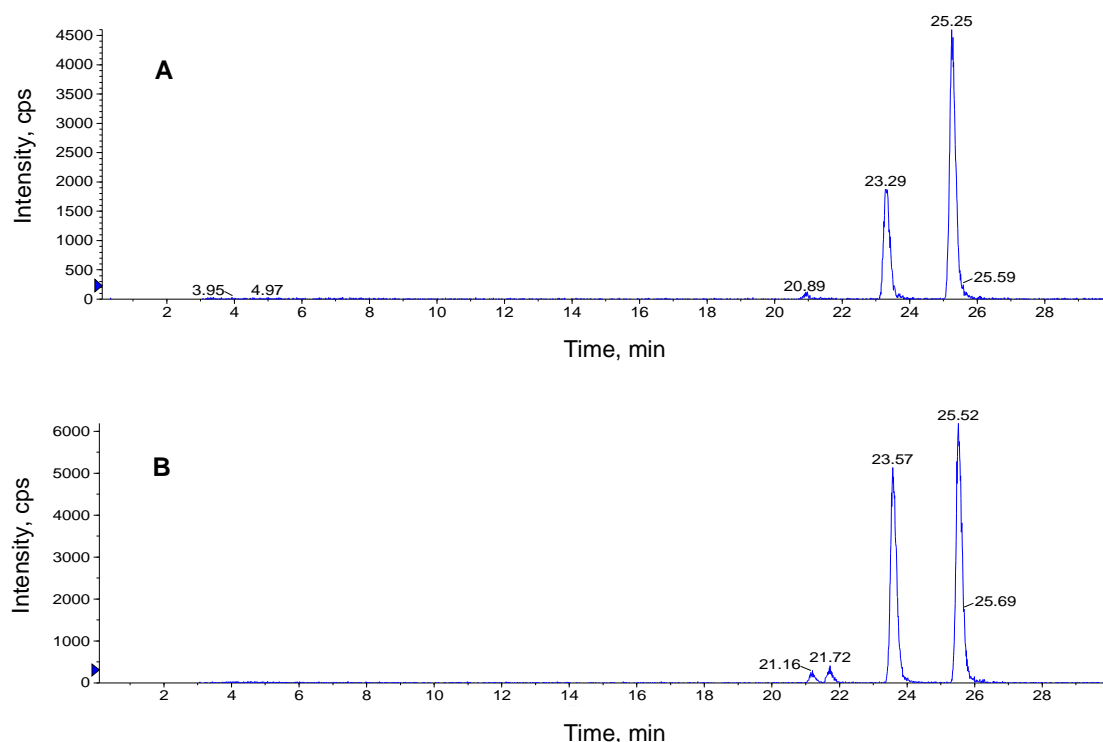
Figure 21. LC-MS/MS chromatogram of 4-OHEN-dC adduct from calf thymus DNA and 4-OHEN incubation. A) Adducts from 100x diluted samples, B) Adducts from spiked 100x diluted samples.

3.8 Conclusions and Future Directions

It has been proposed that DNA adducts formation has been associated with elevated cancer risk. Some epidemiological studies have found the potential of DNA adducts as biomarkers of risk (84). 4-OHEN can be easily oxidized to o-quinone which will react with DNA to form cyclic 4-OHEN-dA, -dC, and -dG stable adducts (55). Various methods such as [32]P-postlabeling, immunoassay, and LC-MS/MS have been developed for the detection of 4-OHEN stable DNA adducts. The [32]P-postlabeling has been a widely used technique with high sensitivity up to 0.1-1 adduct/10^9-10^{10} nucleotides (85). With this high sensitivity, the amount of

samples required for analysis can be as little as 10 μg. It has been applied to 4-OHEN adducts detection in oligomers and calf thymus DNA (76,92). Immunoassay is also a sensitive method with detection limit close to 5 adducts/10^8 nucleotides in 1 μg DNA (100). In addition, the method is rapid since DNA hydrolysis is not required. However, the major disadvantage of ^{32}P-postlabeling and immunoassay is the lack of ability for structural characterization. Moreover, the requirement of radiolabeled compounds and highly specific antibodies is also a concern. The advantage of LC-MS/MS analysis is that it provides structural information, confirming that the correct compounds are studied. The use of stable isotope internal standards allows accurate quantification (105). For many analyzes, ^{32}P-postlabeling still has higher sensitivity than MS (ca. 0.5-5 adducts/10^8 nucleotides) and is more widely applicable when the amount of DNA is limited. Usually more than 200 μg is required for adduct analysis with LC-MS/MS technique.

In order to detect stable adducts formed in calf thymus DNA, cell DNA, or even biological samples, optimization of sample preparation procedure and instrumental condition is needed. The adducts standards were chemically synthesized by incubation of 4-OHEN and 2'-deoxynucleosides. Followed by preparative HPLC purification, the adducts were subject to structural identification by tandem mass spectrometry. Instrumental parameters were finely tuned using standards. The suitable mobile phase among the tested combination is water with 10% methanol and 0.1% formic acid as solvent A, and methanol with 0.1% formic acid as solvent B. Different triple quadrupole instruments have been tested

for the best sensitivity. The results suggested that TSQ Quantum has the best sensitivity, followed by API 3000, Agilent QQQ, and API 4000. The TSQ Quantum was 4 to 5 times more sensitive than API 3000. The unique 90 degree collision cell of TSQ Quantum not only increased the sensitivity but also reduced the noise.

In consideration of instrumental sensitivity, isolation of high amount of DNA and optimized DNA hydrolysis method will be required before analysis. The efficiency of hydrolysis used is about 60% and the efficiency of extraction is about 98%. Taking the two factors into account, the correlation between DNA amount needed and frequency of adduct formation is listed in Table 1.

TABLE I. CORRELATION BETWEEN ADDUCT FREQUENCY (4-OHEN-dA AND -dC) AND DNA AMOUNT REQUIRED

Adduct frequency	DNA amount required (µg)
$1/10^9$	2.57×10^4
$1/10^8$	2.57×10^3
$1/10^7$	2.57×10^2
$1/10^6$	2.57×10^1

The elaboration of LC-MS/MS technique for 4-OHEN-DNA adducts detection has been demonstrated in this study. Amounts of DNA required could

be further decreased with improved hydrolysis and increased instrument sensitivity. The current hydrolysis method takes 8 hours and diethanolamine is used for pH adjustment. Diethanolamine is a high-boiling point compound which causes difficulty in concentrating samples under speed vacuum concentrator. Further optimization of hydrolysis can be studied to achieve the balance between efficiency and time. Nano LC is known for its high sensitivity and can also be integrated with a mass spectrometer in nano LC-MS/MS. With advances in analysis technique, it can be applied for stable adducts detection from cultured cells, which is not fully studied. Since these adducts can be used as biomarkers, formation of stable adducts from different cell lines can be studied to compare mutagenic activities in cell lines. In addition, these cyclic adducts might be removed by nucleotide excision repair (NER) with different rates. To further study the repair mechanism, formation of adducts in NER-deficient cells and specific cofactor knock-out system should be investigated.

CHAPTER 4 Development of an in vivo assay for Anastrozole Measurement

That women exhibit higher risk for Alzheimer's disease than men suggested, in addition to advancing age and genetic factor, gender difference plays a role in AD pathology. In postmenopausal women, improved cognition was also observed in the group receiving HRT. In the trial of anastrozole treatment for women with early-stage breast cancer, the results showed that women with anastrozole treatment have poorer hippocampal-dependent tasks. To evaluate the effect of estrogen on AD pathology in brain, depletion of circulating and/or local brain estrogen was performed in 3xTgAD mice. Amyloid-beta deposition in brain was studied by Overk and alteration of pathology was observed in female mice orally administered with anastrozole. We hypothesize that anastrozole, like its analogue letrozole, can penetrate blood brain barrier and therefore results in change on amyloid-beta deposition. Extraction of anastrozole from the brain was carried out with methanol. LC-MS/MS method was developed and optimized for its quantification. Levels of anastrozole in serum and brain were compared and a positive correlation was shown. This is the first report of detection of anastrozole in the brain, suggesting anastrozole penetrates blood brain barrier after oral administration.

Results and Discussion

4.1 <u>**Calibration Curve**</u>

Qualification and quantification of anastrozole was carried out on TSQ Quantum triple quadrupole coupled to liquid chromatography. For MRM analysis, the precursor → product ion transitions of m/z 294 → 225 and 455 → 165 were employed. A calibration curve was constructed in duplicate by preparing a series of standards each containing various amounts of anastrozole and a constant amount of internal standard verapamil added to blank brain tissue to account for any matrix effects. Each of the standards was then subject to the entire analysis procedure. For brain samples prepared in different batches, the calibration curve constructed as described above was repeated along with every batch. The response was linear over the range of 0.0625 to 2 ng/mL (Figure 22). The calibration line was used to determine the levels of anastrozole in brain samples from treated mice containing 500 pg of the internal standard verapamil. The limit of quantification on column for anastrozole was 0.0625 ng/mL.
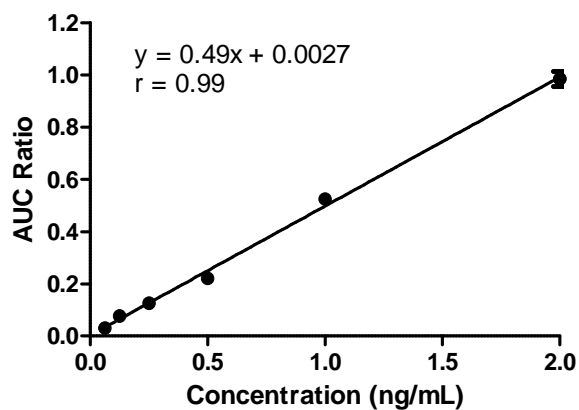
Figure 22. Calibration curve for the determination of anastrozole with verapamil as the internal standard and blank brain tissue matrix.
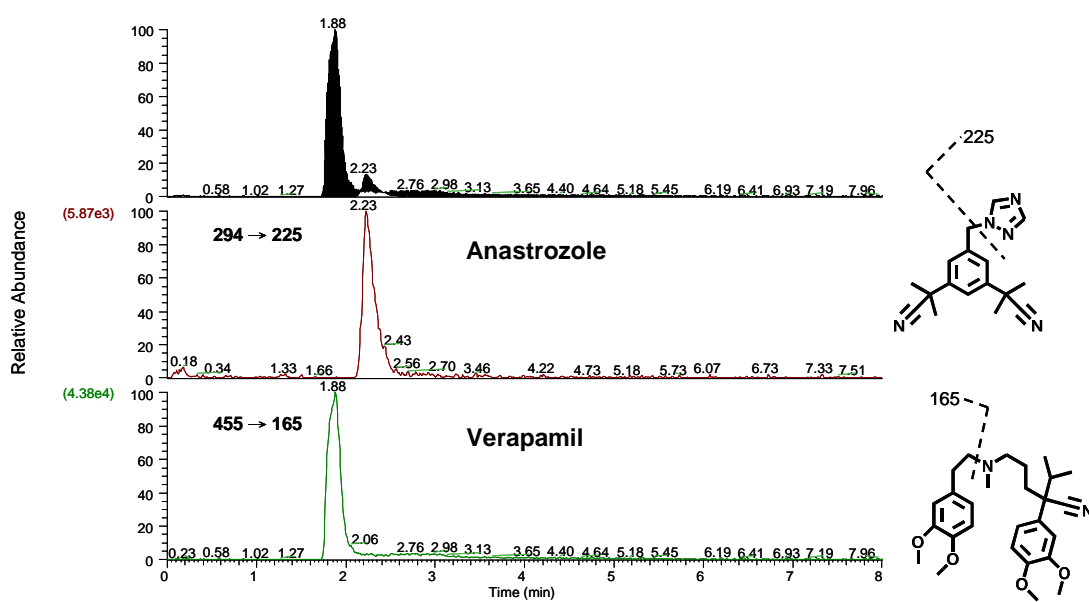
Figure 23. LC-MS/MS chromatogram of anastrozole standard and verapamil.
A) Total ion chromatogram, B) extracted ion chromatogram of anastrozole, MRM 294 → 225, C) extracted ion chromatogram of verapamil, MRM 455 → 165

4.2 <u>**Extraction Method Reproducibility**</u>

The levels of anastrozole in the brain samples were determined by extraction with methanol followed by LC-MS/MS analysis. Therefore, the performance of extraction method was examined with reproducibility. To validate, blank brain tissue was spiked with 10 µL of anastrozole (10 ng/mL) and verapamil (50 ng/mL), followed by the same extraction procedure. Four sets of samples were repeated and LC-MS/MS analysis was performed twice for each sample. The ratio of area under curve of anastrozole over that of verapamil was compared.

There was no significant difference in the ratio among the batch for both injections ($p=0.3664$). Varying the amount of brain tissues from 8 up to 15 mg had no effect on the LC-MS/MS signal observed for anastrozole to verapamil ratio, assuming that the tissues could be adequately removed by centrifugation and anastrozole could be efficiently extracted with methanol.

Reproducibility
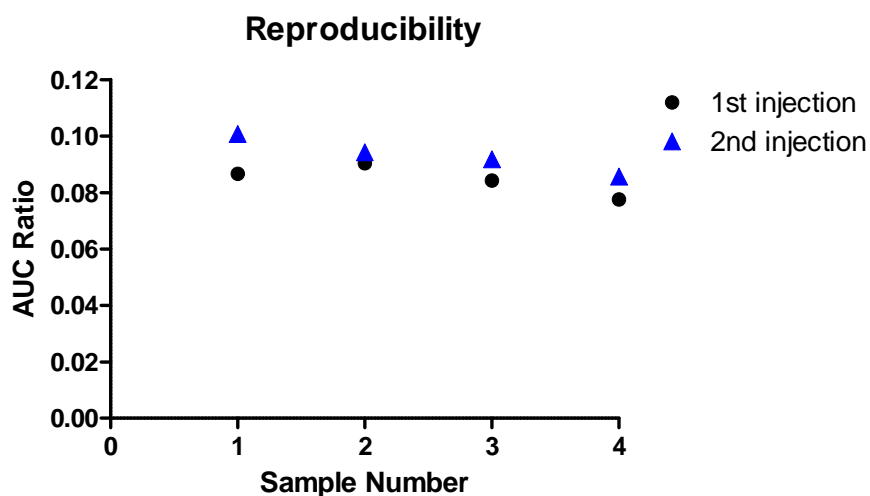
Figure 24. Area under curve ratios for 4 independent sets. The same amounts of anastrozole and verapamil were spiked into blank brain tissues.

The levels of anastrozole in different regions of brain from anastrozole-treated mice were examined to see if the distribution of drug is equal over the brain. Brain regions of brain stem, posterior hippocampus, and striatum from treated animal were used. The concentrations of drug were determined with the calibration curve constructed at the same time. To compare the level of anastrozole, the concentrations calculated were normalized to the amount of brain subject for extraction. The results demonstrated even distribution of anastrozole in the brain.

4.3   <u>Determination of Levels of Anastrozole in Brain Tissues</u>

The mice were treated with anastrozole gel at a dose of approximately 0.4 mg/animal/day for six months. In the control groups vehicle gel was used instead. After tissue collection, the anastrozole in the brains were extracted with methanol, followed by LC-MS/MS analysis. Anastrozole was detected in the tissues from anastrozole-treated animals but not in tissues from vehicle groups. Typical LC-MS/MS MRM ion chromatograms were shown in Figure 25A and 25B for vehicle control and anastrozole-treated groups, respectively. The brain weight was converted into mL brain tissue using the specific gravity for mouse brain (1.05). The mean brain level of anastrozole detected was 5.7 pg/mL. The anastrozole concentrations in serum were compared with those in brain tissues. There was a significant positive correlation between them (Figure 26).
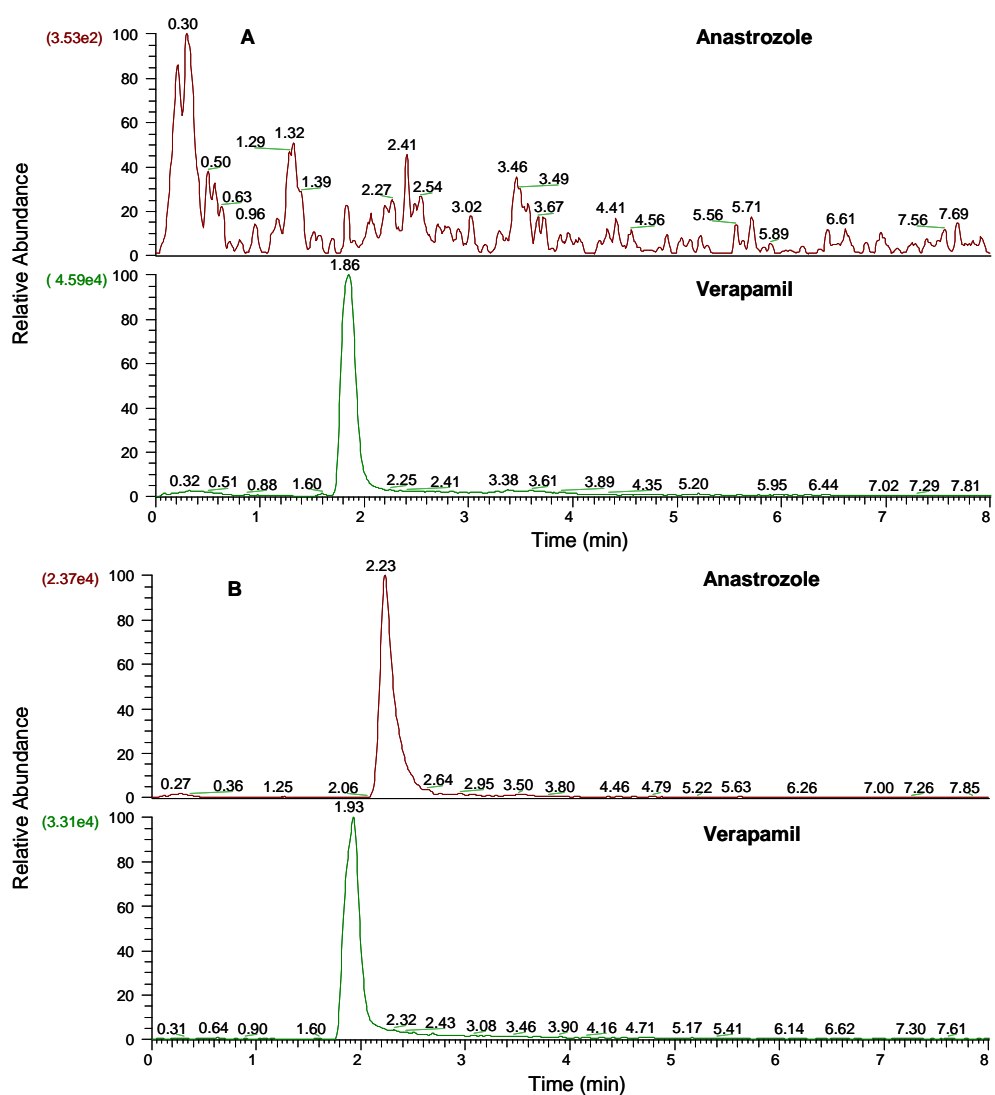
Figure 25. Representative chromatograms of anastrozole from mice brain samples. A) Control group brain sample, B) anastrozole-treated brain sample.
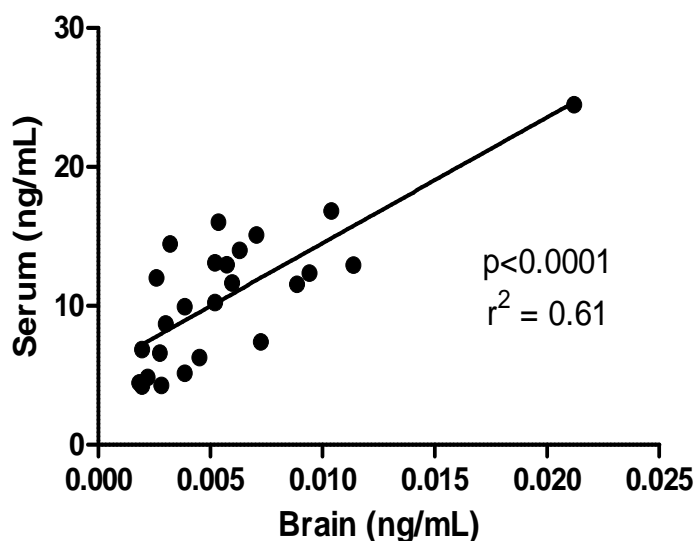
Figure 26. Correlation of the serum and brain anastrozole levels.

4.4   Conclusions and Future Directions

In postmenopausal women, impairment of cognition was found to be reduced in group receiving HRT. Poorer hippocampal-dependent tasks were observed in patients receiving anastrozole for early-stage breast cancer treatment. It can be suggested that AD pathology is affected by estrogen. In our study, anastrozole was orally administered to mice for 6 months and brain AD pathology was studied. Whether anastrozole penetrates blood brain barrier or not was analyzed since no previous studies have reported brain anastrozole levels after oral administration. Method for determination for level of anastrozole was developed. Extraction was performed with methanol and mass spectrometry analysis was carried out on TSQ Quantum triple quadrupole. Anastrozole was detected in brain tissues from treated mice. The mean brain levels were not

significantly different among groups defined by sex and hormone status. A positive correlation between brain and serum levels was shown. The results from analysis of amyloid-beta deposition and anastrozole levels in mice brain tissues suggests estrogen levels in female may be a factor in the development of AD pathology. Further analysis of brain estrogen levels may provide better evidence for effects which estrogens bring on AD.

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APPENDIX

APPENDIX: ANIMAL CARE COMMITTEE APPROVAL

UIC UNIVERSITY OF ILLINOIS
AT CHICAGO

Office of Animal Care and
Institutional Biosafety Committees (MC 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

February 24, 2012

To: University of Illinois Graduate College

From: Dr. Mary Bowman, Director, Office of Animal Care and Institutional Biosafety

Re: Thesis dissertation work involving the use of vertebrate animals conducted by Pei-Yu Lu

This letter is to inform you that the animal work conducted by Pei-Yu Lu for her dissertation was conducted under the Rush IACUC protocol listed below:

ACC Number: 11-002
Title of Application: Age-related Alzheimer's-like Pathology in a Triple Transgenic Mouse Model
Approval Period: 12/15/10 to 12/15/13

This was a collaborative project between a Rush investigator and a UIC investigator and all animal work was conducted at Rush University. Should you have any questions regarding this matter, please contact the Office of Animal Care and Institutional Biosafety at the number listed below.

NAME   PEI-YI LU

EDUCATION   M.S., expected 2012 in Medicinal Chemistry,
                  Department of Medicinal Chemistry and Pharmacognosy,
                  University of Illinois at Chicago

                  B.S., Pharmacy, School of Pharmacy
                  College of Pharmacy, 2003-2007
                  Taipei Medical University, Taiwan

EMPLOYMENT   Research Assistant, June 2010 to
HISTORY   Department of Medicinal Chemistry and Pharmacognosy,
                  University of Illinois at Chicago

                  Teaching Assistant, January 2009 to June 2010
                  Department of Medicinal Chemistry and Pharmacognosy,
                  University of Illinois at Chicago

                  Research Assistant, October 2007 to Jun 2008
                  Department of Medicinal Chemistry
                  Taipei Medical University, Taiwan

PUBLICATION & PRESENTATION

- Cassia R. Overk, Pei-Yi Lu, Yue-Ting Wang, Jaewoo Choi, James W. Shaw, Gregory R. Thatcher, Elliott J. Mufson. Effects of aromatase inhibition versus gonadectomy on hippocampal complex amyloid pathology in triple transgenic mice. *Neurobiol. Dis.* **2012**, 45, 479-487.

- Kim, H.Y., Sohn, J., Wijewickrama, G.T., Edirisinghe, P., Gherezghiher, T., Hemachandra, M., Lu, P.Y., Chandrasena, R.E., Molloy, M.E., Tonetti, D.A., Thatcher, G.R. "Click synthesis of estradiol-cyclodextrin conjugates as cell compartment selective estrogens." *Bioorg. Med. Chem.* **18**, 809-821 (2010).

- Jing-Ping Liou, Zi-Yi Wu, Ching-Chuan Kuo, Chi-Yen Chang, Pei-Yi Lu, Chi-Ming Chen, Hsing-Pang Hsieh, Jang-Yang Chang. Discovery of 4-amino and 4-hydroxy-1-aroylindoles as potent tubulin polymerization inhibitors. *J. Med. Chem .***2008,** 51, 4351-4355.

- Zi-Yi Wu, Pei-Yi Lu, Chi-Yen Chang, Chi-Ming Chen, Hsing-Pang Hsieh, Jang-Yang Chang, Jing-Ping Liou. Synthesis and structure-activity relationships of 1-aroylindoles as anticancer agents. *2008 PST Medicinal Chemistry Symposium*, Si-Tou, Taiwan. **2008**