Quantitative Analysis of Estrogenic Xenobiotics in Humans Using Liquid Chromatography-Mass Spectrometry

By

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THESIS

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This thesis is dedicated to my parents, Bin Gao and Shi Yuan without whom this work would never have been accomplished.

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LIST OF ABBREVIATIONS

6-PN	6-prenylnaringenin
8-PN	8-prenylnaringenin
AUC	Area under the curve
BDS	Botanical dietary supplements
BPA	Bisphenol A
Cmax	Maximum concentration
CID	Collision-induced dissociation
CL/F	Apparent clearance
CYP	Cytochrome P450
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetra-acetic acid
EFSA	European Food Safety Authority
EIA	Enzyme Immunoassay
ER	Estrogen receptor
ESI	Electrospray ionization
EU	European Union
GC-MS	Gas chromatography-mass spectrometry
HLM	Human liver microsomes
HPLC	High performance liquid chromatography
HRT	Hormone replacement therapy
IX	Isoxanthohumol
KI	Kupperman index

LIST OF ABBREVIATIONS (Continued)

LC-MS	Liquid chromatography-mass spectrometry
LC-MS-MS	Liquid chromatography-tandem mass spectrometry
LC-UV	Liquid chromatography-ultraviolet-visible spectroscopy
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantitation
LOD	Limit of detection
m/z	Mass-to-charge ratio
MAPK	Mitogen-activated protein kinase
MRS	Menopause rate scale
MTBE	Methyl *tert*-butyl ether
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cell
NOAEL	No observed adverse effect level
PBS	Phosphate buffered saline
PK	Pharmacokinetics
RDA	Retro Diels-Alder
RSD	Relative standard deviation
ROS	Reactive oxygen species
SIM	Selected ion monitoring
SPE	Solid phase extraction
SRM	Selected reaction monitoring
SULT	Sulfotransferase
Tmax	Time to reach peak concentration

LIST OF ABBREVIATIONS (Continued)

TNFα	Tumor neurosis factor alpha
$T_{1/2}$	Half-life
TDI	Tolerable daily intake
UDPGA	Uridine diphosphate glucuronic acid
UGT	Uridine-5' diphosphate (UDP)- glucuronosyltransferases
UHPLC	Ultra high performance liquid chromatography
Vd/F	Apparent volume of distribution
WHI	Women's health initiative
XN	Xanthohumol
β-NADPH	*β*-Nicotinamide adenine dinucleotide phosphate
λ_z	Terminal elimination constant

SUMMARY

Herbs, botanical products and phytomedicines are commonly used as dietary supplements intended to maintain or improve health. A botanical dietary supplement must meet the following criteria for marketing in the United States: 1) intended to supplement the diet; 2) contains one or more dietary ingredients including vitamins, herbs or other botanicals; 3) is taken orally as a pill, tablet, capsule or liquid; 4) is labeled appropriately as a dietary supplement.

Hormone replacement therapy (HRT) is a medical treatment for menopausal and postmenopausal women. HRT involves the use of one or more groups of medications designed to boost hormone levels such as estrogens, progesterone or progestins. Often given for short-term relief from menopausal symptoms, the prolonged use of HRT has been associated with an increased risk of breast cancer. As alternatives to conventional HRT, women are trying botanical dietary supplements for the possible relief of menopausal symptoms.

Standardization of a botanical dietary supplement is an essential step to achieve a safe and reliable product. Typically, chemical standardization is used to provide consistent levels of active constituents and predictable pharmacological effects to consumers. *Humulus lupulus* L. (hops) is a source of prenylated flavonoids, and hop dietary supplements are often standardized to the phytoestrogen 8-prenylnaringenin. In this dissertation, a HPLC-MS-MS method is described and validated for the standardization of a hop extract, which was used for a Phase I clinical trial.

To determine if prenylflavonoids from a standardized hop extract are orally bioavailable, a Phase I clinical trial was carried out. Five menopausal women subjects were recruited and received 3 escalating dosages of the hop extract with a one-month washout period between

each dosage. Using a newly developed and validated UHPLC-MS-MS method that was approximately 10-fold faster than the HPLC-MS-MS method used to standardize the hop extract, serum and urine levels of prenylflavonoids from hops were determined. These prenylflavonoids included xanthohumol, isoxanthohumol, 8-prenylnaringenin, and 6-prenylnaringenin. These data suggest that hop prenylflavonoids are absorbed rapidly after oral administration and that the major circulating forms are glucuronic acid conjugates.

Bisphenol A (BPA) is used as a material for the production of epoxy resins and polycarbonate plastics. BPA has been reported to migrate from can surface coatings and polycarbonate plastics into food and water. Leaching of BPA can be facilitated when canned food is cooked or sterilized at high temperatures. Although the environment (water, air and soil) can be one source of human BPA exposure, the primary route is considered to be intake of BPA in food. Since BPA is a weak estrogenic compound, chronic human exposure to low levels of BPA might have undesirable effects, especially in infants or children. Without a robust, accurate and precise bioanalytical method, human exposure to BPA is difficult to assess. Therefore, a sensitive, selective and rapid analytical method based on UHPLC-MS-MS was developed and validated for the measurement of BPA and its primary metabolite, BPA-glucuronide, in human serum. Then, this method was tested as part of a round robin analysis conducted by the National Institute of Environmental Health Sciences. Spiked samples and samples from human subjects were collected and analyzed by our newly developed and validated UHPLC-MS-MS method for BPA and BPA-glucuronide. Our method was the fastest and among the most sensitive and accurate of those tested in the round robin.

CHAPTER 1

INTRODUCTION

1.1 Botanical Dietary Supplements

Botanical dietary supplements (BDS) and herbal medicine are particularly important for people in the developing countries, but even in the United States, the use of complementary and alternative medicines and BDS are common (42% of the population) (1). The use of BDS by American consumers has increased by 380% since 1990, and this market continues to grow (2). The reasons the popularity of BDS appear to be as follows: 50-60% of the public view herbal therapies as effective, while 70-80% believe that since BDS are natural, they are safer than conventional drug therapy (3). However, evidence for efficacy and safety of most BDS have not been well characterized and documented. No toxicology studies are required for BDS, such as how they interact with drugs, whether constituents of BDS undergo metabolic activation, or what their maximum tolerated dosages might be. The safety of these products is only self-regulated by manufacturers. Hence, there is considerable need for routine quality assurance including control of authentication, standardization, and minimization of contamination. The recent implementation of good manufacturing practice (GMP) for BDS by the US Food and Drug Administration will help with some of these safety issues (4).

Batch to batch consistency in levels of active ingredients of BDS may be ensured by the use of chemical standardization, which uses specific chemicals or markers during manufacturing. With standardization, pharmacological and physiological effects and the overall safety of BDS might become more predictable. For example, the UIC/NIH Center for Botanical Dietary Supplements Research standardized an extract of hops (from *Humulus lupulus* L.) intended for use by menopausal women to 8-prenylnaringenin (8-PN), since this

is the most potent phytoestrogen in this botanical product . This extract was then used in a Phase I clinical trial to study the safety and pharmacokinetics of hop prenylflavonoids.

For some BDS, the active ingredients might not be identified or clearly known in the literature. In such cases, marker compounds that are characteristic or unique to the specific BDS may be used for standardization. For example, black cohosh (*Cimicifuga racemosa*) is standardized to the triterpene glycoside 23-epi-26 deoxyacetin since active constituents responsible for the serotonergic activity are unknown (5).

In summary, the standardization of BDS is essential to improve safety and to facilitate additional studies of safety and efficacy.

1.1.1 Botanical Dietary Supplements as Alternatives to Hormone Replacement Therapy

Menopause is the permanent cessation of the primary functions of the human ovaries and is defined as the end of menstruation for six months or more (6). Menopause usually occurs in women during the late 40s or early 50s. Menopause is associated with some symptoms such as hot flashes, osteoporosis, cardiovascular disease, vaginal dryness, muscle pain, etc. Among these symptoms, hot flashes are the most common and are typically described as a feeling of intense heat with sweating and rapid heartbeat lasting from 2 to 30 min for each occurrence. The frequency and intensity of hot flashes may vary (6).

Hormone replacement therapy (HRT) is a pharmacological treatment for menopausal and postmenopausal women. Based on the concept that menopausal discomfort is caused by decreased levels of circulating estrogen and progesterone hormones, HRT involves the use of medications designed to boost hormone levels such as estrogens, progesterone or progestins. HRT is often administered for the short-term relief from menopausal symptoms. The most commonly prescribed products for HRT are Premarin® and Prempro®. The year 2002

marked a change of attitude towards HRT since a slightly higher incidence of breast cancer, heart attack, stroke, and pulmonary embolism was detected in women receiving HRT during a study conducted by the Women's Health Initiative (WHI) funded by the National Institutes of Health (NIH) (7). The WHI recommended that women should take the lowest feasible dose of HRT for the shortest possible time to minimize these risks. As a result, increasing numbers of women have turned to BDS or other alternative medicines for the relief of menopausal symptoms instead of using traditional HRT.

Various BDS have become popular as alternatives to HRT in menopausal women. Examples include *Humulus lupulus* (hops), *Cimicifuga racemosa* (black cohosh), *Trifolium pratense* (red clover), *Glycine max* (soy), *Angelical sinensis* (dong quai), and *Glycyrrhiza uralensis* (licorice) (8-12).

Recently, hops has attracted attention as an alternative to HRT. Several clinical trials have been conducted to examine the safety and efficacy of hop extracts for the relief of menopausal discomforts (13-17). Heyerick et al. reported in 2006 a prospective, randomized, double-blinded, and placebo-controlled study on the use of a standardized hop extract to alleviate menopausal symptoms (13). The study included 67 menopausal women who were administered a hop extract standardized on 8-PN at 100 or 250 µg for 12 weeks. The outcome were determined by means of a modified Kupperman index (KI) and a patients' questionnaire. All groups showed a significant reduction of KI after 6 weeks and 12 weeks although no dose response was established. A trend for a more rapid decrease of KI was observed for the women randomized to receive a hop extract. The conclusion from this study was that daily intake of hop extract produced favorable effects on vasomotor symptoms and other menopausal discomforts. A recent clinical trial based on a 16-week randomized, double-blinded, placebo-controlled, cross-over study was conducted with 36 menopausal

women in 2010 (14). The subjects were treated with either placebo or a hop extract for a period of 8 weeks after which treatments were switched for another 8 weeks. Similarly, the KI, the menopause rate scale and a mutifactorial visual analogue scale were used to assess outcomes at baseline, 8 weeks and 16 weeks. Interestingly, the first 8-week treatment showed similar reductions in menopausal discomforts in both treatment groups whereas women receiving the hop extract showed reduced menopausal symptoms compared with placebo after the second treatment period.

1.1.2 In Vitro and In Vivo Activity of Hops Prenylflavonoids

Milligan et al. reported that among the hop compounds, 8-PN displayed most estrogenic activity based on the stimulation of alkaline phosphatase activity in Ishikawa cells, and binding to estrogen receptors (ER) in a radio ligand binding assay using rat uterine cytosol (18, 19). The estrogenic activity of 8-PN was later confirmed by using a yeast screen expressing the human estrogen receptor (19). At the UIC/NIH Center for Botanical Dietary Supplements Research, Liu et al. reported that hop extract showed strong binding affinity to both ERα and ERβ which lead to up-regulated progesterone receptor mRNA in Ishikawa cells, induced alkaline phosphates activity in Ishikawa cells, and up-regulated presenelin-2 (20). The high estrogenic activity of 8-PN was also confirmed in different in vivo experiments by other researchers. Subcutaneous administration of 8-PN (30 mg/kg/day) for 2 weeks was shown to slow the reduction in uterine weight ovariectomized rats and the decrease in bone mineral density (21). 8-PN was also reported to induce a characteristic estrogenic response in ovariectomized female rats (22). Sehmisch et al. compared 8-PN with two other phytoestrogens (genistein and resveratrol) as agents to prevent osteoporosis (23). They treated ovariectomy female rats with three phytoestrogens including 8-PN for 12 weeks and

found that 8-PN showed very good biomechanical properties and increased bone mineral density which is comparable to estradiol. Vaginal dryness in postmenopausal women was significantly reduced by topical application of a gel containing hyaluronic acid, liposomes, vitamin E and hop extract (24) .

Rad *et al*. carried out a randomized, double-blind, placebo-controlled study in which a single dose of 8-PN of 50 mg, 250 mg or 750 mg was administered orally to healthy menopausal women (25). They found decreased luteinizing hormone (LH) in serum which showed the ability of 8-PN to exert endocrine effects in menopausal women. The data strongly suggest that 8-PN prenylated flavonoids from hops might provide an alternative to estrogen replacement therapy for the relief of menopausal symptoms in women.

In the past two decades, many in vitro studies have reported the activities of hops and hop constituents as potential chemopreventive agents. Among hop components, XN showed the most chemopreventive activity against various cancer cell lines in the in vitro experiments, such as prostate cancer cells, brain cancer cells, liver cancer cells, etc. Besides that, XN also appeared to be a broad spectrum chemoprevention agent in the in vivo animal studies.

Festa et al. reported that XN induced apoptosis in human malignant glioblastoma cells by increasing reactive oxygen species (ROS) generation in the cells and activating mitogen-activated protein kinase (MAPK) pathway, suggesting XN might be a potential chemotherapeutic agent for the treatment of glioblastoma (26). Zajc et al. reported that XN was able to induce apoptosis in glioblastoma cells at higher rate than in normal astrocytes with p53 activation in glioblastoma cells, and this effect was linked to higher expression of the cell cycle inhibitor p21 in glioblastoma cells. Their conclusion for the study was that XN can induce different cytotoxicity and apoptotic pathways in malignant astrocytes compared with normal cells (27).

Viegas et al. showed that XN suppressed mutations by known carcinogens in a dose dependent manner in bacteria. In HepG2 cells, XN completely prevented DNA breaks at nanomolar concentrations of carcinogens. qRT-PCR mRNA showed that XN up-regulated the expression of CYP1A1 and CYP1A2 and UGT1A1 enzymes which provided a mechanism of XN as a chemoprevention agent (28). Moreover, Hemachandra et al. showed that hops extracts possessed cancer chemopreventive activity through attenuation of estrogen metabolism by inhibiting estrogen-induced expression of CYP1B1 and 1A1 in MCF-10A cells (29).

Recently, Deeb et al. demonstrated that XN induced apoptosis in prostate cancer cells by inhibiting the protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and NF-kB regulated anti-apoptotic proteins B-cell lymphoma (30). Szliszka et al. reported that XN significantly enhanced tumor neurosis factor alpha (TNFα) induced apoptosis and cytotoxicity in prostate cancer cells. Delmulle et al. showed that XN inhibited the growth of DU145 cells and PC-3 cells with IC_{50} values of 12.3 μM and 13.2 μM respectively (31). Colgate et al. demonstrated that XN decreased cell viability in a dose dependent manner from 2.5 μM to 20 μM in BPH-1 and PC3 cell. XN induced cell cycle changes in both cells lines with elevated sub G1 peak at 48 h treatment, activated pro-apoptotic proteins Bax and p53, and decreased activity of NF-κB. All these activities suggest that XN can prevent prostate hyperplasia and prostate carcinogenesis (32).

XN also showed chemopreventive activities during in vivo animal studies. Benelli et al. treated acute lymphocytic leukemia cell lines with XN leading to growth arrest and apoptosis induction. Moreover, 50 μg/day (5 days/week) XN treatment significantly increased mouse life span by delaying the occurrence of neurological disorders due to leukemic cell dissemination.

XN also significantly down regulated AKT and NF-kB signaling pathways in their study (33).

Albini et al. showed that when mice were treated with 2 μM XN (in drinking water), it strongly inhibited angiogenesis in mice implanted with a matrigel sponge (34). At 200 μM treatment, XN displayed significant angiogenesis inhibition without adverse effects in mice. If male nude mice were administrated XN orally at 20 μM, starting from the 20th day of treatment it significantly inhibited the growth of KS-IMM tumors in those animals. In female immuno-deficient mice implanted with human breast cancer tumor xenografts, subcutaneous administration of XN at 1000 mg/kg/day for 14 days strongly inhibited tumor angiogenesis and growth.

All the above evidence strongly supports that XN, a prenylated flavonoid from hops, is able to inhibit cancer cell growth, induce apoptosis in cancer cell lines through different mechanisms and also inhibit NF-κB activation to function as a chemoprevention agent.

1.1.3 Metabolism and Pharmacokinetics of Active Compounds in Hops

The female flowers of hops (*Humulus lupulus* L.) are used in the brewing industry to add aroma and bitterness to beer. Most of the recent research has been focused on the potential estrogenic properties of hop extracts. Among the possible active constituents, prenylated flavonoids including the prenylated chalcone XN and prenylated flavanones 8-PN, 6-prenylnaringenin (6-PN) and isoxanthohumol (IX) have received the most attention. In hop cones, the most abundant prenylated chalcone is XN which can constitute up to 1% of dry weight (35, 36). XN exhibits strong antiproliferative activity against breast, colon and ovarian cancer cell lines and is a potent inducer of quinone reductase (37-39). In contrast to chalcones, prenylated flavanones are minor constituents of hops occurring at 10-100 fold lower concentrations relative to XN (35, 36). This class of compounds has been primarily

investigated for their estrogenic properties. Among prenylated flavanones, 8-PN has been identified as one of the most potent phytoestrogens (18), and its estrogenic properties have been confirmed in numerous in vitro and animal studies (40-42). IX has much weaker estrogenic activity (40).

In vitro phase I metabolism of XN by human liver microsomes has been reported by Nikolic et al., and a total of 12 metabolites were identified (43). It was shown that the biotransformation occurred on the prenyl group and on the flavonone skeleton. Hydroxylation cis or trans on the prenyl methyl group of 8-PN was found to be the major metabolite in vitro (Figure 1.1). The CYP isoform responsible for this oxidation was subsequently determined, and CYP 2C19 was found to carry out the hydroxylation reaction by using both chemical inhibitors and monoclonal antibody to specific CYP450s (44). Similar to 8-PN, hydroxylation of the prenyl methyl group was the primary route of oxidative metabolism forming either *cis* or *trans* hydroxylated metabolites of IX but only the trans isomer of XN (Figure 1.1) (43). Besides the hydroxylated metabolites, demethylation of IX to form the potent phytoestrogen 8-PN was detected (Figure 1.2). Interestingly, the conversion of IX to 8-PN was also discovered to occur by intestinal microbiota (45-47). Thus, IX can be considered a pro-estrogen, which provides an important rationale for inclusion of this compound in the standardization of various hop extracts. Although the demethylation of XN was not observed in these studies, XN can be converted to IX through acid-catalyzed cyclization in the stomach (48). This cyclization reaction followed first-order kinetics with a half-life of 37 min. Thus, XN might also contribute to the in vivo level of 8-PN affecting the overall estrogenic activity after consumption of hop extracts. Another source of 8-PN might be from isomerization of desmethylxanthohumol (DMX) which is much less abundant in hops (35, 36, 49, 50). Although this compound may have chemopreventive activities on its own (49), the interest in this compound primarily

comes from its propensity to isomerize into flavanones 8-PN and 6-PN (See Figure 1.2). It was found that the ratio of 8-PN and 6-PN varies depending on the experimental conditions. However, 6-PN is typically the major isomer.

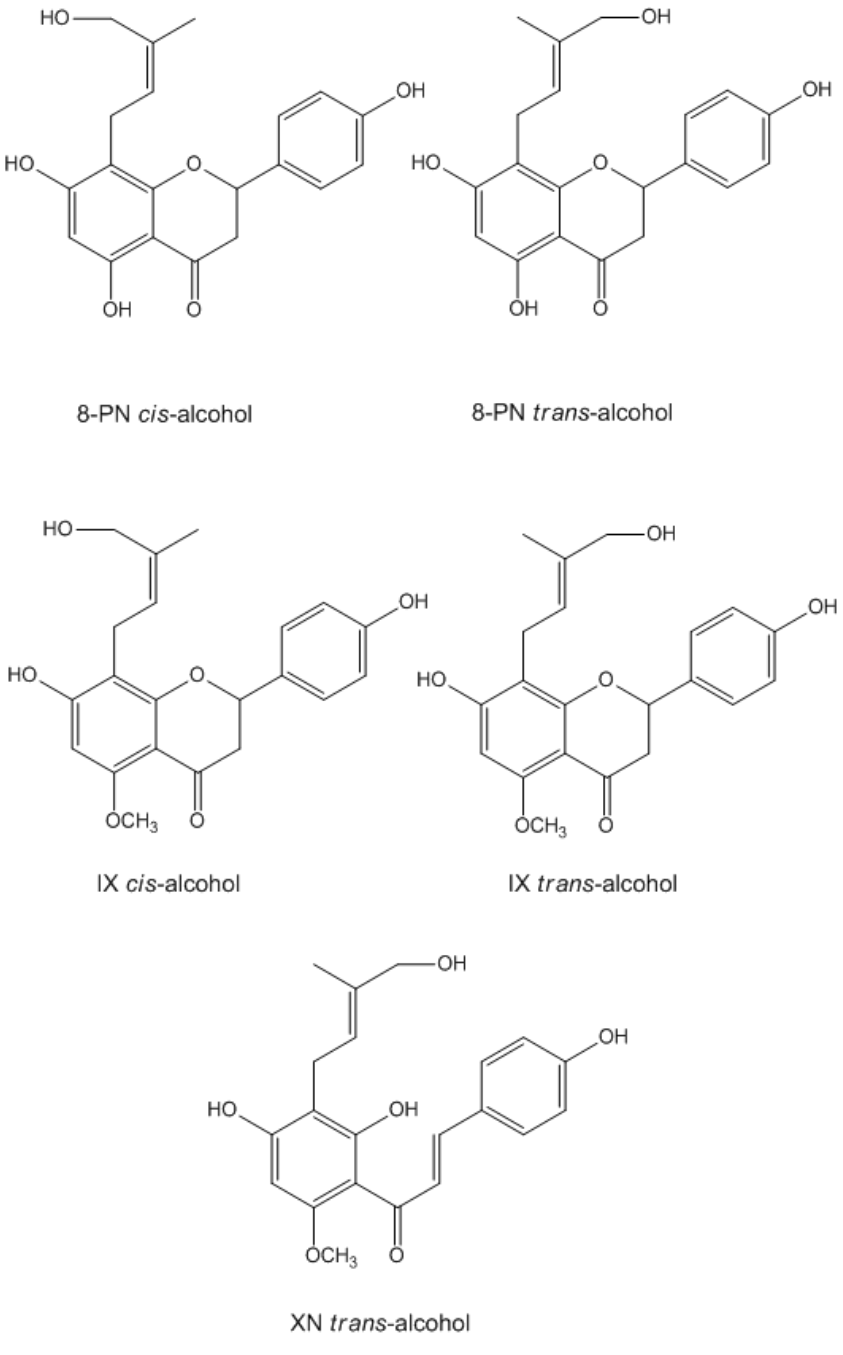


Figure 1.1 Hydroxylation to form *cis* and *trans* alcohols of major prenylated flavonoids

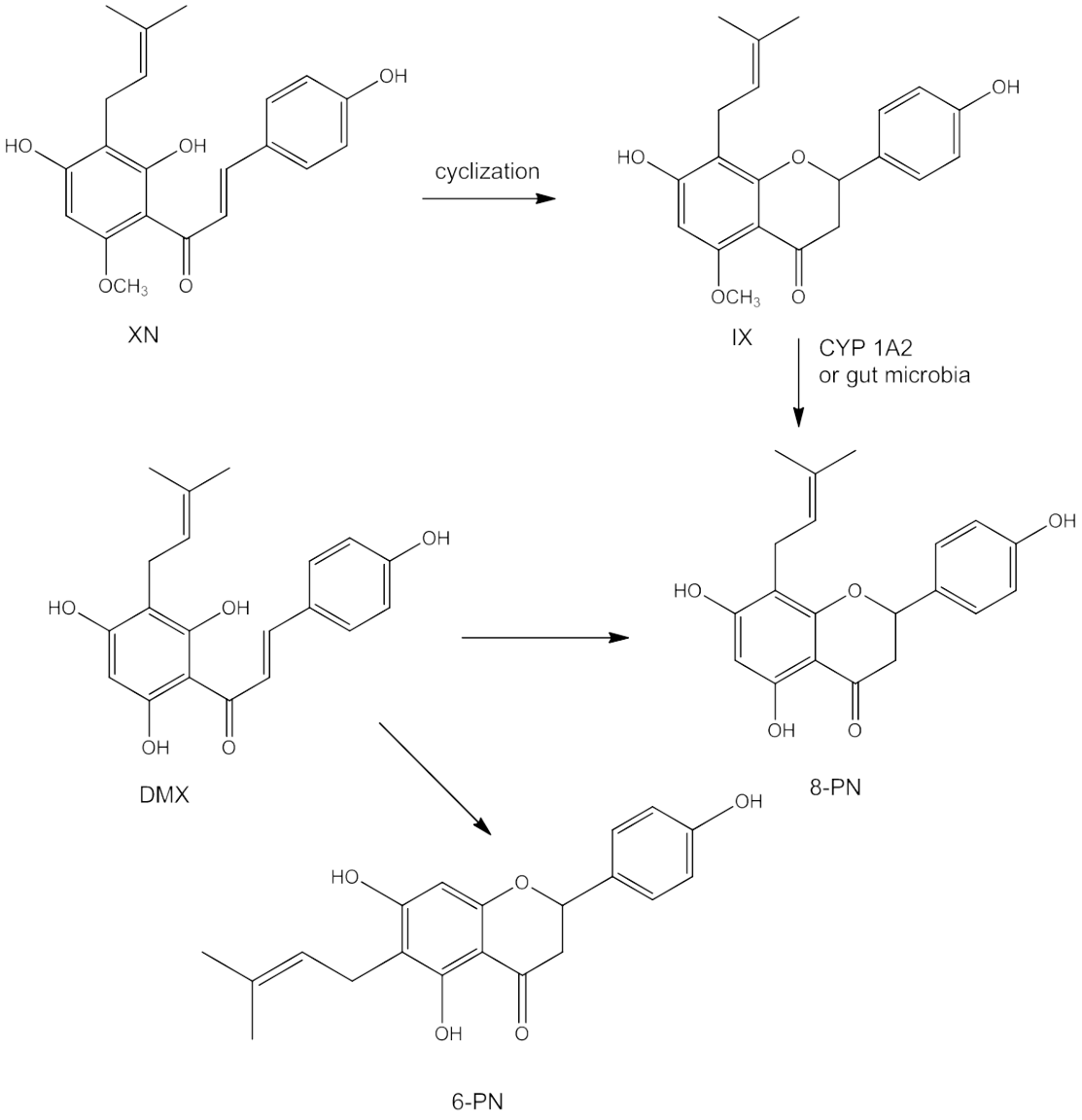


Figure 1.2. Isomerization of XN to IX and conversion of DMX to 8-PN and 6-PN

Phase II metabolites of 8-PN were identified by Nikolic et al. using monolayers of human intestinal epithelial cancer cell line Caco-2 and human hepatocytes (48). Both glucuronic acid and sulfate conjugates of 8-PN were detected in the Caco-2 cell incubations while only glucuronides were found in human hepatocyte incubations. Various 8-PN glucuronides were formed, and the 4'-*O*-glucuronide was the predominant Caco-2 cell metabolite, followed by 8-PN 7-*O* sulfate and 8-PN 4'-*O* sulfate (see structure in Figure 1.3) (48). Ruefer et al. reported that incubations of XN with various cDNA-expressed UDP glucuronosyltransferases can effectively lead to the formation of XN glucuronide conjugates by UGT1A8, UGT1A9 and UGT 1A10 (51, 52). SULT 1A3 and 2A1 were responsible for the minor phase II metabolites, sulfate conjugates. Overall, three mono glucuronides and three mono-sulfates of XN have been reported.

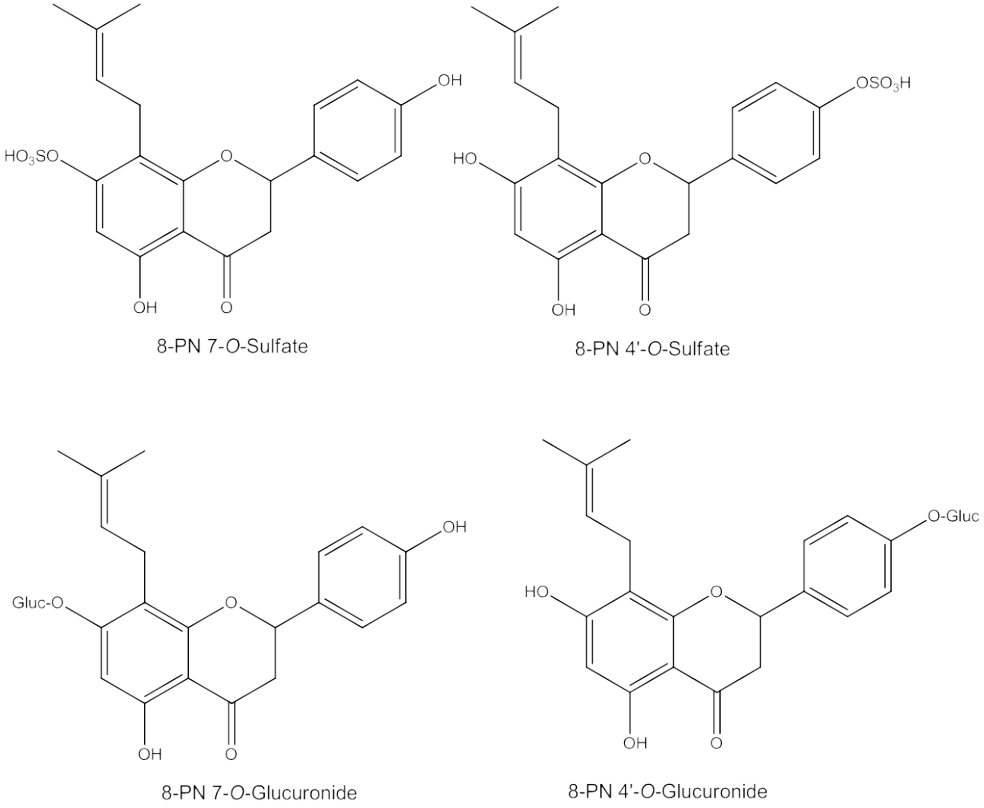


Figure 1.3. Structures of 8-PN glucuronides and 8-PN sulfates

To evaluate bioavailability, especially absorption, metabolism, tissue distribution, and excretion of these important prenylflavonoids in vivo, several studies have been carried out in animals and humans. Overk et al. measured plasma and tissue levels of 8-PN and their major metabolites in rats after intraperitoneal injection of pure compound (40). Also, IX was injected subcutaneously to monitor its conversion into estrogenic 8-PN. It was found that 8-PN glucuronide was the major circulating metabolite. Mono-glucuronides of phase I metabolites were also detected. This study indicated that local deconjugation by glucuronidases might occur in liver and mammary tissues since 8-PN aglycone was detected at low levels in tissues. However, no conversion of IX into 8-PN was detected in rats which differs from several studies in humans. For example, Bolca et al. reported in vivo conversion of IX into 8-PN in a dietary intervention study with women who received a commercially available hop supplement containing 2.4 mg XN, 1.2 mg IX and 0.1 mg of 8-PN per capsule daily for 5 days. Significant inter-individual differences in IX conversion to 8-PN were observed and attributed to differences in microbial phenotype among the study subjects. Together with previous in vitro studies, this study suggested formation of 8-PN from IX by microbial activities.

Legette et al. recently reported a PK study for XN in rats and determined the PK parameters and its major metabolites in urine (53). Three escalating dosages of XN were orally or intravenously administered to male Sprague-Dawley rats. Bioavailability of XN was found to be dose-dependent and approximately was 33%, 13% and 11% in rats for the low, medium, and high dose groups, respectively. A high amount of circulating IX at early time points suggested rapid isomerization of XN to IX. Hepatic demethylation of IX produced 8-PN which was detected at later time points (12-48 h). XN was poorly absorbed but had a long elimination half-life of 32 h. It also confirmed that glucuronides were the main circulating metabolites of XN.

Rad et al. reported a pharmacokinetic study of 8-PN after single oral administration of up to 750 mg pure 8-PN to post menopausal women. The pharmacokinetic profile of 8-PN was characterized by rapid and probably complete absorption, high metabolic stability, pronounced enterohepatic recirculation, and dose linearity (25).

Shion et al. applied matrix-assisted laser desorption ionization (MALDI) mass spectrometry to image the spatial distribution of XN and its metabolites in the rat liver, small intestine and colon after repeated oral dosing (54). XN was administered by oral gavage for 5 consecutive days at 100 mg/kg dose. Animal tissues were sectioned and analyzed using a hybrid quadrupole/time of flight instruments. XN was shown to accumulate in the liver and intestine and concentrate in the colon folds. Another interesting finding was that distribution of XN glucuronides in the colon was similar to that of XN aglycone but differed in the small intestine where glucuronides were predominantly located in the epithelial portion of the tissue. This study suggested that XN might be particularly beneficial for the prevention of cancer in the GI tract.

1.2 Endocrine Disruptor Bisphenol A (BPA)

BPA is composed of two unsaturated phenol rings (Figure 1.4). BPA is mainly used as a material for the production of epoxy resins and polycarbonate plastics (55). Epoxy resins are used as food-contact surface lacquer coatings for cans, metal jar lids and polycarbonate is used in tableware, and re-usable water bottles and baby bottles (55). BPA migrates from epoxy coatings and has been detected in canned food (56). Leaching of BPA is accelerated when canned food is cooked or sterilized at high temperatures, and the primary route of human exposure is through the food supply.

Most BPA exposure estimates have been based on the BPA concentration in food or in water (56-62). For example, Thomson et al. has reported levels of BPA in canned foods, and Fujimaki et al. estimated the intake level of BPA in Japanese pregnant women based on measurement of urinary excretion of the BPA glucuronide (63, 64). A number of analytical methods have been developed to measure BPA in biological samples and environmental media. A highly sensitive and selective method is critical for the assessment of human exposure to BPA from environmental sources.

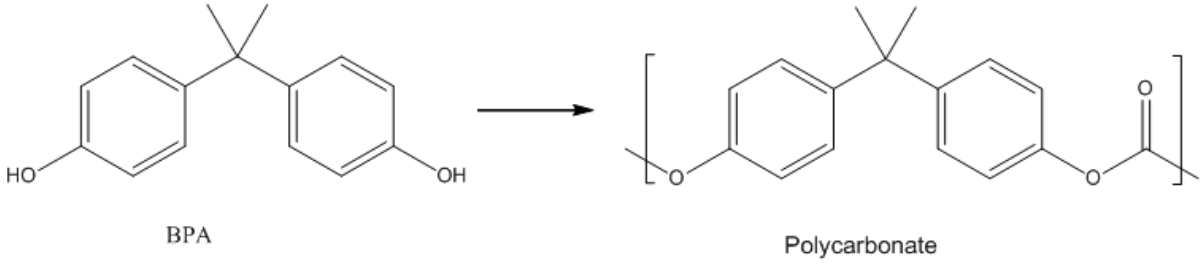


Figure 1.4. Structure of BPA and polycarbonate

1.2.1 Toxicology of Bisphenol A

Studies on the potential toxicity of BPA have focused on its endocrine disruptor action since BPA can mimic estrodiol and interfere with endocrine organ functions. BPA has been reported as non genotoxic and carcinogenic. Adverse effects have been observed at doses > 50 mg/kg/bw/day (65). However, low dose studies have indicated that due to its weak estrogenicity, BPA can have reproductive and developmental toxicity (66, 67). Vom et al. reviewed that 115 published in vivo studies on low-dose effects of BPA, and found that 94 of these reported significant effects (67). In the review, it was pointed out that some industry-funded studies have ignored the results of positive controls and many studies reporting no significant effects used a strain of rat that was inappropriate for the study of estrogenic responses. Since the relevance of low dose effects are still controversial and

humans are exposed to other endocrine disruptors that are weakly estrogenic, the European Food Safety Authority (EFSA) concluded that low dose effects of BPA posed no significant risk to human health. EFSA reported that the no-observed-adverse-effect-level of BPA induced effects in rats and mice was 5 mg/kg bw/day, and that the tolerable daily intake was 0.05 mg/kg bw/day.

1.2.2 Exposure Pathways to BPA

Inhalation exposure to BPA has generally been considered to be minor (EU, 2003) because the particle sizes of household dust are too small to result in significant uptake of BPA from lung. Wilson et al. reported an observational study of the potential exposures of preschool children to BPA at home and in daycare (68). BPA was detected in >50% of indoor air, hand wipes, solid food, and liquid food samples. Inhalation of BPA was estimated to contribute only 0.008 to 0.014 μg/person/day. In contrast, food ingestion resulted in a daily uptake of 1.7 to 2.7 μg/person/day. Therefore, children are exposed to BPA predominately through dietary ingestion.

BPA is added to thermal printing papers and receipts in the g/kg concentration range (69-71). As a result, dermal absorption of BPA was tested in skin cell models and was determined to account for 10% to 60% of the applied dose (72-74). However, this estimated high level of absorption could be due to the impaired skin integrity in the in vitro model. Daily intake of BPA through dermal absorption was estimated based on the BPA concentration and handling frequency of paper products, and it was 17.5 and 1300 ng/day for the general population and occupationally exposed individuals, respectively (71). Mielke et al. applied a physiologically based pharmacokinetic modeling to simulate BPA concentration in blood, liver and kidney after a dermal BPA dose of 0.97 μg/kg/day (worst case assumption by

Biedermann et al. (74)) (75). It was shown that even at this high level of exposure (orders of magnitude higher than the currently estimated aggregate exposure levels) dermal exposure to BPA should be safe (75). Therefore, skin contact with BPA is considered only a minor contributor to BPA levels in humans (EU, 2003)(76).

Another source of oral exposure is the use of BPA-based resins in dentistry (77-79). Fung et al. collected saliva and blood samples from male subjects immediately before dental sealant placement and at 1 h, 3 h, 1 day, 3 days, and five days after sealant placement. After measurement by HPLC, 5.8-105.6 ppb BPA was detected in saliva samples at 1 h and 3 h. BPA was not detectable in serum after 3 h. This PK study of BPA released from dental sealant indicated that BPA leaching from the sealant does not contribute significantly to the systemic circulation.

Food appears to be the main source of BPA exposure in the general. EFSA estimated that the daily intake of BPA from food ranges from 0.2 µg/kg bw/day to 13 µg/kg bw/day in 3 month old infant fed breast milk only and in 6 month old infants fed with polycarbonate bottles and commercial food, respectively. The dietary exposure to BPA in children (1.5-4.5 years old) is estimated to be 5.3 µg/kg bw/day. In adults, the dietary exposure was 1.5 µg/kg bw/day (80, 81).

1.2.3 Biotransformation of BPA

A thorough understanding of the biotransformation of BPA can help guide 1) decision making on whether urine or serum/plasma samples are more appropriate to determine the pharmacokinetics of BPA in humans; 2) analytical method development to measure parent compound or metabolites; 3) translation of serum/plasma and urine concentrations to daily intake estimation. Estimation of BPA exposure based on serum/plasma concentration would

be inappropriate if extensive metabolism occurs. Similarly, estimation based on urinary concentration would be irrelevant for hydrophobic compounds like BPA or if it is eliminated largely by biliary excretion. Generally, urine samples are more appropriate for biomonitoring since collecting urine samples is non invasive and large volumes of urine samples can be processed quickly by using solid phase extraction (SPE).

Biotransformation of BPA has been studied in laboratory animals and humans (82-93). The liver plays an essential role in BPA metabolism, and BPA is extensively metabolized to BPA glucuronide (BPA-G) by UDP-glucuronosyltransferases (UGT) isoforms in rats, monkeys and humans (Figure 1.5). Incubation of BPA with human intestine, kidney, or liver microsomes but not lung microsomes resulted in glucuronidation of BPA (94). Compared with liver, kidney and intestine contribute to less than 1% of tissue intrinsic clearance of BPA although an early study suggested that BPA was mainly glucuronidated by UGT 2B1 in rat liver microsomes (95). A recent study found that human UGT 2B15 showed the highest activity for BPA glucuronidation, followed by human UGT 1A9, UGT 2B7, UGT 1A1, UGT 2B4 and UGT 1A3 (96). These findings indicate that UGT isoforms are important for the detoxification and elimination of BPA and suggest that UGT polymorphisms might affect BPA metabolism.

Another phase II metabolite of BPA has been identified as BPA-sulfate (BPA-S) and has been found in incubation of BPA with human hepatocytes, in plasma from rats dosed intraperitoneally with BPA, and in human urine (90, 91, 97). However, the formation of BPA-S was much lower than BPA-G in cryopreserved hepatocytes from rats, monkeys and humans (91). This finding was also confirmed by studies showing that the major circulating metabolite of BPA was BPA-G. Human studies of single doses of BPA ranging from 0.31 μg/kg bw to 80 μg/kg bw showed that BPA is quantitatively recovered as BPA-G in urine within 42 h (82, 98).

Other than phase II conjugates, BPA has been shown to be metabolized in vitro to

3-hydroxy BPA (BPA catechol) (see Figure in 1.5) and to BPA-ortho-quinone by rat CYP450 enzyme (99, 100). Electrophilic BPA-ortho-quinone can react with biological nucleophiles and might result in the formation of DNA adducts. There is a possibility that BPA conjugates may be deconjugated locally in various body tissues to release BPA. For instance, treatment of human breast cells with BPA sulfate and BPA disulfate leads to desulfation via estrone sulfatases and uptake of unconjugated BPA. Because these arylsulfatases are ligand- and organ-specific, deconjugation of BPA is likely to be different throughout the body (101). Studies that have attempted to determine the pharmacokinetics of BPA metabolites in human subjects have used relatively insensitive methods, leading to additional controversy. That is because these studies have used rodent models with acute exposures to relatively high BPA doses, which do not reflect the situation in humans, where exposure is usually chronic and at low levels (67).

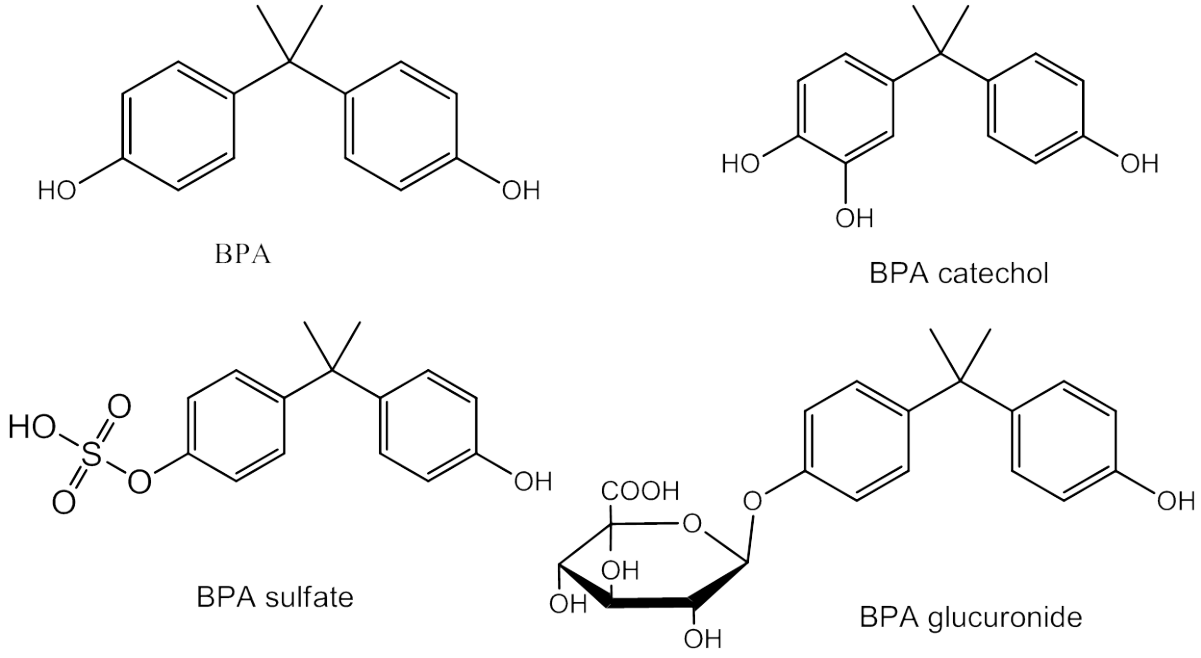


Figure 1.5 Structure of BPA, BPA catechol, BPA-sulfate and BPA-glucuronide

1.2.4 Analytical Approaches for the Measurement of BPA

Multiple analytical methods have been developed and validated for the measurement of BPA. These included enzyme-linked immunosorbent assay (ELISA), gas chromatography-mass spectrometry (GC-MS), HPLC-UV, liquid chromatography-mass spectrometry (LC-MS) and LC-tandem mass spectrometry (LC-MS-MS). The ELISA is simple, rapid and inexpensive and has been used for the measurement of BPA in environmental samples (102-104). The limit of detection (LOD) of BPA using ELISA is 1.3 nM (103). However, ELISA suffers from 1) low selectivity when used to measure BPA in complex matrices; 2) cross-reactivity with metabolites and synthetic derivatives of BPA and equol, which is a common phytoestrogen present in many clinical specimens at relatively high concentrations (105). Several studies have directly compared ELISA to LC-MS (56, 106) and concluded that ELISA is not suitable for BPA measurement in human serum because of its low concentrations in humans, interference caused by matrix and lack of specificity. During ELISA, anti-BPA antibody is not able to distinguish between BPA and BPA metabolite such as BPA-G.

Several studies report the use of GC-MS to measure BPA in environmental samples (107-111). Elaborate sample preparation including derivatization is required prior to analysis since BPA is not volatile enough in GC analysis. GC-MS analysis of BPA metabolites has not been reported due to derivatization limitations. As a result, samples containing BPA metabolites must be hydrolyzed prior to analysis in order to obtain the total concentration of BPA. Trimethylsilylation, fluorobenzylation and pentaflurobenzylation are the most commonly used derivatization methods (70, 109-112). For example, on column derivatization with pentafluorobenzyl bromide followed by GC-MS analysis was reported to show a LOD > 0.1 ng/mL in urine (112). Another method, based on stir bar sorptive extraction with in situ

derivatization and thermal GC-MS, gave LOD values of 1, 20, 100, 200 pg/mL for river water, urine, plasma and saliva samples (113, 114).

HPLC-UV is not appropriate for the detection of low level BPA in complex matrices due to the lack of sensitivity and selectivity(115). Instead of UV, HPLC has also been coupled with an electrochemical detector for the quantitation of BPA in water and human serum (115-117). The LOD obtained was quite low at 0.01 ng/mL in water and 0.05 ng/mL in serum which was more than 3000 times better than UV and 200 times better than fluorescence (115). This method has been successfully applied to measurement of BPA serum concentration in healthy human with a mean value of 0.32 ng/mL. However, whether this method might be suitable for measurement of BPA metabolites and the specificity of this method has not been addressed.

Several LC-MS and LC-MS-MS methods for the analysis of BPA and its metabolites have been reported and have shown superior sensitivity and selectivity compared with other analytical approaches (61, 82, 118-123). A summary of published LC-MS and LC-MS-MS methods for analysis of BPA is presented in Table I. Separation of BPA, BPA metabolites and matrix compounds is usually carried out using C_{18} reversed phase stationary phases with conventional 3.5 μm particle sizes. Gradient elution has been used for most LC-MS and LC-MS-MS due to the complexity of the matrix, and the MS-compatible mobile phases have contained either ammonia or ammonium acetate (pH > 7). An exception is the method of Inoue et al. (115, 120), which used 0.01% acetic acid in the mobile phase A. Separation under slightly alkaline conditions enhances the formation of deprotonated BPA due to ionization of the phenols which enhances the sensitivity of mass spectrometer detection using negative ion electrospray. A high pH mobile phase also provides good chromatographic peak shape.

BPA ionizes efficiently to form a deprotonated molecule during either electrospray or

atmospheric pressure chemical ionization (APCI). Generally, electrospray is used since APCI is unsuitable for the analysis of BPA-G and BPA-S. During APCI, BPA-G and BPA-S fragment extensively in the ion source.

Quadrupole mass spectrometers with selected ion monitoring (SIM) have been used for LC-MS analysis of BPA (120, 121). One of the major disadvantages of SIM is possible interference from co-eluting matrix compounds with the same nominal mass. To minimize matrix interference, extensive sample preparation and/or excellent chromatographic separation are required for the accurate quantitation of BPA using LC-MS with SIM.

In contrast to SIM, selected reaction monitoring (SRM) provides higher selectivity by adding the dimension of ion fragmentation and higher sensitivity by significantly lowering the background noise. During SRM, transitions from specific precursor ion and product ion pairs are recorded continuously over time during negative ion electrospray SRM analysis of BPA, the deprotonated molecule of *m/z* 227 is used as the precursor ion and is selected during the first stage of mass spectrometry. Then, collision-induced dissociation (CID) is used to fragment the ion of *m/z* 227. The most abundant fragment ion of *m/z* 212, (loss of $\cdot CH_3$) is then measured during the second stage of mass spectrometry. The transition of *m/z* 227 to *m/z* 212 is used for quantitative analysis and is called the "quantifier". For quality control, the transition of *m/z* 227 to *m/z* 133, $[M\text{-}H\text{-}C_6H_5O]^-$, is monitored as a "qualifier". The ratio of signals of the quantifier and qualifier SRM transitions should remain constant throughout the LC-MS-MS analysis of BPA standards and unknowns. Alteration in the ratio indicates matrix interference or instrument failure.

A less common fragment ion of BPA of *m/z* 211 has been reported during negative ion electrospray with CID (124). This ion corresponds to the loss of oxygen, $[M\text{-}H\text{-}O]^-$; and has

been observed only with mobile phase containing methanol. The fragment ion of *m/z* 211 is not observed when acetonitrile is substituted for methanol.

Whether using HPLC-UV, HPLC with electrochemical detection, GC-MS, LC-MS, or LC-MS-MS, the use of an internal standard enhances the accuracy and precision of the method by correcting for any analyte loss during sample preparation or changes in detector performance between analyses. It should be noted that ELISA cannot use internal standard. Mass spectrometry has an advantage over all other analytical techniques used for BPA analysis, since stable isotopically labeled internal standards-known as surrogate standards, may be used. Surrogate standards for BPA include d_{16}-BPA, d_6-BPA and $^{13}C_{12}$-BPA and have nearly identical chemical and physical properties. Deuterium labeled BPA can show small difference in HPLC retention times compared with BPA, especially when the deuterium labels are located on the rings, but ^{13}C labeled BPA co-elutes with unlabeled BPA during LC-MS-MS (82).

Although existing LC-MS-MS methods have shown LOD values as low as 0.2 nM, BPA derivatization has been evaluated to improve the LOD. Derivatization of the phenolic group of BPA with either dansyl chloride or 2,4-difluorobenzoyl chloride was carried out (119, 125). Both derivatization methods showed a 5-10 fold increase in sensitivity. However, an extra sample purification step was required to remove excess reagents and the stability of each type of derivatives needs to be evaluated.

Various sample preparation methods have been used for the analysis of BPA in serum/plasma or urine. Liquid-liquid extraction (LLE), solid phase extraction (SPE) and protein precipitation (PPT) are the most commonly used methods. For urine samples, simple dilution followed by direct injection onto LC-MS system has been used (82). To facilitate automation, on-line sample preparation with column switching has been reported (97).

However, the loading and washing buffer needs to be selected carefully to improve the robustness and sensitivity of this method. Another issue with the automated on-line sample preparation has been a carryover problem (97).

Although most analytical methods have been used only for the analysis of BPA, distinguishing BPA and BPA metabolites is essential because only unconjugated BPA is estrogenic, and BPA metabolites are also indicative of total BPA exposure. BPA is stable in serum and plasma, but some have questioned the stability of BPA-G and its possible susceptibility to nonenzymatic deconjugation during storage, sample shipping and handling (82, 126, 127). This question was answered by Volkel et al. who showed that BPA-G is stable in human plasma during storage and sample preparation (82). In order to measure the free BPA and BPA phase II conjugates, one approach has been used to analyze a sample for free BPA, treat the duplicate sample with glucuronidase and sulfatase and then analyzed the hydrolyzed sample for total BPA (118). Alternatively, direct and simultaneous measurement of BPA and BPA metabolites without enzymatic hydrolysis has been reported (128). The direct measurement approach has obvious advantages over enzymatic hydrolysis in that it is faster, simpler and less prone to contamination. However, authentic reference standards of the BPA metabolites are necessary for construction of standard curves. Ye et al. addressed the issue of BPA-G stability during sample storage by comparing the stability of BPA conjugates in human urine stored up to 6 months under different temperatures (129). BPA-G was stable at -70 °C for at least 180 days. However, significant degradation of BPA-G in urine was observed at room temperature for a short period of time. BPA-G was found to be stable in human serum during storage at 37 °C for at least 30 days (128). These findings suggest that BPA-G is more stable in serum than urine.

BPA contamination is a significant problem. Background contamination with BPA can have a huge impact on the accuracy and reproducibility of BPA quantitation. Contamination can be introduced in almost every step from the sample collection, sample storage, sample preparation or analysis. BPA contamination may result from use of tainted collection syringes, pipets, containers and solvents. At the method development stage, all solvents and reagents which will in contact with the biological samples need to be tested for possible contamination. Water generated in house by purification systems has also reported to have trace level of BPA (63, 82, 122). In summary, having a reliable source of BPA free solvents and reagents are essential for the analysis of BPA.

TABLE I. SUMMARY OF LC-MS AND LC-MS-MS METHODS FOR ANALYSIS OF BPA

Ref	Sample Matrix	Analytes	Sample Prep	Mobile Phase	Ionization	MS	Internal standard	LOD
Twaddle 2010	Rats serum Tissues, urine, feces	d_6-BPA	Enzymatic hydrolysis LLE[1] SPE[2]	Water MeOH	ESI-	QQQ[3] SRM	$^{13}C_{12}$-BPA	0.2 nM 0.4 pmol/g
Zhang 2011	Surface water	BPA	MCX (SPE)	2mM Ammonium acetate/0.5% ammonia ACN	ESI-	QQQ SRM	DES-d_8	8 nM
Chen 2011	Human amniotic fluid	BPA	SPE (Silica based bonded C_{18}) enzymatic hydrolysis	Water ACN/10%Methanol	ESI-	Ion trap SIM	$^{13}C_{12}$-BPA	0.4 nM
Gallart 2011	Soft drink	BPA, BPF,BPE, BPB,BPS	On-line SPE	Water MeOH	ESI-	QQQ SRM	d_{16}-BPA	0.8 nM
Jimenez 2010	Placental tissue	BPA/Chlorinated derivatives	LLE with ethyl acetate	0.1%ammonia 0.1%ammonia in MeOH	APCI-	QQQ SRM	d_{16}-BPA	0.8 nmol/g
Yi 2010	Breast milk	BPA	LLE with 2-propranol	Water ACN	ESI-	QQQ SRM	BPB	1.7 nM
Chang 2010	Plasma	BPA / 10 OH-PBDEs 15 BRPs	Dansyl chloride derivatization LLE 3-Propanol hexane/MTBE	Water ACN	ESI+	QQQ SRM	d_{16}-BPA	0.4 nM
Geens 2009	Indoor dust	BPA TCS TBBPA	Liquid solid extraction with n-hexane/acetone 3:1	Water ACN	ESI-	QQQ SRM	$^{13}C_{12}$-BPA	13 nmol/g
Tominaga 2008	Rats,monkey serum	BPA	SPE Oasis HLB Derivatization with 3,4-difluorobenzoyl chloride	Water ACN	ESI-	QQQ SRM	$^{13}C_{14}$-BPA	0.8 nM
Inoue 2002	Human semen	BPA	SPE Shodex SPEC EDS-1	0.01% acetic acid in water ACN	ESI-	Q^4 SIM	d_{16}-BPA	0.4 nM
Volkel 2002	Human blood and urine	d_{16}-BPA / d_{16}-BPA-G	Enzymatic hydrolysis/Protein precipitation	Water MeOH with 2mM ammonium acetate	ESI-	QQQ SRM	bis-(4-hydroxyphenyl)-methane	6nM in urine 10nM in blood

TABLE I. (CONTINUED) SUMMARY OF LC-MS AND LC-MS-MS METHODS FOR ANALYSIS OF BPA

Ref	Sample Matrix	Analytes	Sample Prep	Mobile Phase	Ionization	MS	IS	LOD
Inoue 2003	Human urine	BPA	Size exclusion flow extraction	0.01% Acetic acid ACN	ESI-	Q SIM	$^{13}C_{12}$-BPA	0.4 nM
Maragou 2006	Milk	BPA	SPE	Water/MeOH	ESI-	Ion trap SIM	d_{16}-BPA	3 nM
Volkel 2005	Human plasma/urine	BPA/BPA-G	Enzymatic hydrolysis/Protein precipitation	Water/ACN	ESI-	QQQ SRM	d_{16}-BPA-G d_{16}-BPA	5 nM

Abbreviations:

FA = formic acid; AA= acetic acid; IS= internal standard; LOD= limit of detection,

1. LLE = liquid-liquid extraction

2.SPE = solid phase extraction

3.QQQ = triple quadrupole MS

4. Q = single quadrupole MS

CHAPTER 2

QUANTITATIVE ANALYSIS OF HOP PRENYLFLAVONOIDS IN HUMAN SERUM USING UHPLC-MS-MS

2.1 Introduction

Hops (*Humulus lupulus* L.) are used in the brewing industry as a preservative and as a flavoring agent for beer. Among the active constituents of hops, prenylated polyphenols have attracted the most interest due to their broad biological activities (35, 130). XN, a prenylated chalcone, is abundant in hop cones (female inflorescences) and is under investigation as a cancer chemoprevention agent (37, 131, 132). Hop preparations containing the prenylflavonoids 8-PN, 6-PN and IX are estrogenic (18-20, 133) and are under investigation as alternatives to traditional hormone replacement therapy for the management of menopausal symptoms in women (22).

Since intramolecular cyclization and metabolic transformation can interconvert these hop prenylflavones (43), a selective and sensitive analytical method is needed for the measurement of all these species during a single analysis. Previous methods for the quantification of hop prenylflavonoids in plasma or serum have used immunoassay (134), high performance liquid chromatography with ultraviolet absorbance detection (LC–UV) (17, 46, 47), liquid chromatography–mass spectrometry (LC-MS) (135), or LC-tandem mass spectrometry (LC-MS-MS) (40, 53, 136). Immunoassays require extensive sample preparation to remove interfering, cross-reactive compounds and are unable to detect the low concentrations of prenylflavonoids often occurring in serum or plasma. LC-UV and LC-MS are suitable for the measurement of prenylflavonoids in serum or plasma at high concentrations, but lack sufficient sensitivity to measure prenylflavonoids at low concentrations such as those

required for pharmacokinetics studies. We have reported a LC-MS-MS assay for the measurement of XN, IX and 8-PN in human serum, but this assay did not include 6-PN and required more than 20 min per analysis (40).

In the current study, we developed an ultra high pressure LC-MS-MS (UHPLC-MS-MS) method for the simultaneous quantitation of 8-PN, 6-PN, IX, and XN in human serum. This simple method uses enzymatic hydrolysis followed by liquid-liquid extraction and chromatographic separation within 2.5 min using a sub 2 μm reversed-phase C_{18} column. The use of 8-isopentylnaringenin as an internal standard, that closely resembles the analytes, together with two SRM transitions (quantifier/qualifier) to monitor each analyte helped provide superior selectivity, precision and accuracy than previous methods.

2.2 Materials and Methods

2.2.1 Chemicals and Reagents

Acetonitrile, methanol, methyl-*t*-butyl ether were purchased from ThermoFisher (Rockford, IL). Water was prepared in house using Milli-Q water purification system (Billerica, MA). Formic acid (ACS reagent grade) and β-glucuronidase and sulfatase from *Helix pomatia* was obtained from Sigma-Aldrich (St. Louis, MO). Reference standards for 8-PN, 6-PN, IX, XN, and 8-pentylprenylnaringenin (IS) were obtained from the UIC/NIH Center for Botanical Dietary Supplements Research, Chicago, IL, USA. (see structures in Figure 2.1.) The purity of each reference standard was checked by qNMR. Blank human serum matrix was obtained from Bioreclamation (Hicksville, NY). Serum samples from women who had been administered a single dose of an extract of hops were obtained from the UIC/NIH Center for Botanical Dietary Supplements Research. These serum samples contained no patient identifying information.

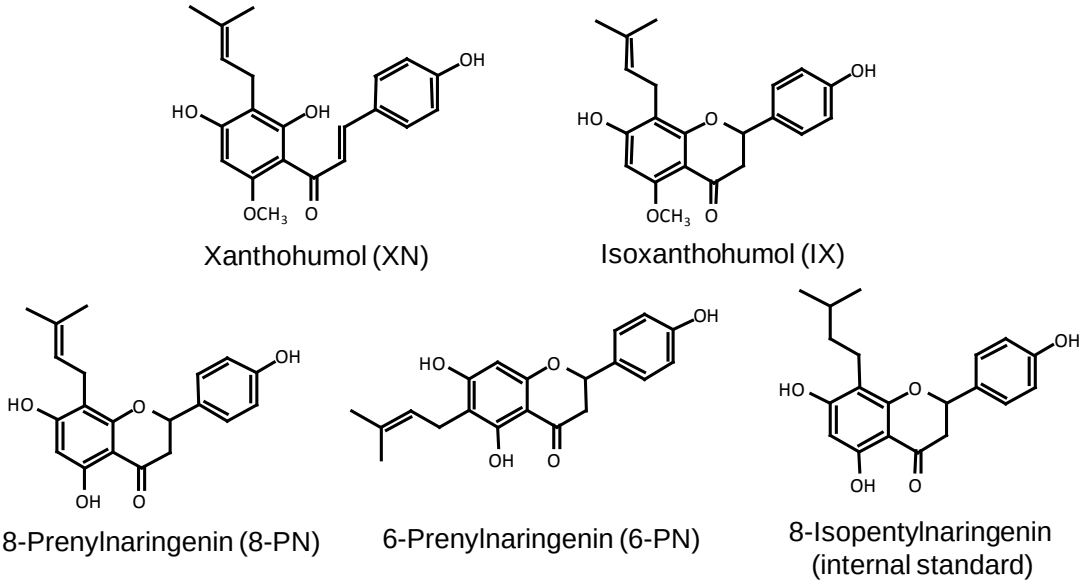


Figure 2.1. Structures of hop prenylflavonoids and internal standard.

2.2.2 Sample Preparation

Human serum samples from women participating in a Phase I clinical trial of hops were collected and stored at -80 °C until analysis. After thawing and vortex mixing, 300 µL aliquots of serum were spiked with 300 µL of 8-isopentylnaringenin (IS) (20 ng/mL) in 100 mM sodium acetate (pH 5.0) as an internal standard. Calibration standards and quality control (QC) samples were prepared by mixing 60 µL of each standard or QC solution with 300 µL blank serum and 300 µL of IS (20 ng/mL) in 100 mM sodium acetate (pH 5.0). The mixtures were extracted and processed as described below. Liquid-liquid extraction (LLE) of prenlyflavonoids from each sample was carried out twice using 2 mL portions of methyl-*t*-butyl ether. After centrifugation for 5 min at 4000 x *g* at 4 °C, the organic layers were removed, combined and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 100 µL of 70% aqueous methanol. For comparison, protein precipitation using

3-volumes of acetonitrile or solid phase extraction (Waters HLB Oasis, 3 cc, 60 mg) was also evaluated. To quantify the total amount of each prenylflavonoid, enzymatic hydrolysis of conjugates was carried out. Serum samples (300 µL) were hydrolyzed using 15 units of sulfatase and 400 units of β-glucuronidase for 1 h at 37 °C followed by liquid-liquid extraction.

2.2.3 UHPLC-MS-MS

UHPLC separations were carried out using a 1.5 min linear gradient from 45-70% acetonitrile in 0.1% aqueous formic acid followed by a hold at 70% for 0.1 min. The UHPLC column (Shimadzu XR-ODS III, 1.6 µm, 2.1 x 50 mm) (Kyoto, Japan) was re-equilibrated at 45% acetonitrile for 0.9 min before the next injection. The total run time including equilibration was 2.5 min. The flow rate was 0.5 mL/min. Shimadzu LCMS 8030 mass spectrometer (Kyoto, Japan) parameters were optimized using reference standards and flow injection. Positive ion and negative ion electrospray for the ionization of the prenylflavonoids were compared, and negative ion electrospray was selected for all subsequent experiments. Data were acquired using collision-induced dissociation and SRM of two transitions (quantifier and qualifier) for each analyte as follows: *m/z* 353 to 119 and *m/z* 353 to 233 for IX and XN; and *m/z* 339 to 119 and *m/z* 339 to 219 for 8-PN and 6-PN. The SRM transition of *m/z* 341 to 119 was monitored for the IS, and the SRM dwell time was 20 ms/ion for all species. The DL temperature was 300°C, the spray voltage was 3500 V, the nebulizing gas flow was 3 L/min, and the drying gas flow was 20 L/min.

2.2.4 Method Validation

Selectivity and Specificity

The specificity of the assay was evaluated by analyzing 6 different lots of blank human serum to check for chromatographic interference. Any peak detected using SRM at the retention time of the corresponding analyte with an area greater than 20% (5% for the IS) of the analyte at the LLOQ was considered significant interference. The LLOQ was defined as a signal to noise ratio of 10.

Precision and Accuracy

The accuracy and precision of the assay were evaluated using 3 separate analyses on 3 consecutive days. Each set of analyses included a standard curve and 5 replicates of low, medium and high QC samples. The measured concentrations of the QC samples were determined based on the corresponding calibration curves. The accuracy of the assay was defined as the percent of the mean of multiple measurements of the 3 QC samples compared with the true value. The precision of the assay was defined as the coefficient of variation calculated from multiple measurements of the QC samples at 3 different concentrations.

Recovery

The recovery of each analyte at low and high concentrations was determined 3 times and was calculated by comparing the peak area ratios of the analyte/internal standard in the extracted samples with blank matrix spiked with analyte after extraction. Internal standards were post-spiked into both the extracted samples and the extracted matrix blanks.

Matrix effects

The potential for matrix effects was investigated by spiking extracted matrix blanks (obtained from 6 different lots of human serum) with analytes and the internal standard followed by UHPLC-MS-MS analysis. The percent coefficient of variation (%CV) was calculated for the peak area ratio of each analyte at each concentration across the lots of serum. Alternatively, matrix effects were evaluated using the method of post column infusion

(137). Briefly, a 100 ng/mL solution of each analyte was constantly infused at 100 µL/min into a tee connecting the analytical column to the MS ion source. An extract of pooled blank serum after liquid-liquid extraction was injected onto the column to observe any ion suppression effects on the infused analyte signal during elution.

Stability

Bench-top stability of prenylflavonoids was examined using 3 replicates of QC samples at low and high concentrations that were stored at room temperature on the laboratory bench for 24 h. After sample preparation and analysis, the mean concentrations of each analyte were calculated and compared with their nominal concentrations. Freeze-thaw stability was tested using 3 replicates of low and high QC samples by freezing the samples at -20°C followed by thawing at room temperature for 3 cycles. Stability in the autosampler was evaluated by re-injecting extracted low and high QC samples (3 replicates each) which had been stored in the autosampler at 4 °C for 24 h. The long-term storage stability of each analyte in human serum at -80 °C was examined for 60 days. The stabilities of stock solutions at 1 mg/mL in methanol stored at -20 °C were evaluated for up to 30 days. The previously stored solutions were compared with freshly prepared stock solutions. Five replicate injections of each analyte and their internal standards were made for each stock solution.

2.2.5 HPLC-UV comparison

The new UHPLC-MS-MS method was compared with our HPLC-UV. During HPLC-UV, HPLC was carried out using a flow rate of 0.2 mL/min and a gradient from 0.1% aqueous formic acid to acetonitrile as follows: 0-3 min, 30% acetonitrile; 3-10 min, 30%-70% acetonitrile; 10-20 min 30% acetonitrile. 8-PN, 6-PN and IX were monitored at 296 nm, and

XN was measured at 368 nm. Human serum samples were analyzed that had been prepared following the same procedure as described for UHPLC-MS-MS.

2.3 Results and Discussion

Sample preparation

Three extraction approaches were evaluated including LLE, protein precipitation by acetonitrile and solid-phase extraction. Among these, protein precipitation was the fastest, but the recoveries were lower than for the other approaches (85% compared with 93% using LLE). For the low QC standards, the recovery of prenylflavonoids using SPE (91%) was similar to LLE (93%) at low QC levels but was significantly lower at high QC levels (75% compared with 96% for LLE). Since LLE consistently (±5.7) produced the highest recoveries (93%) of prenylflavonoids from human serum at all concentration levels, LLE was selected for sample extraction.

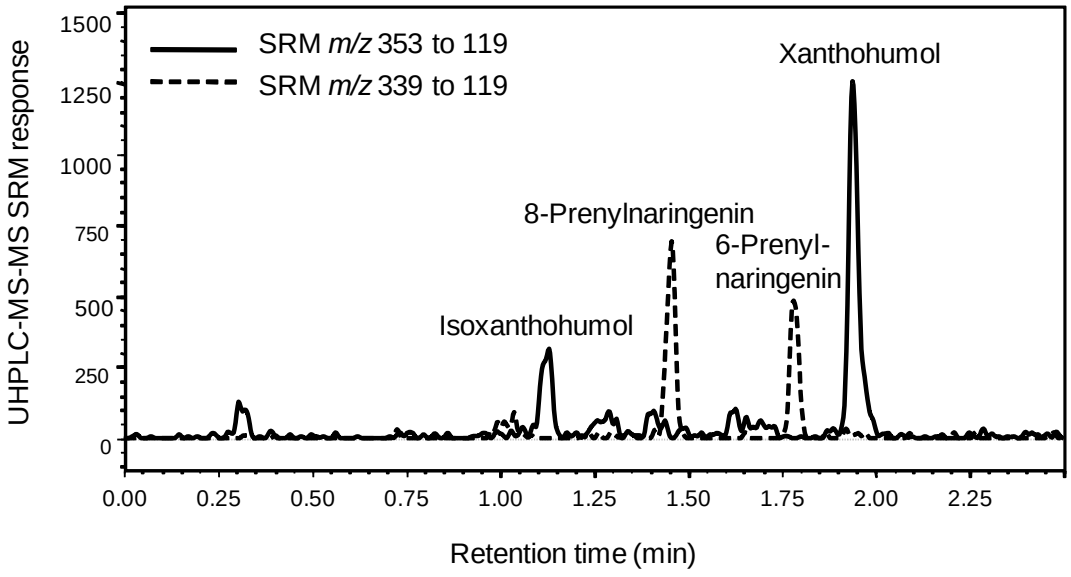


Figure 2.2. Negative ion electrospray UHPLC-MS-MS SRM chromatograms of 8-PN, 6-PN, IX, and XN standards at 1.0 ng/mL in human serum which is the LLOQ for 8-PN, 6-PN and IX.

UHPLC-MS-MS Optimization

Different organic solvents and modifiers were evaluated with respect to both sensitivity and chromatographic separation. Acetonitrile provided superior LLOQ values than did methanol and produced lower UHPLC backpressure. Adding 0.1% formic acid to the mobile phase was found to enhance ion abundance during positive ion electrospray. Using these optimized UHPLC-MS-MS parameters, baseline separation of all analytes was obtained in less than 2.5 min (Figure 2.2), which was almost 10-times faster than our previous LC-MS-MS method (40).

Although all of the prenylflavonoids could be ionized using positive ion or negative ion electrospray, negative ion mode provided 1.5-fold lower LLOQ values. The optimized ionization and MS-MS parameters are summarized in Table II for each analyte. Data were acquired by monitoring two SRM transitions (quantifier and qualifier) for each analyte, and no cross-talk between SRM channels was observed. Product ion mass spectra of each prenylated flavonoids are shown in Figure 2.3. Proposed fragmentation pathways of these prenylated flavanones in negative electrospray are shown in Figure 2.4.

TABLE II. UHPLC-MS-MS PARAMETERS FOR ANALYSIS OF HOP PRENYLFLAVONOIDS AND 8-ISOPENTYLNARINGENIN (IS).

Analyte	Retention time (min)	Precursor *(m/z)*	Product *(m/z)*	Q1 bias	Collision energy (eV)	Q3 bias
8-PN	1.4	339	119	26	35	22
		339	219	26	20	15
6-PN	1.8	339	119	28	35	23
		339	219	26	20	15
IX	1.1	353	119	28	25	24
		353	233	28	25	20
XN	2.0	353	119	28	35	23
		353	233	28	28	23
IS	1.6	341	119	28	34	23

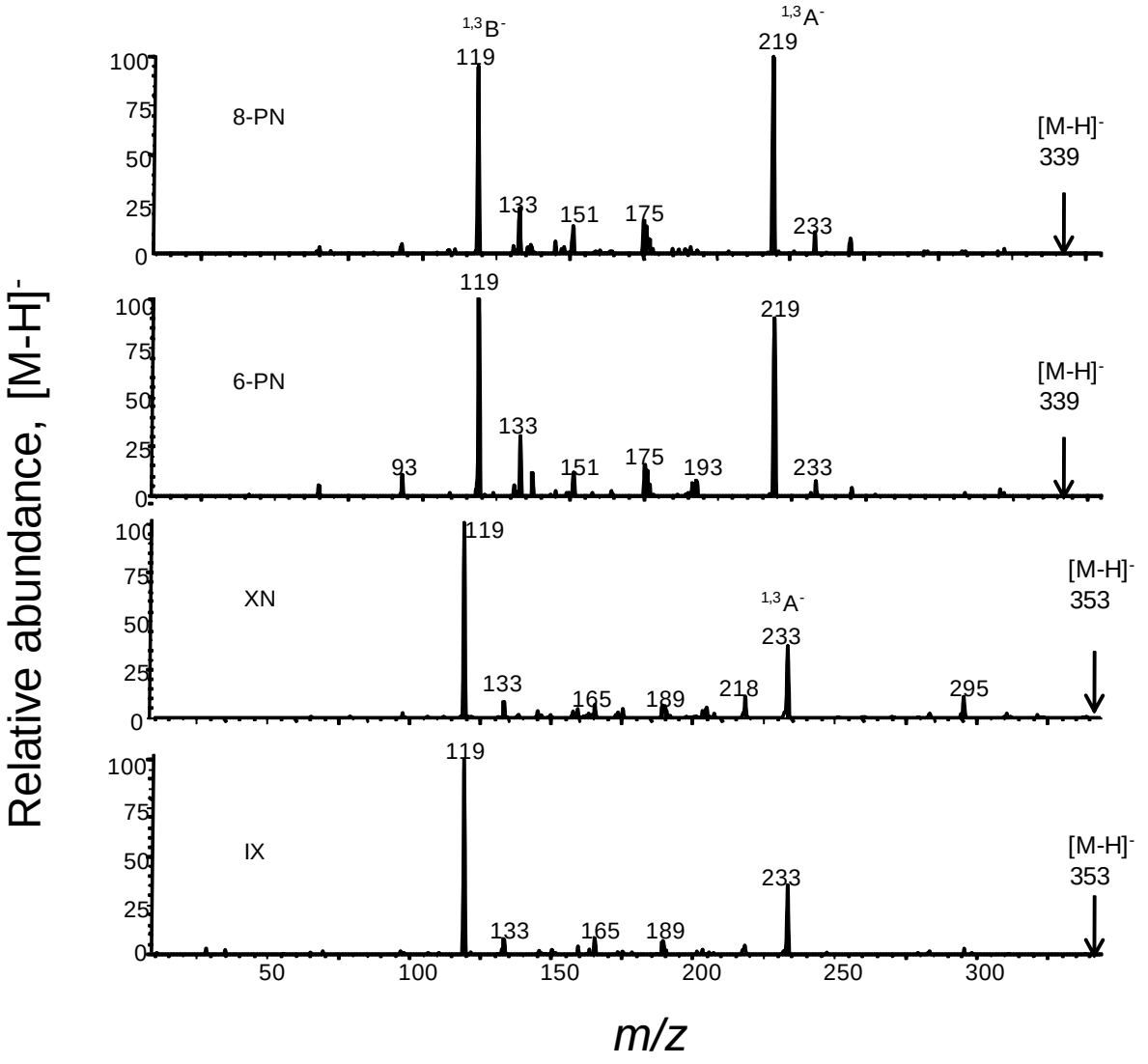


Figure 2.3: Negative ion electrospray CID product ion tandem mass spectra of prenylated flavonoids obtained using a Shimadzu LCMS-8030 triple quadrupole mass spectrometer; collision energy 35 eV; collision gas: argon. The most abundant fragmentation pathway is retro Diels-Alder (RDA) reaction to produce a $^{1,3}A^-$ ion at *m/z* 219/233 (for 6-PN/8-PN and IX/XN, respectively) and a $^{1,3}B^-$ at *m/z* 119.

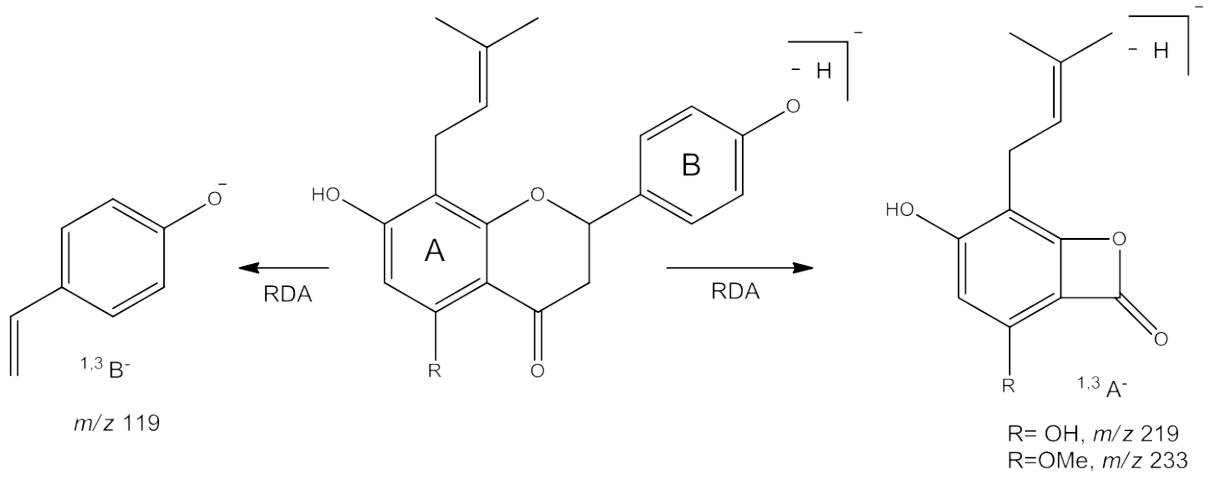


Figure 2.4. Proposed fragmentation pathways for prenylated flavanones in negative ion electrospray. Due to cyclization in the gas phase, XN showed similar fragmentation to IX. RDA = retro Diels-Alder fragmentation.

In negative ion mode, retro Diels-Alder (RDA) cleavage is the dominant pathway (see Figure 2.3). Briefly, the letter denotes the ring that retains the charge and the numbers denotes the bond broken during CID. The $^{1,3}A^-$ ion at *m/z* 219/233 (for 6-PN/8-PN and IX/XN, respectively) and the $^{1,3}B^-$ ion at *m/z* 119 were typically used for quantitative analysis (proposed fragmentation pathway is shown in Figure 2.4).

Method Validation

The linearity of the calibration curves was excellent from 1 ng/mL to 500 ng/mL (see Figure 2.5). The calibration curve for each analyte showed a coefficient of determination (R^2) >0.997 with a %Bias within ±12.5% at all concentrations (Table III). During the analysis of 6 different lots of blank human serum, no peaks were observed that could cause significant interference at the retention time of any analyte or internal standard.

Accuracy and precision were assessed over three days (Table IV). The intra-assay and inter-assay %CV were ≤15%, and the %Bias was <±13%. Overall, the %CV of the calculated concentrations from the three runs demonstrated excellent reproducibility. The lower limit of quantitation (LLOQ) for 8-PN, 6-PN, IX was 1 ng/mL (2.94 nM for 8PN and 6PN; 2.82 nM for IX) and 0.5 ng/mL for XN (1.41 nM). The limit of detection (LOD) for 8-PN, 6-PN, IX was 0.2 ng/mL (0.58 nM for 8-PN/6-PN, 0.56 nM for IX) and 0.1 ng/mL for XN (0.28 nM).

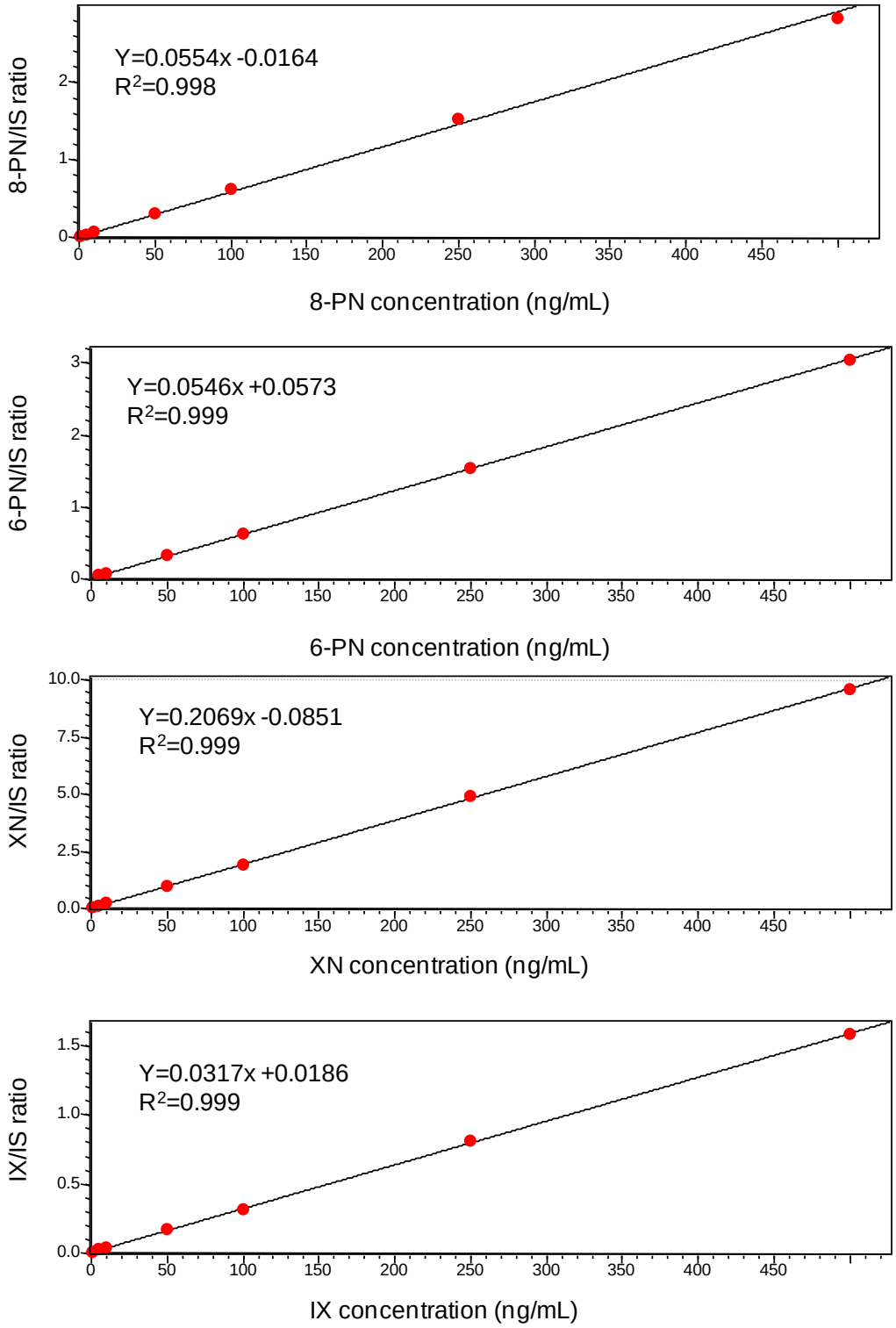


Figure 2.5. Calibration curves for 8-PN, 6-PN, XN, and IX in human serum ranging from 1 ng/mL to 500 ng/mL.

TABLE III. LINEARITY, ACCURACY AND PRECISION OF CALIBRATION STANDARDS.

	Std1 1 ng/mL	Std2 5 ng/mL	Std3 10 ng/mL	Std4 50 ng/mL	Std5 100 ng/mL	Std6 250 ng/mL	Std7 500 ng/mL	Slope	R^2
8-PN									
Mean	0.8	4.8	10.4	54.5	102.7	262.7	480.1	0.0554	0.9970
S.D	0.013	0.209	0.232	0.755	4.730	10.869	12.406	0.0049	0.0026
%CV	1.45	4.34	2.24	1.39	4.60	4.14	2.58	8.78	0.26
%Bias	-12.5	-3.9	3.5	9.0	2.7	5.1	-4.0		
6-PN									
Mean	0.9	5.1	10.9	50.2	94.9	264.3	489.7	0.0546	0.9978
S.D	0.059	0.140	0.282	2.551	4.246	10.119	7.630	0.0058	0.0020
%CV	6.52	2.75	2.59	5.08	4.48	3.83	1.56	10.56	0.20
%Bias	-9.6	2.0	8.6	0.4	-5.2	5.7	-2.1		
IX									
Mean	0.9	4.8	10.1	54.0	102.0	261.7	482.4	0.0317	0.9980
S.D	0.022	0.137	0.155	0.232	3.271	5.199	3.313	0.0047	0.0007
%CV	2.38	2.85	1.54	0.43	3.21	1.99	0.69	14.83	0.07
%Bias	-8.4	-3.9	0.9	8.1	2.0	4.6	-3.6		
XN									
Mean	0.9	5.1	9.9	51.4	103.3	265.1	480.3	0.2069	0.9973
S.D	0.031	0.153	0.405	1.026	3.890	10.170	6.622	0.0168	0.0013
%CV	3.41	3.00	4.08	2.00	3.77	3.84	1.38	8.11	0.13
%Bias	-9.1	1.5	-0.8	2.8	3.3	6.0	-4.0		

TABLE IV. INTRA-ASSAY AND INTER-ASSAY ACCURACY AND PRECISION FOR THE QUANTITATIVE ANALYSIS OF 8-PN, 6-PN, XN, AND IX.

Analyte	Nominal value (ng/mL)	Measured value (ng/mL)	Intra day N=5		Inter day N=15	
			CV%	Accuracy	CV%	Accuracy
8-PN						
Low	3	3.12	10.5	102	12.1	104
Med	50	51.6	4.7	101	7.8	103
High	160	156	5.3	95	6.2	97
6-PN						
Low	3	3.23	10.9	108	11.7	107
Med	50	48.7	4.7	96	7.8	97
High	160	163	5.3	103	4.4	101
IX						
Low	3	3.42	12.9	103	13.5	113
Med	50	56.2	4.7	94	7.8	112
High	160	169	5.3	102	4.4	105
XN						
Low	3	3.2	9.5	102	12.1	106
Med	50	52.9	4.7	99	7.8	105
High	160	152	5.3	93	4.4	95

All 4 analytes were stable on the bench-top (room temperature) for 24 h, during 3 freeze/thaw cycles, in the autosampler (4 °C) for 24 h, and during long-term storage for up to 60 days at -80 °C (Table V). The stock solution in methanol was also stable (>92%) for up to 30 days. Samples containing low and high concentrations of glucuronic acid conjugates of XN and 8-PN were hydrolyzed enzymatically for 30 min, 1 h, 2 h, or 4 h. By monitoring the appearance of each aglycone and the disappearance of each glucuronide using UHPLC-MS-MS, incubation with deconjugating enzymes for 1 h at 37 °C was found to produce complete hydrolysis of the glucuronides. Although no sulfate conjugates of hop prenylflavonoids were detected in the human serum samples, sulfatase was included during enzymatic hydrolysis just in case.

Possible matrix effects were investigated by calculating the %CV of the peak area ratio of the analyte/internal standard spiked at low and high concentrations into extracts of 6 lots of blank human serum. The %CV values were ≤ 9.5%, ≤ 10.8%, ≤ 8.2%, and ≤ 11.6% for 8-PN, 6-PN, IX, and XN, respectively. These results indicated that there was no significant matrix effect. Post-column infusion was also performed to check any ionization suppression or enhancement caused by matrix components. No significant signal suppression/enhancement was observed at the retention time of each analyte (data not shown).

TABLE V. STABILITY OF 8-PN, 6-PN, IX, AND XN DURING SAMPLE STORAGE AND HANDLING.

Storage condition	QC concentration (ng/mL)	Remaining %			
		8-PN	6-PN	IX	XN
Freeze/thaw 3 cycles	3	95.7±5.6	90.4±4.1	94.7±6.3	100.0±12.3
	160	103.2±12.1	94.6±11.9	99.9±2.2	96.4±2.2
Autosampler 4°C 24 h	3	98.5±8.2	92.6±5.4	99.5±8.2	95.5±6.2
	160	101.5±3.7	103.1±4.2	101.5±3.7	94.8±6.5
Bench top 24 h	3	96.9±1.1	92.6±1.9	95.4±2.6	93.2±6.8
	160	97.5±1.5	93.5±3.6	93.6±6.2	92.8±7.4
Long term -80°C 60 days	3	93.5±2.5	94.3±6.0	91.5±3.7	94.5±2.9
	160	94.8±3.8	95.2±8.2	90.5±8.6	91.3±5.6

Comparison of UHPLC-MS-MS with HPLC-UV

To illustrate the utility of the new UHPLC-MS-MS method, HPLC-UV was used to analyze the same extracts of human serum spiked with hop prenylflavonoids. The LLOQ of the HPLC-UV approach was 100 ng/mL (294 nM for 8PN and 6PN, 282 nM for XN and IX) which was 100-fold higher than that obtained using UHPLC-MS-MS. The linear range was 100 ng/mL to 5000 ng/mL. The HPLC-UV separation required 25 min (20 min plus column re-equilibration of 5 min), which was 10-fold slower than UHPLC-MS-MS.

HPLC-UV (Figure 2.6) and UHPLC-MS-MS (Figure 2.7) were then used to analyze an extract of human serum from a volunteer who had consumed capsules containing a hop extract standardized to 1.0 mg of 8-PN. Many peaks were detected during HPLC-UV analysis of the serum extract, and either no peaks eluted at the expected retention times of the hop prenylflavonoids or interfering substances with similar retention times prevented their measurement. In contrast, all 4 hop prenylflavonoids were detected during UHPLC-MS-MS (Figure 2.7), including XN (24.35 ng/mL; 68.78 nM), IX (31.98 ng/mL; 90.34 nM), 6-PN (7.47 ng/mL; 21.97 nM), and 8-PN (7.32 ng/mL; 21.53 nM). Chromatographic peak shape was excellent, and no interfering peaks were observed during UHPLC-MS-MS. Finally, the UHPLC-MS-MS method was used to analyze serum from 5 women who each consumed a single dose of a hop extract in a Phase I pharmacokinetics study. The time-concentration profile of 8-PN, 6-PN, IX, and XN in serum for the first 24 h following administration of the hop extract is shown in Figure 4.4 in Chapter 4. Except at baseline, all of the analytes were detected at levels above the LLOQ in each serum sample for up to 24 h after enzymatic hydrolysis.

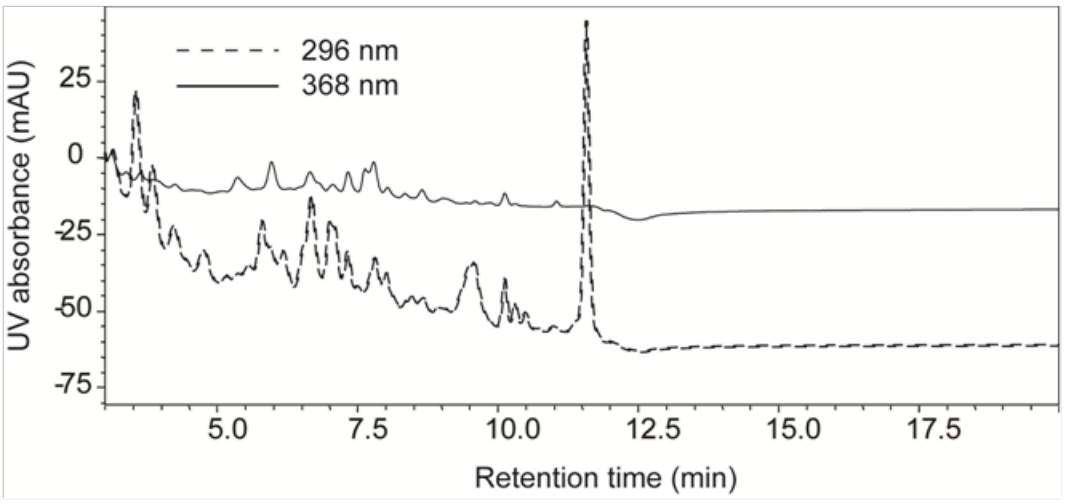


Figure 2.6. HPLC-UV measurement of serum obtained from a volunteer 2 h after consumption of a hop extract standardized to 1 mg 8-PN. Although many peaks were detected, none corresponded to the retention times of XN, IX, 6-PN, or 8-PN. The solid line represents UV absorption at 368 nm (for XN), and the dashed line represents UV absorption at 296 nm (for IX, 6-PN and 8-PN).

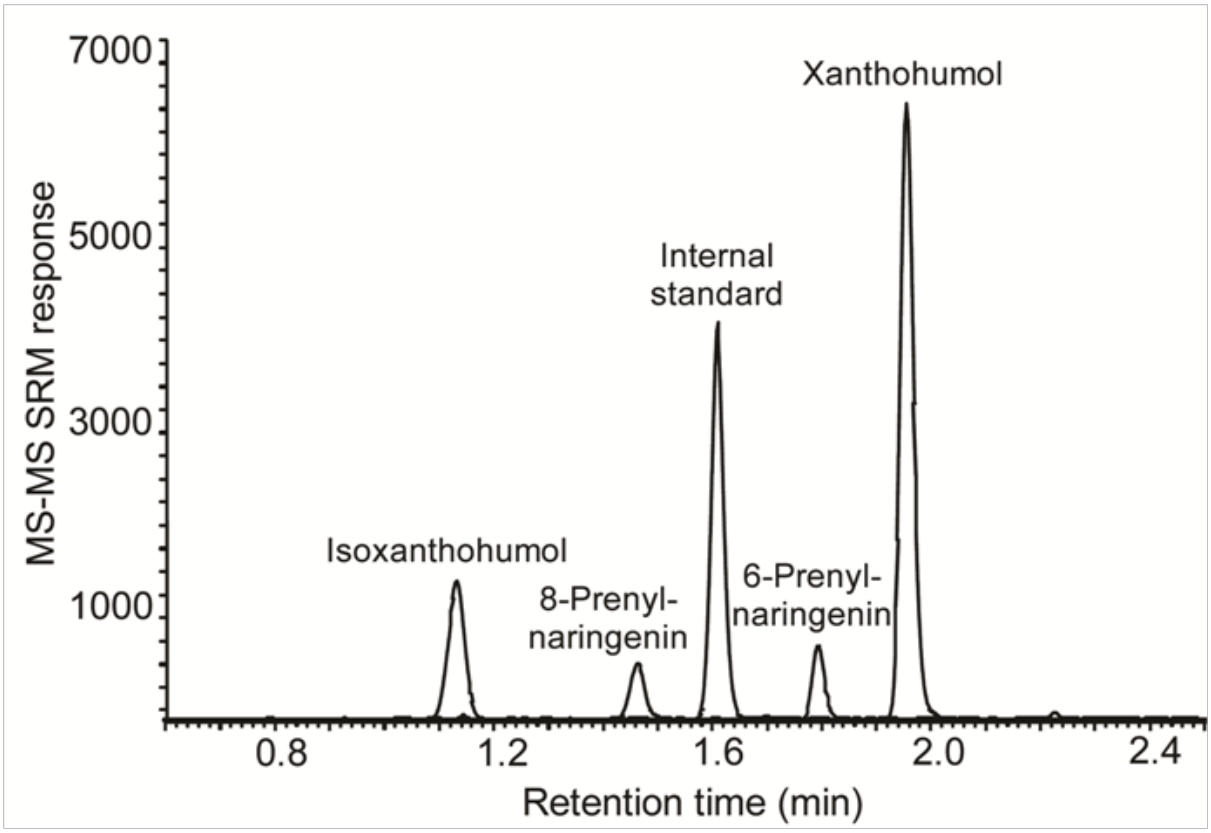


Figure 2.7 UHPLC-MS-MS chromatogram of the same human serum extract analyzed using HPLC-UV in Figure 2.6 that was obtained from a volunteer 2 h after consuming a hop extract standardized to 1 mg 8-PN. Unlike HPLC-UV, all 4 prenylflavonoids produced signals above the LLOQ during UHPLC-MS-MS.

In conclusion, an analytical method was developed and validated based on UHPLC-MS-MS for the measurement of 4 hop prenylflavonoids in human serum. Compared with other existing methods, UHPLC-MS-MS significantly increases the speed and sensitivity of analysis while maintaining good accuracy and precision. The new UHPLC-MS-MS method was found to be suitable for the measurement of prenylflavonoids in the serum of human subjects in support of a Phase I clinical trial.

CHAPTER 3

STANDARDIZATION OF HOP EXTRACT FOR PHASE I CLINICAL STUDIES

3.1 Introduction

Standardization of a botanical dietary supplement is an essential step to achieve a safe and reliable product. Typically, chemical standardization is used to provide a consistent level of active constituents and predictable pharmacological effects. *Humulus lupulus* L. (hops) is a rich source of prenylated flavonoids (138), and standardization is usually based on 8-PN which exhibits estrogenic effects in a number of in vitro and in vivo studies (13, 14). Most commercially available hop extracts are aqueous ethanol or methanol extracts of spent hops, which is what remains after extraction of bitter acids from hop cones using supercritical carbon dioxide. Česlová et al reported a HPLC-UV and LC-MS method for quantitative analysis of hop prenylflavonoids and bitter acids in beer and hop extracts (138). The limit of detection for LC-UV was 2-10 fold less of the LC-MS analysis. In this Chapter, a new method using LC-tandem mass spectrometry for the quantitative analysis of hop prenylflavonoids were developed for the standardization of a hop extract for Phase I clinical study and described herein.

3.2 Materials and Methods

XN was isolated from hops and purified as previously described (133). The purity was determined to be >99.5% purity by qHNMR. IX (> 99% pure by qNMR) was prepared by cyclization of XN as described previously (50). 8-PN was chemically synthesized, and 6-PN was purified as previously reported (18). Organic solvents were HPLC grade and purchased from Fisher Scientific (Fair Lawn, NJ). The hop extract for a clinical phase I study (BC#402)

was developed by collaboration of the UIC/NIH Botanical Center with Hopsteiner (New York, NY) and was supplied by Hopsteiner.

3.2.1 Sample Preparation

IX, XN, 8-PN and 6-PN (see structures in Figure 2.1) were dissolved in methanol at a concentration of 1 mg/mL to make the stock solutions. Further dilution to the desired concentration was made by using 50% aqueous methanol. The calibration curves for XN, IX, 8-PN and 6-PN ranged from 10 to 1000 ng/mL. Quality control (QC) samples were prepared similarly to the calibration standards at low (30 ng/mL), medium (200 ng/mL) and high (800 ng/mL) concentrations.

The test hop extract was dissolved in methanol to make the final concentration of 1 mg/mL. The solution was further diluted with 50% aqueous methanol to a final concentration of 10 µg/mL. A structural analogue of 8-PN, 8-isopentylnaringenin (see structure in Figure 2.1) was selected as an internal standard and spiked into the calibration standards, QCs and hops extract solution before LC-MS-MS analysis to compensate any loss during sample injection and ionization.

3.2.2 LC-MS-MS

An Applied Biosystems (Foster City, CA) API 4000 triple quadrupole mass spectrometer coupled with a Shimadzu (Kyoto, Japan) LC-20AD HPLC system was used for sample analysis. A Waters (Milford, MA) reversed-phase C_{18} column YM-AQ (3.5 µm, 2.1 x 100 mm) was used for the chromatographic separation of the 4 analytes and internal standard. The HPLC solvent system consisted of a 10 min linear gradient from water to acetonitrile, each containing 0.1% formic acid. The flow rate was 0.25 mL/min, and the column oven temperature was 35°C. The column was equilibrated for 10 min between analyses..

Mass spectrometer parameters were optimized for sensitivity by using infusion of reference standard solutions at 1 µM in 50% aqueous methanol. Negative ion electrospray tandem mass spectrometry was used, and the optimized parameters an ion source temperature of 350°C, spray voltage of -4200 V, curtain gas flow of 10 L/min, a gas I flow of 20 L/min, a declustering potential of -66, an exit potential of -10, and a collision energy of -31V were used for data acquisition. Data were acquired using collision-induced dissociation and selected reaction monitoring (SRM) of two transitions (quantifier and qualifier) for each analyte as follows: *m/z* 353 to 119 (quantifier) and *m/z* 353 to 233 (qualifier) for IX and XN; and *m/z* 339 to 119 (quantifier) and *m/z* 339 to 219 (qualifier) for 8-PN and 6-PN. The SRM transition of *m/z* 341 to 119 was monitored for the internal standard. The SRM dwell time was 150 ms/ion.

3.3 Results

The LC-MS-MS method showed excellent accuracy with bias <15% for the QCs and calibration standards over the range of 10 to 1000 ng/mL. The accuracy and precision of QCs are shown in Table VI.

Different dilution factors were used since analytes were in various concentrations ranges. Since the level of XN was so high in the spent hops preparation, a 10-fold dilution was required to bring the concentration of XN onto the calibration curve. The levels of the 4 hop prenylflavonoids are expressed as weight percent values of the hop extract and are summarized in Table VII.

TABLE VI. ACCURACY AND PRECISION OF QCS FOR 8-PN, 6-PN, XN, AND IX

Analyte	Nominal value (ng/mL)	Measured value (ng/mL)	N=3	
			CV%	Accuracy
8-PN				
Low	30	33.2	8.2	110
Med	200	215	7.5	107
High	800	831	9.7	104
6-PN				
Low	30	31.1	11.5	103
Med	200	204	8.4	102
High	800	758	7.3	95
IX				
Low	30	28.5	14.5	95
Med	200	186	5.8	93
High	800	842	6.7	105
XN				
Low	30	26.4	8.4	88
Med	200	224	7.2	112
High	800	830	11.1	103

TABLE VII. CONTENT OF PRENYLFLAVONOIDS IN A CLINICAL HOP EXTRACT.

Analyte	8-PN	6-PN	IX	XN
% in extract	0.42 ± 0.02	2.18 ± 0.09	1.35 ± 0.08	35.78 ± 0.31

HPLC-MS-MS chromatograms of calibration standards and the hop extract are shown in Figure 3.1 and Figure 3.2, respectively. The retention times for IX, 8-PN, 6-PN, and XN are 6.97 min, 8.98 min, 10.66 min, and 11.58 min, respectively. The 4 analytes and the internal standard were chromatographically resolved to base line under the current chromatographic conditions within 12 min. Because molecular masses and tandem mass spectra are nearly identical for the isomeric pairs XN/IX and 6-PN/8-PN, a good chromatographic separation is essential for accurate quantitation.

Calibration curves for the 4 prenylflavonoids are shown in Figure 3.3 and Figure 3.4. The calibration curves were linear from 10 ng/mL to 1000 ng/mL for all 4 analytes. The calibration curve for each analyte showed a good coefficient of determination (R^2) >0.99 with less than ±15% bias.

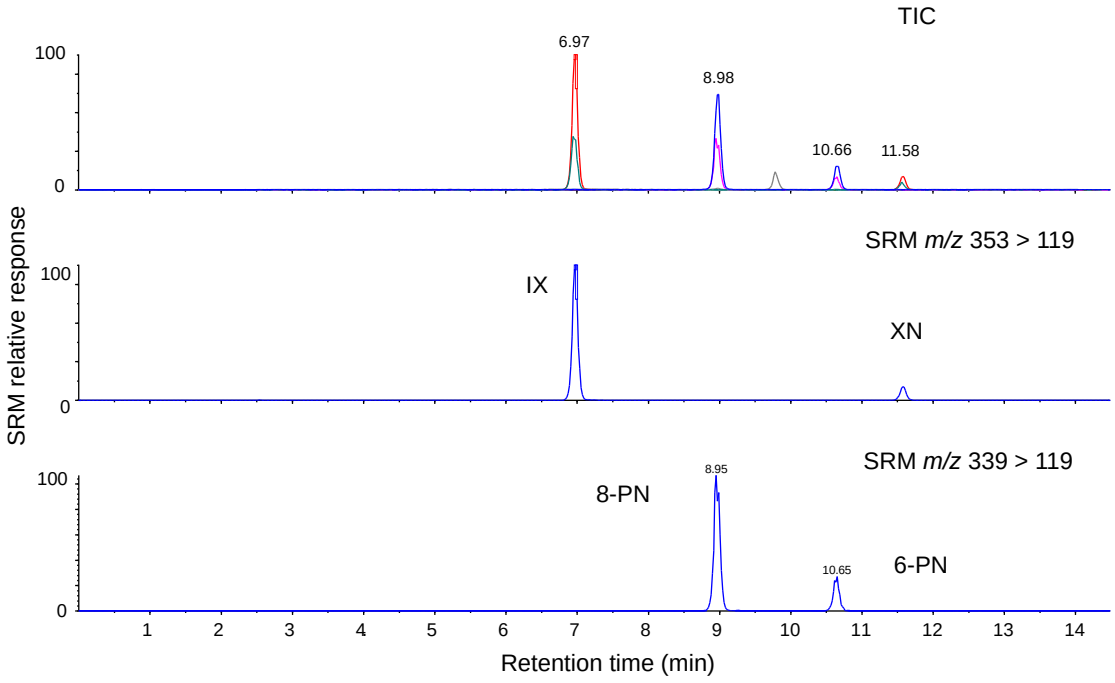


Figure 3.1. Negative ion electrospray LC-MS-MS chromatograms of IX, XN, 8-PN, and 6-PN calibration standards at 100 ng/mL each.

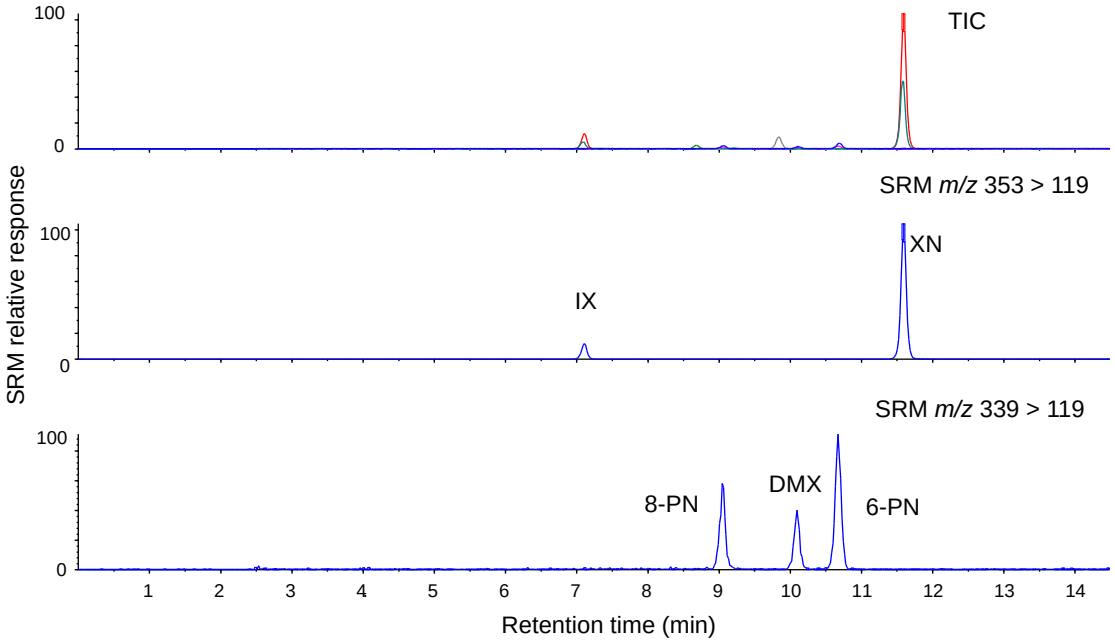
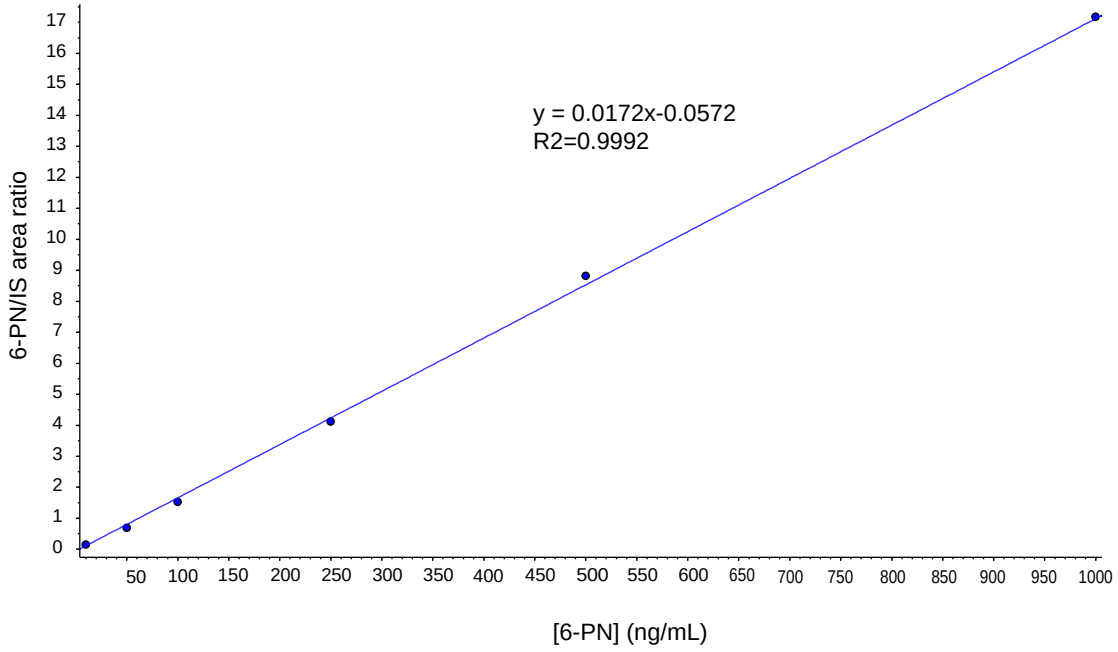


Figure 3.2. Negative ion electrospray LC-MS-MS chromatograms of the hop extract at 10 µg/mL.



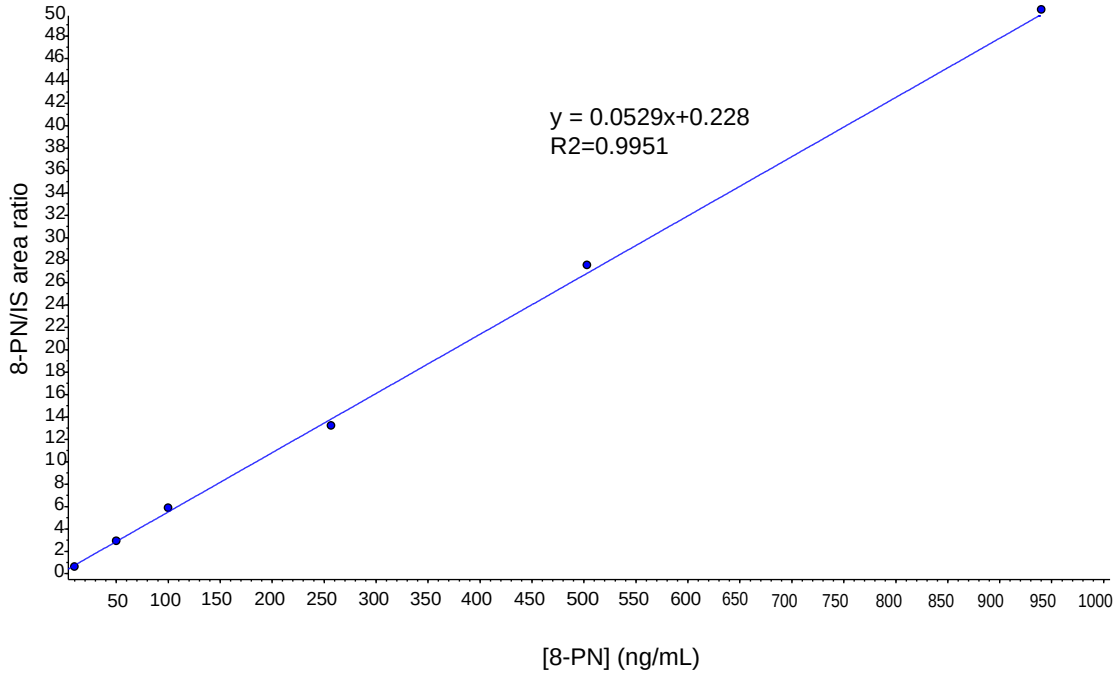
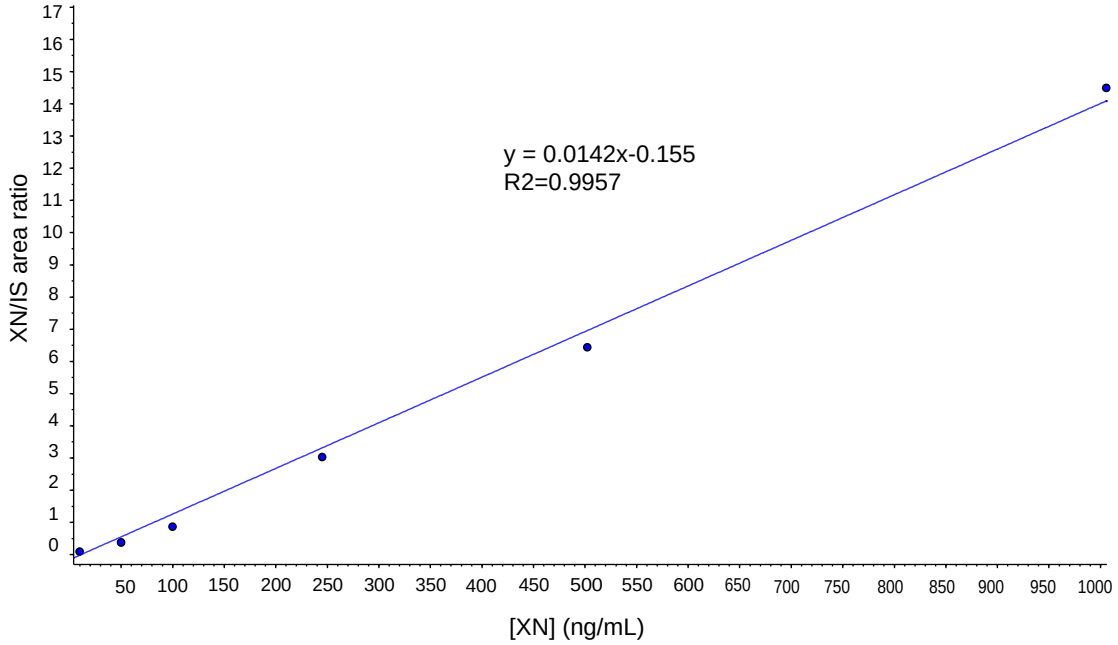


Figure 3.3. Calibration curves for 6-PN and 8-PN



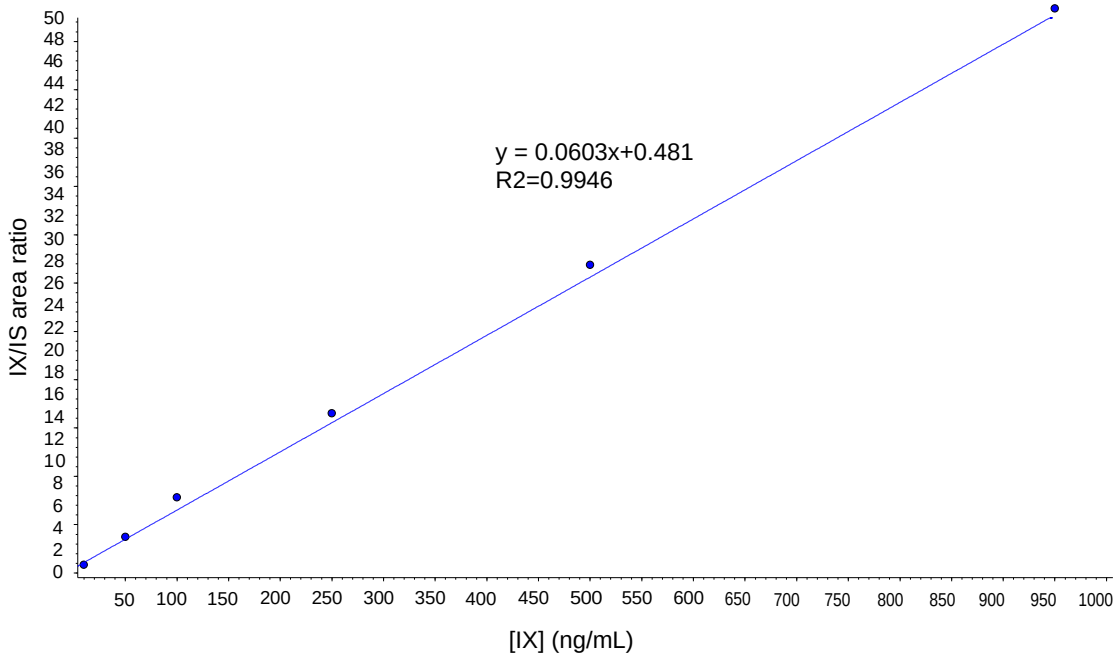


Figure 3.4. Calibration curves for XN and IX.

CHAPTER 4

PHARMACOKINETICS STUDY OF HOP PRENYLFLAVONOIDS IN POSTMENOPAUSAL WOMEN FOLLOWING ORAL ADMINISTRATION

4.1 Introduction

The female flowers of hops (*Humulus lupulus* L.) are used in the brewing industry to add flavor to beer. Recent research on hops has focused on their potential estrogenic and chemopreventive properties. Prenylated flavonoids including the prenylated chalcone XN and the prenylated flavanones 8-PN, 6-PN, and IX have received the most attention. In hop cones, the most abundant prenylated chalcone is XN which can comprise up to 1% of the dry weight (35, 36). XN exhibits strong anti-proliferative activity against breast, colon and ovarian cancer cell lines and is a potent inducer of the chemoprevention marker quinone reductase (37-39).

Prenylated flavanones are minor constituents of hops and occur at 10-100 fold lower concentrations relative to XN (35, 36). This class of compounds has been investigated primarily for their estrogenic properties. Among the prenylated flavanones, 8-PN has been identified as one of the most potent phytoestrogens (18), and its estrogenic properties have been confirmed in numerous in vitro and animal studies (40-42). IX has much weaker estrogenic activity than 8-PN (40), but enzymatic *O*-demethylation of IX to form 8-PN can be catalyzed in the human liver by cytochrome P450 1A2 (44). Interestingly, intestinal microflora have also been reported to convert IX to 8-PN (42, 46, 47). Thus, IX can be considered to be a pro-estrogen, which provides an important rationale for inclusion of this compound in the standardization of various hop extracts.

Although enzymatic demethylation of the chalcone XN has not been observed, XN can be converted to IX through acid-catalyzed cyclization in the stomach, and then IX can be

O-demethylated to form 8-PN (48). This cyclization reaction follows first-order kinetics with a half-life of 37 min. Other than that, another source of 8-PN might be isomerization of the chalcone desmethylxanthohumol (DMX), which is much less abundant in hop extract (35, 36, 49, 50). Although DMX may have chemopreventive activity (49), interest in this compound primarily comes from its conversion to the flavanones 8-PN and 6-PN.

To evaluate the absorption, metabolism, tissue distribution, and excretion of these important prenylflavonoids in vivo, several studies have been carried out in animals and humans. Overk et al. measured plasma and tissue levels of 8-PN and its major metabolites in rats after intraperitoneal injection of pure 8-PN (40). In the same study, IX was injected subcutaneously to monitor its in vivo conversion into estrogenic 8-PN. Rad et al. reported a pharmacokinetics study of 8-PN after a single oral administration of up to 750 mg pure 8-PN to post menopausal women (25). The pharmacokinetics of 8-PN was characterized by rapid and probably complete enteral absorption, high metabolic stability, pronounced enterohepatic recirculation, and tight dose linearity. Bolca et al. studied the in vivo conversion of IX into 8-PN using a commercially available supplement in capsule form administered to 21 healthy female subjects, and the disposition of XN, IX and 8-PN in human breast tissue was determined (139). Sowell et al. reported the pharmacokinetic properties of XN in humans after oral consumption of pure XN (140). Their study showed that XN was poorly absorbed, had a very long elimination half-life (32 h) and that XN-glucuronides were the main circulating forms. The same research group reported on the pharmacokinetics of XN in rats after intravenous and oral dosing (141), and their conclusions were similar to those observed in the human study, including poor absorption, long elimination half-life and extensive glucuronidation.

In this chapter, pharmacokinetics data are reported from a Phase I clinical study carried out at the UIC/NIH Center for Botanical Dietary Supplements Research using a standardized

hop extract. This is perhaps the first study examining the pharmacokinetic properties of all major hop prenylflavonoids after oral administration of a standardized hop extract to human subjects.

4.2 Materials and Methods

4.2.1 Materials

Reference standards for 8-PN, 6-PN, IX, XN, and 8-pentylprenylnaringenin (internal standard) were obtained from the UIC/NIH Center for Botanical Dietary Supplements Research (Chicago, IL). The structure and purity of each reference standard was confirmed by qNMR and mass spectrometry. Blank serum matrix was obtained from Bioreclamation Inc (Hicksville, NY). β-Glucuronidase and sulfatase from *Helix pomatia* were purchased from Sigma-Aldrich (St. Louis, MO). All solvents were HPLC grade or better and were purchased from ThermoFisher Scientific (Hanover Park, IL). The hop raw material that was used to prepare the extract, was botanically authenticated, and then the hop extract was chemically standardized to 8-PN, 6-PN, XN, and IX.

4.2.2 Clinical Design

Informed consent was obtained from each subject and the clinical protocol was approved by the Institutional Review Board (#2009-0477) and the General Clinical Research Center of the University of Illinois at Chicago. Five healthy post-menopausal women were recruited for the study. Subjects on hormone replacement therapy were required to wash out for 2 months before the study. No beer consumption was allowed up to 1 month before or during the study. One capsule containing the hop extract standardized to contain 0.25 mg 8-PN, 1.30 mg 6-PN, 0.80 mg IX, and 21.3 mg XN (low dose) was administered to each subject once daily for 5

days. The subjects were followed for 7 more days to observe any delayed effects. After a 1 month washout, the study was repeated with the same subjects using a dosage of 2 capsules/day (0.5 mg 8-PN; medium dose). After another month washout, the study was repeated a third time using the same subjects at a dosage of 4 capsules/day (1 mg 8-PN; high dose). Blood samples were obtained hourly from each subject for the first 24 h, and then once a day for the next 4 days. Urine was collected during the first 24 h post-dose. Serum and urine samples were frozen at -80°C until analysis.

4.2.3 Sample Preparation

After thawing and vortex mixing, 300 µL aliquots of serum were spiked with 300 µL of 100 mM sodium acetate (pH 5.0) containing 8-isopentylnaringenin (IS) (20 ng/mL) as an internal standard. Calibration standards and quality control (QC) samples were prepared by mixing 60 µL of each standard or QC solution with 300 µL blank serum and 300 µL of internal standard (20 ng/mL) in 100 mM sodium acetate (pH 5.0). Liquid-liquid extraction (LLE) of prenlyflavonoids from each sample was carried out twice using 2 mL portions of methyl-*t*-butyl ether. After centrifugation for 5 min at 4000 x *g* at 4 °C, the organic layers were removed, combined and evaporated to dryness under a stream of nitrogen. Each residue was reconstituted in 100 µL of 70% aqueous methanol. To quantify the total amount of each prenylflavonoid, enzymatic hydrolysis of conjugates was carried out. Serum samples (300 µL) were hydrolyzed using 15 units of sulfatase and 400 units of β-glucuronidase for 1 h at 37 °C followed by liquid-liquid extraction describe above.

Urine samples were thawed at room temperature. After vortex mixing, 1 mL of urine was mixed with 0.5 mL of 100 mM sodium acetate buffer (pH 5.0) containing internal standard (20 ng/mL) and extracted twice with 4.5 mL of methyl-*t*-butyl ether in a glass tube.

Calibration standards and quality control (QC) samples were prepared by mixing 100 µL of each standard or QC solution with 0.9 mL blank urine and 0.5 mL of internal standard (20 ng/mL) in 100 mM sodium acetate (pH 5.0) followed by liquid-liquid extraction. After centrifugation for 5 min at 4000 x *g* at 4 °C, the organic layers were removed, combined and evaporated to dryness under a stream of nitrogen. Each residue was reconstituted in 100 µL of 70% aqueous methanol. Urine samples (1 mL) were hydrolyzed using 15 units of sulfatase and 400 units of β-glucuronidase for 1 h at 37 °C followed by liquid-liquid extraction describe above.

4.2.4 UHPLC-MS-MS

A sensitive and selective UHPLC-MS-MS method was developed and validated for the quantitative analysis of XN, IX, 8-PN, and 6-PN in human serum and urine. Chromatographic separations were carried out using a Shimadzu Nexera UHPLC with a Shimadzu Shim-pack XR-ODS III (2.0 x 50 mm, 1.6 µm) C_{18} column. The solvent system consisted of a 1.5 min linear gradient from 45-70% acetonitrile/0.1% aqueous formic acid followed by a hold at 70% for 0.1 min, and then re-equilibration at 45% acetonitrile/0.1% aqueous formic acid for 0.9 min before the next injection. The total run time including equilibration was 2.5 min. The flow rate was 0.5 mL/min.

Shimadzu LCMS 8030 triple quadrupole mass spectrometer (Kyoto, Japan) parameters were optimized for sensitive detection of the reference standards using flow injection. The analytes could be ionized using either positive or negative electrospray, but the sensitivity was 1.5 times better in negative ion mode. As a result, negative ion electrospray mass spectrometry with CID and SRM were used for the measurement of each analyte. Data were acquired by monitoring two transitions (quantifier and qualifier) for each analyte as follows: IX

and XN: *m/z* 353 to 119 and *m/z* 353 to 233; 8-PN and 6-PN: *m/z* 339 to 119 and *m/z* 339 to 219; and IS: *m/z* 341 to 119. The SRM dwell time was 20 ms per transition. The DL temperature was 300°C, the spray voltage was 3500 V, the nebulizing gas flow was 3 L/min, and drying gas flow was 20 L/min.

4.2.5 Pharmacokinetics

The mean plasma concentration over time profile for each prenylflavonoid was generated using Graphpad Prism (Version 5.0, San Diego, CA). The pharmacokinetics parameters were derived using WinNonlin 6.2 (Pharsight, Mountain View, CA) for each dosage group and expressed as mean ± S.D. A non-compartment analysis was selected to calculate the kinetic parameters including area under curve (AUC), the peak serum concentration (Cmax), time to reach peak concentration (Tmax), the apparent volume of distribution (Vd/F), the elimination half-life ($T_{1/2}$), and the apparent clearance (CL/F). The AUC from time 0 to the last measureable time point defined by the LLOQ (defined as a signal-to-noise level of 10:1) was calculated using the log-linear trapezoidal rule. The half-life was calculated using the terminal elimination constant λ_z, which was derived from linear regression analysis of the serum concentrations. The half-life equation was determined using the following equation: $T_{1/2} = 0.693/\lambda_z$.

4.2.6 Desmethylxanthohumol Stability in Biological Matrix

To investigate the possible contribution of DMX to the formation of 8-PN and 6-PN during sample preparation and analysis, DMX was spiked into blank human serum and blank urine, and the subsequent formation of 8-PN and 6-PN under various sample handling conditions was monitored. DMX was dissolved in methanol to a concentration of 1 mg/mL and diluted to

working standards using methanol. A 10 µL aliquot of a working standard of 30 µM or 100 µM was spiked into 290 µL blank serum (pH 7.4) or 990 µL blank urine to make a final concentration of 1 µM. The spiked samples were incubated at 37°C for 1 h. Control samples were extracted immediately using methyl-*t*-butyl ether without any incubation. Another set of control samples without matrix were also included in the evaluation. DMX at 1 µM was left on bench top at room temperature for 2 h, at 4°C in the UHPLC autosampler for 2 h and at 37°C for 1 h. After each incubation, serum and urine samples were extracted as described above. After evaporation under a stream of nitrogen, samples were reconstituted in 100 µL of 10% aqueous methanol. Control samples were immediately extracted without incubation. Aliquots of 10 µL were injected on the UHPLC-MS-MS system for quantitative analysis of 8-PN and 6-PN as described in section 4.2.4.

4.3 Results

4.3.1 Method Validation

The validation of the UHPLC-MS-MS method for the analysis of XN, IX, 6-PN, and 8-PN in human serum is described in Chapter 2. Similar method validation for the analysis of these prenylflavonoids in human urine was carried out based on the FDA guidelines (142). The inter-day and intra-day assay accuracy and precision of the method was evaluated by using two levels of QC samples. The results are summarized in Table VIII. The intra day and inter day assay accuracy for the QCs was from 94% to 107%. The CV% values were within 15% showing the method is robust and reproducible. The calibration curves of all 4 prenylflavonoids in urine were linear from 0.5 ng/mL to 500 ng/mL with regression coefficient $R^2 > 0.99$. The LLOQ was 0.5 ng/mL for 8-PN, 6-PN, IX, and XN in human urine. The recovery of low QC (3 ng/mL) and high QC (160 ng/mL) for 8-PN, 6-PN in human urine was ~ 90% and

the recovery of low QC (3 ng/mL) and high QC (160 ng/mL) for IX and XN was ~93%. Matrix effects were examined using the post column infusion methods (method was described in details in Chapter 2.2.4). No significant ion suppression or ion enhancement was observed at the retention time for each prenylflavonoid. Blank urine sample was checked for any chromatographic interferences (see figure 4.1, bottom panel). No interference peak was detected at the retention time for each analyte.

TABLE VIII. INTRA-ASSAY AND INTER-ASSAY ACCURACY AND PRECISION FOR THE QUANTITATIVE ANALYSIS OF 8-PN, 6-PN, XN, AND IX IN HUMAN URINE.

Analyte	Nominal value (ng/mL)	Measured value (ng/mL)	Intra day N=3		Inter day N=9	
			CV%	Accuracy	CV%	Accuracy
8-PN						
	3	2.90	8.5	94	10.1	96
	160	154	4.3	92	7.4	96
6-PN						
	3	3.15	8.9	101	10.5	105
	160	165	4.2	95	5.8	103
IX						
	3	3.22	10.2	98	12.8	107
	160	170	8.5	102	10.5	106
XN						
	3	2.92	10.5	95	13.5	97
	160	154	7.8	104	8.2	96

4.3.2 Pharmacokinetics of Total Prenylflavonoids

All serum samples were analyzed using the validated UHPLC-MS-MS method. At time zero for each dosage group, no hop prenylflavonoids were detected in any of the serum samples (Figure 4.1, top panel). At some time points in the high dose subjects, unconjugated IX and XN were observed, but not unconjugated 6-PN or 8-PN . However, 8-PN, 6-PN, XN, and IX were observed in most post-dose serum samples after enzymatic deconjugation. For example, UHPLC-MS-MS analysis (Figure 4.2) of a serum sample obtained from a subject 2 h after administration of a high dose (1 mg 8-PN) of the standardized hop extract shows the presence of unconjugated IX and XN, but not 6-PN or 8-PN. UHPLC-MS-MS analysis of an aliquot of a 24-h urine collection from the same subject after a single high dose of the standardized hop extract showed unconjugated XN, IX and 8-PN (Figure 4.3). After enzymatic deconjugation, strong signals for all 4 prenylflavonoids were detected during UHPLC-MS-MS analysis of both serum and urine samples (Figure 4.2 and Figure 4.3).

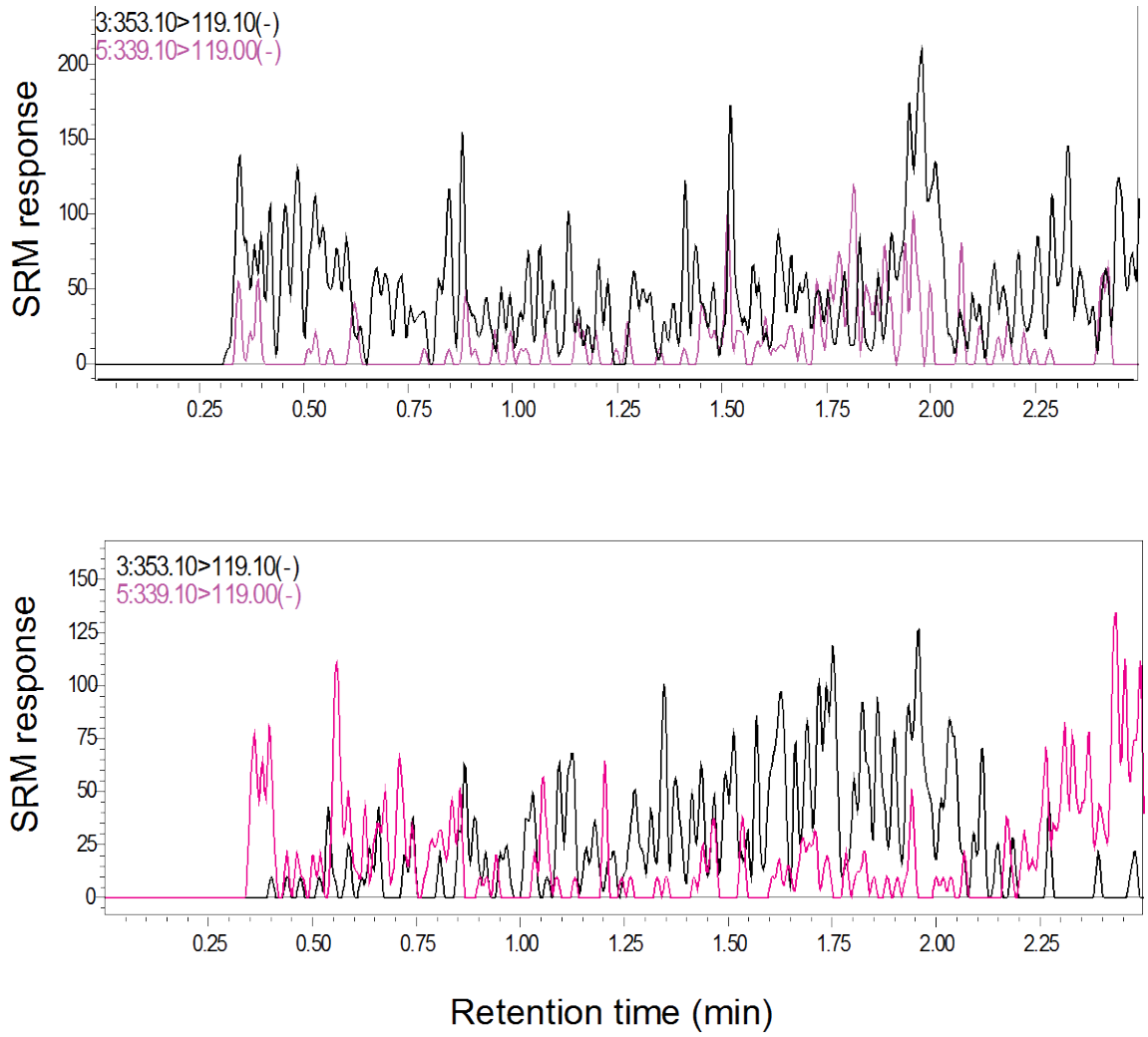


Figure 4.1. Negative ion electrospray UHPLC-MS-MS SRM chromatograms of (top) human serum obtained at baseline (t=0 h); and (bottom) blank urine.

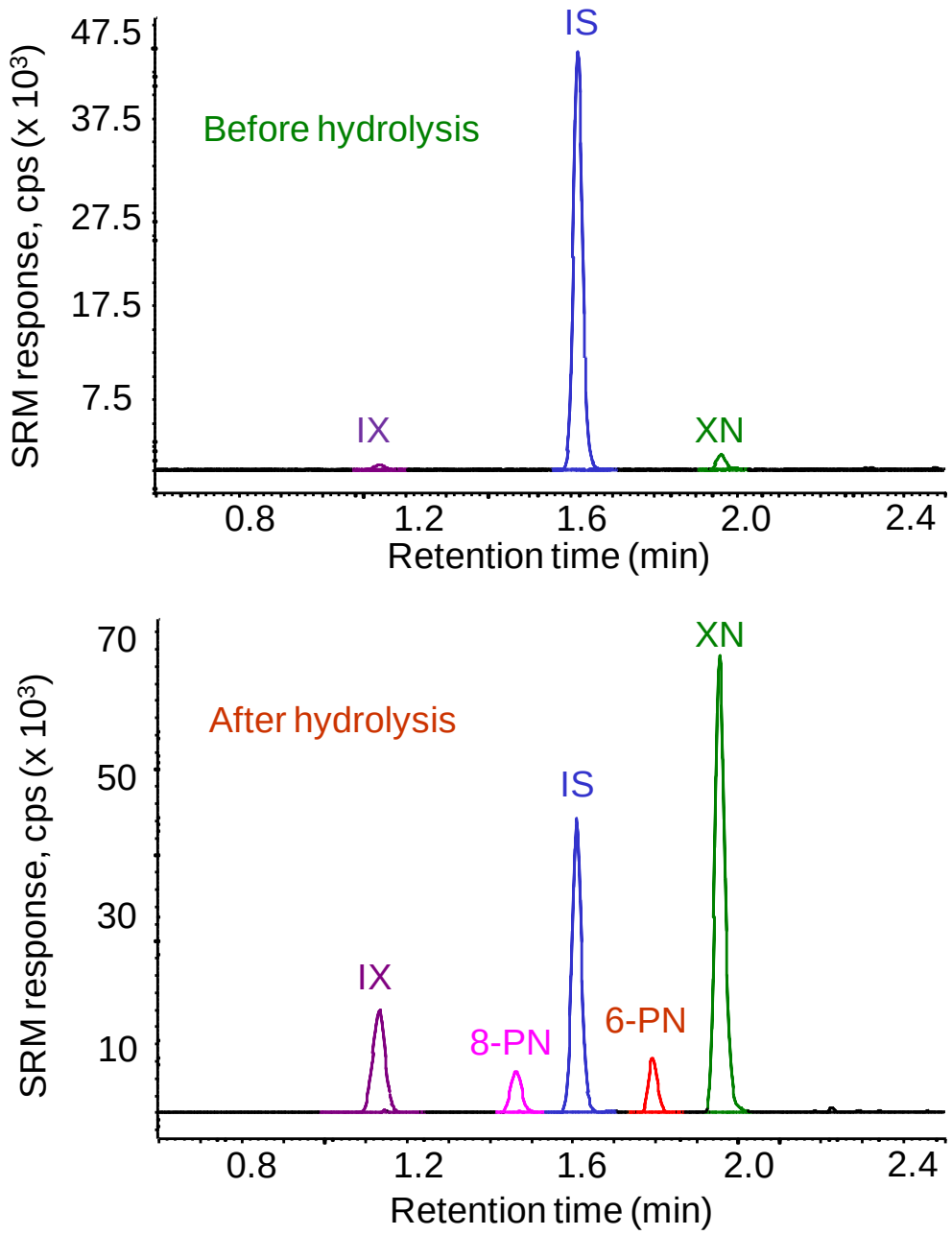


Figure 4.2. Negative ion electrospray UHPLC-MS-MS chromatograms from a serum sample obtained 2 h after administration of the highest dose (1 mg 8-PN) of the standardized hop extract. Note the large increase in the levels of prenylflavonoids after enzymatic deconjugation, indicating that conjugates were the major circulating form of these compounds.

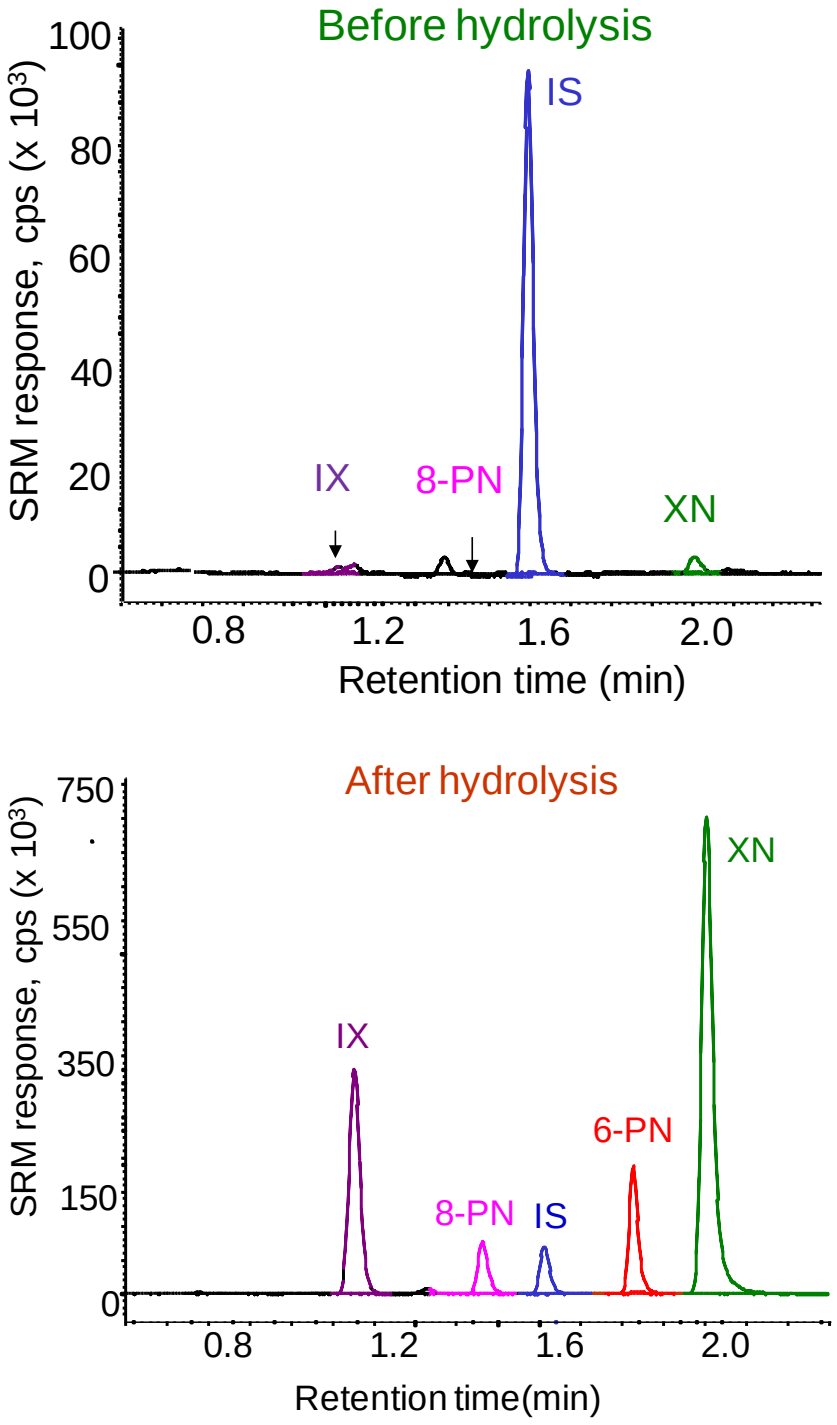


Figure 4.3. Negative ion electrospray UHPLC-MS-MS chromatograms from a 24-h urine collection obtained after consumption of the highest dose of the standardized hop extract (data are from the same patient as in Figure 4.2) before (top) and after (bottom) enzymatic deconjugation.

The mean serum concentration over time profiles for each hop prenylflavonoid after enzymatic deconjugation are shown in Figure 4.4 and the corresponding data for each individual are shown in Table IX through Table XIV. The serum concentration of the free hop prenylflavonoids before deconjugation for each individual are shown in Table XV through Table XVI. None of the serum samples from the low dose group subjects have detectable peaks for unconjugated prenylflavonoids. The pharmacokinetic parameters for each compound were calculated using a non-compartmental analysis, and the data are summarized in Table XVII through Table XX. A non-compartmental analysis was used because it provided good values for the initial estimation of PK parameters while a two-compartment model was not sufficient to fit all of the curves due to the complexity of pharmacokinetics profiles, for example, the secondary peak or the lag time in absorption. These data indicate that oral administration of the hop extract, prenylflavonoids were rapidly absorbed into the bloodstream, followed by slow elimination during the next 24 h. No elimination data are available beyond 24 h after the first dose, since second doses were administered on day 2.

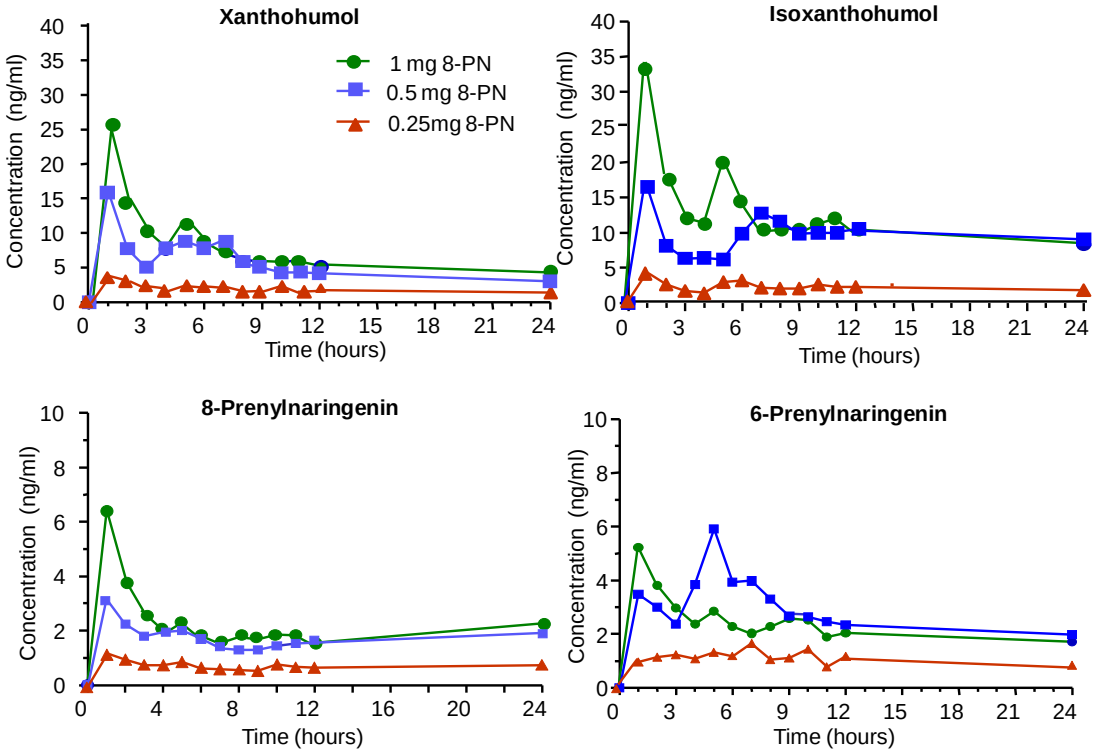


Figure 4.4. Pharmacokinetic curves of the 4 major hop prenylflavonoids following oral administration of single doses to 5 women. The curves represent total flavonoid content (free + conjugates) and are obtained by averaging concentrations from all 5 subjects. Although large inter-individual variation was observed (see Table IX to Table XIV) the error bars are not shown in these graphs to preserve clarity. Secondary peaks occurring approximately 5-6 h post-dose suggest enterohepatic recirculation. Note an increase in the concentration of 8-PN beginning12 h post-dose, indicative of in vivo formation of 8-PN.

TABLE IX. TOTAL SERUM CONCENTRATIONS OF HOP PRENYLFLAVONOIDS FOR EACH INDIVIDUAL SUBJECT AT EACH TIME POINT UP TO 24 H AFTER ORAL ADMINISTRATION OF HOP EXTRACT CONTAINING 1 MG 8-PN, 5.2 MG 6-PN, 3.2 MG IX, AND 85.2 MG XN (HIGH DOSE GROUP).

Time(h)	3001	3002	3003	3004	3006	3001	3002	3003	3004	3006
	8-PN (ng/mL)					6-PN (ng/mL)				
0	<LOD[1]	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
1	8.9	3.1	0.6	9.7	9.6	7.9	2.1	<LOD	9.1	7.0
2	5.3	1.3	0.4	4.7	7.3	5.7	2.0	<LOD	7.5	3.9
3	2.5	0.9	1.0	2.7	6.0	2.4	1.5	1.5	7.4	2.2
4	1.4	1.0	1.3	2.6	3.4	1.6	1.1	1.0	5.8	2.4
5	2.6	1.8	2.0	2.6	3.0	2.6	1.9	2.6	4.4	2.7
6	1.4	1.0	1.0	2.4	3.3	2.0	1.3	1.3	4.0	2.7
7	1.2	0.7	1.0	2.1	2.7	2.4	1.1	1.0	3.5	2.2
8	1.5	0.7	1.7	1.6	3.4	2.2	1.0	<LOD	6.7	1.7
9	1.4	0.9	1.9	1.3	3.0	1.6	1.1	1.5	7.4	1.4
10	1.5	1.1	2.1	1.4	3.2	1.8	1.6	1.8	6.3	1.1
11	1.4	1.5	1.6	1.6	3.0	1.2	1.3	1.2	4.4	1.3
12	1.2	1.0	1.2	1.5	2.6	1.6	1.2	1.5	4.8	1.0
24	1.4	1.0	1.6	2.3	4.8	1.3	1.2	1.7	3.1	1.2
	XN (ng/mL)					IX (ng/mL)				
0	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
1	32.0	24.4	5.0	38.0	29.3	53.0	16.2	4.1	52.8	43.0
2	15.4	8.1	4.9	20.1	24.3	28.5	5.6	2.8	20.2	32.0
3	5.3	4.9	5.5	10.7	24.3	11.2	3.8	3.6	13.8	28.5
4	3.1	4.9	6.7	11.2	12.7	7.0	4.7	3.9	26.1	15.5
5	10.9	11.7	14.2	13.6	9.3	19.7	27.0	5.6	38.8	11.2
6	4.6	7.3	8.3	13.3	10.8	10.3	15.2	3.1	31.0	13.3
7	4.6	5.4	5.2	11.4	9.2	7.8	9.9	2.7	23.6	8.5
8	4.0	4.3	5.7	8.6	8.2	8.1	10.4	4.9	17.5	13.1
9	3.3	3.9	7.5	5.9	8.2	7.7	11.0	7.2	13.3	12.2
10	3.1	5.5	5.7	6.1	7.8	10.8	14.0	7.7	14.8	10.6
11	2.8	7.8	5.4	7.5	7.2	16.1	13.9	5.7	18.2	8.2
12	3.4	3.2	10.8	4.8	5.3	8.0	8.5	12.3	16.2	6.6
24	2.5	2.6	6.1	4.2	6.2	7.8	3.8	8.4	16.8	4.7

1. LOD: limit of detection (0.2 ng/mL for 8-PN, and 6-PN, IX; 0.1 ng/mL for XN)

TABLE X. MEAN VALUES OF TOTAL SERUM CONCENTRATIONS OF HOP PRENYLFLAVONOIDS FOR THE 5 SUBJECTS AT EACH TIME POINT UP TO 24 H AFTER ORAL ADMINISTRATION OF HOP EXTRACT CONTAINING 1 MG 8-PN, 5.2 MG 6-PN, 3.2 MG IX, AND 85.2 MG XN (HIGH DOSE GROUP).

Post dose time (h)	8-PN	6-PN	IX	XN
	Mean ± SD (ng/mL)			
0	<LOD [1]	<LOD	<LOD	<LOD
1	6.4 ± 4.2	5.2 ± 4.0	33.8 ± 22.4	25.7 ± 12.6
2	3.8 ± 2.9	3.8 ± 2.9	17.8 ± 13.2	14.6 ± 8.1
3	2.6 ± 2.1	3.0 ± 2.5	12.2 ± 10.2	10.1 ± 8.3
4	1.9 ± 1.0	2.4 ± 2.0	11.4 ± 9.4	7.7 ± 4.1
5	2.4 ± 0.5	2.9 ± 0.9	20.5 ± 13.1	11.9 ± 2.0
6	1.8 ± 1.0	2.3 ± 1.1	14.6 ± 10.3	8.9 ± 3.3
7	1.5 ± 0.8	2.0 ± 1.1	10.5 ± 7.8	7.2 ± 3.0
8	1.8 ± 1.0	2.3 ± 2.6	10.8 ± 4.8	6.2 ± 2.1
9	1.7 ± 0.8	2.6 ± 2.7	10.3 ± 2.7	5.8 ± 2.1
10	1.9 ± 0.8	2.5 ± 2.1	11.6 ± 2.9	5.7 ± 1.7
11	1.8 ± 0.7	1.9 ± 1.4	12.4 ± 5.3	6.1 ± 2.1
12	1.5 ± 0.6	2.0 ± 1.6	10.3 ± 3.9	5.5 ± 3.1
24	2.2 ± 1.5	1.7 ± 0.8	8.3 ± 5.1	4.3 ± 1.8

1. LOD: limit of detection (0.2 ng/mL for 8-PN, and 6-PN, IX; 0.1 ng/mL for XN)

TABLE XI. TOTAL SERUM CONCENTRATION OF HOP PRENYLFLAVONOIDS FOR EACH INDIVIDUAL SUBJECT AT EACH TIME POINT UP TO 24 H AFTER ORAL ADMINISTRATION OF HOP EXTRACT CONTAINING 0.5 MG 8-PN, 2.6 MG 6-PN, 1.6 MG IX, AND 42.6 MG XN (MEDIUM DOSE GROUP).

Time (h)	2001	2002	2003	2004	2006	2001	2002	2003	2004	2006
	8-PN (ng/mL)					6-PN (ng/mL)				
0	<LOD [1]	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
1	1.3	<LOD	1.7	8.2	4.6	1.6	ND	1.7	4.4	9.7
2	1.0	<LOD	1.2	5.7	3.3	1.4	1.2	1.0	2.0	9.8
3	1.4	<LOD	0.9	4.9	2.0	1.4	1.3	1.0	1.6	6.8
4	1.8	1.0	1.6	2.9	2.5	1.8	8.1	1.0	1.0	7.5
5	1.0	2.0	2.4	2.0	2.6	1.5	16.9	1.0	1.0	9.2
6	1.0	1.0	2.4	1.4	2.9	1.8	5.6	1.6	1.0	9.7
7	1.0	0.3	1.4	1.5	2.8	1.5	4.2	3.0	0.9	10.3
8	1.0	0.5	1.7	1.2	2.2	2.0	4.6	1.8	1.1	7.0
9	1.0	0.7	1.5	0.9	2.6	1.6	3.9	1.3	0.9	5.5
10	1.0	1.1	1.8	0.7	2.8	1.4	4.4	1.9	0.7	4.6
11	1.0	0.9	1.9	1.0	3.1	1.4	4.4	1.7	0.7	4.0
12	1.2	0.6	1.5	1.0	3.5	1.9	3.6	1.2	0.7	4.4
24	1.1	<LOD	2.9	1.4	4.2	1.6	3.0	1.0	0.7	3.6
	XN (ng/mL)					IX (ng/mL)				
0	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
1	6.8	<LOD	6.0	28.2	39.4	6.9	<LOD	7.3	42.4	26.0
2	2.8	1.2	4.0	11.8	18.8	2.7	<LOD	3.8	21.3	13.0
3	5.8	1.3	3.1	7.5	7.5	6.6	<LOD	2.9	17.2	5.3
4	7.4	8.1	4.7	5.5	13.4	6.6	3.8	3.2	10.1	8.4
5	4.2	16.9	3.9	3.9	15.2	3.5	5.9	3.1	10.7	7.8
6	4.6	5.6	9.9	4.9	14.2	4.9	1.8	8.6	26.8	7.3
7	4.9	4.2	19.0	4.2	12.3	4.4	1.0	12.9	40.1	5.7
8	4.3	4.6	8.9	3.8	6.4	3.5	1.0	14.2	36.5	3.1
9	3.3	3.9	9.2	2.7	6.0	2.7	1.5	11.5	30.9	2.7
10	4.7	4.4	7.0	2.2	2.9	3.2	2.2	13.2	27.4	3.8
11	3.9	4.4	7.7	2.7	3.0	3.2	3.8	11.6	27.9	3.3
12	6.2	3.6	4.6	2.2	4.3	5.7	2.4	10.1	30.3	3.6
24	4.1	3.0	2.3	1.1	4.4	6.4	3.6	4.9	28.4	2.1

1. LOD: limit of detection (0.2 ng/mL for 8-PN, and 6-PN, IX; 0.1 ng/mL for XN)

TABLE XII. MEAN VALUES OF TOTAL SERUM CONCENTRATIONS OF HOP PRENYLFLAVONOIDS FOR THE 5 SUBJECTS AT EACH TIME POINT UP TO 24 H AFTER ORAL ADMINISTRATION OF HOP EXTRACT CONTAINING 0.5 MG 8-PN, 2.6 MG 6-PN, 1.6 MG IX, AND 42.6 MG XN (MEDIUM DOSE GROUP).

Post dose time (h)	8-PN	6-PN	IX	XN
	Mean ± S.D. (ng/mL)			
0	<LOD[1]	<LOD	<LOD	<LOD
1	3.2 ± 3.3	3.5 ± 3.8	16.5 ± 17.4	16.1 ± 16.9
2	2.2 ± 2.3	3.0 ± 3.8	8.2 ± 8.8	7.7 ± 7.4
3	1.8 ± 1.9	2.4 ± 2.5	6.4 ± 6.5	5.0 ± 2.8
4	1.9 ± 0.7	3.9 ± 3.7	6.4 ± 2.9	7.8 ± 3.4
5	2.0 ± 0.6	5.9 ± 7.1	6.2 ± 3.2	8.8 ± 6.6
6	1.7 ± 0.9	3.9 ± 3.7	9.9 ± 9.8	7.8 ± 4.2
7	1.4 ± 0.9	4.0 ± 3.7	12.8 ± 15.9	8.9 ± 6.6
8	1.3 ± 0.7	3.3 ± 2.4	11.7 ± 14.8	5.6 ± 2.1
9	1.3 ± 0.8	2.7 ± 2.0	9.9 ± 12.4	5.0 ± 2.6
10	1.4 ± 0.9	2.6 ± 1.8	10.0 ± 10.7	4.2 ± 1.9
11	1.5 ± 1.0	2.5 ± 1.7	10.0 ± 10.6	4.4 ± 2.0
12	1.6 ± 1.2	2.3 ± 1.6	10.4 ± 11.5	4.2 ± 1.5
24	1.9 ± 1.6	2.0 ± 1.3	9.1 ± 10.9	3.0 ± 1.4

1. LOD: limit of detection (0.2 ng/mL for 8-PN, and 6-PN, IX; 0.1 ng/mL for XN)

TABLE XIII. TOTAL SERUM CONCENTRATION OF HOP PRENYLFLAVONOIDS FOR EACH INDIVIDUAL SUBJECT AT EACH TIME POINT UP TO 24 H AFTER ORAL ADMINISTRATION OF HOP EXTRACT CONTAINING 0.25 MG 8-PN, 1.3 MG 6-PN, 0.8 MG IX, AND 21.3 MG XN (LOW DOSE GROUP).

Time (h)	1001	1002	1003	1004	1006	1001	1002	1003	1004	1006
	8-PN (ng/mL)					6-PN (ng/mL)				
0	<LOD[1]	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
1	1.0	1.3	0.8	1.6	<LOD	<LOD	1.5	0.4	<LOD	<LOD
2	<LOD	1.1	0.8	0.9	<LOD	<LOD	1.9	0.5	<LOD	<LOD
3	<LOD	1.1	0.5	0.6	<LOD	<LOD	2.1	0.4	<LOD	<LOD
4	<LOD	1.2	0.5	0.5	<LOD	<LOD	1.7	0.5	<LOD	<LOD
5	<LOD	1.5	0.5	0.6	<LOD	<LOD	2.2	<LOD	0.4	<LOD
6	<LOD	1.1	0.4	0.4	<LOD	<LOD	1.9	0.5	<LOD	<LOD
7	<LOD	1.0	0.4	0.4	<LOD	<LOD	1.6	<LOD	<LOD	<LOD
8	<LOD	0.8	0.5	0.4	<LOD	<LOD	1.4	0.7	<LOD	<LOD
9	<LOD	0.9	0.6	0.4	0.2	<LOD	1.7	0.5	<LOD	<LOD
10	<LOD	0.9	1.1	0.4	<LOD	<LOD	1.9	1.0	<LOD	<LOD
11	<LOD	0.8	0.7	0.5	<LOD	<LOD	0.8	0.8	<LOD	<LOD
12	<LOD	0.8	0.6	0.5	<LOD	<LOD	1.7	0.6	<LOD	<LOD
24	0.9	0.9	0.7	0.5	<LOD	<LOD	1.8	0.5	0.2	<LOD
	XN (ng/mL)					IX (ng/mL)				
0	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
1	2.8	6.0	6.5	4.6	0.1	2.4	4.3	2.5	8.5	<LOD
2	1.8	6.2	2.9	2.3	<LOD	1.2	2.8	2.1	4.7	<LOD
3	1.3	5.3	2.2	1.1	0.1	0.6	2.9	1.0	3.5	0.6
4	1.5	4.1	2.1	0.5	0.1	1.4	3.1	0.7	1.8	0.4
5	1.7	8.0	1.9	1.2	0.1	1.4	7.2	1.4	4.8	0.3
6	2.0	5.7	2.8	1.0	0.1	1.9	4.8	2.2	4.0	<LOD
7	2.0	4.7	2.9	0.8	0.1	1.6	3.4	2.2	3.4	0.5
8	1.3	2.5	2.2	0.8	0.2	1.2	2.9	2.6	3.3	0.5
9	1.5	2.8	2.8	0.6	0.2	1.3	3.0	2.9	2.9	0.5
10	1.8	2.9	5.8	0.9	0.2	1.4	3.3	4.8	3.3	0.5
11	<LOD	2.7	2.5	0.7	0.4	1.5	2.8	3.3	3.3	0.8
12	1.6	2.3	2.1	0.7	0.3	1.1	2.9	2.5	4.2	0.7
24	<LOD	2.8	1.6	0.2	0.2	1.0	3.5	1.4	2.5	0.8

1. LOD: limit of detection (0.2 ng/mL for 8-PN, and 6-PN, IX; 0.1 ng/mL for XN)

TABLE XIV. MEAN VALUES OF TOTAL SERUM CONCENTRATIONS OF HOP PRENYLFLAVONOIDS FOR THE 5 SUBJECTS AT EACH TIME POINT UP TO 24 H AFTER ORAL ADMINISTRATION OF HOP EXTRACT CONTAINING 0.25 MG 8-PN, 1.3 MG 6-PN, 0.8 MG IX, AND 21.3 MG XN (LOW DOSE GROUP).

Post dose time (h)	8-PN	6-PN	IX	XN
	Mean ± S.D. (ng/mL)			
0	<LOD[1]	<LOD	<LOD	<LOD
1	1.2 ± 0.4	1.0 ± 0.8	4.4 ± 2.9	4.0 ± 2.6
2	0.9 ± 0.2	1.2 ± 1.0	2.7 ± 1.5	3.3 ± 2.0
3	0.7 ± 0.3	1.2 ± 1.2	1.7 ± 1.4	2.0 ± 2.0
4	0.7 ± 0.4	1.1 ± 0.9	1.5 ± 1.1	1.7 ± 1.6
5	0.8 ± 0.5	1.3 ± 1.3	3.0 ± 2.9	2.6 ± 3.1
6	0.6 ± 0.4	1.2 ± 1.0	3.2 ± 1.4	2.3 ± 2.2
7	0.6 ± 0.3	1.6 ± 0.0	2.2 ± 1.3	2.1 ± 1.8
8	0.6 ± 0.3	1.0 ± 0.5	2.1 ± 1.2	1.4 ± 1.0
9	0.5 ± 0.3	1.1 ± 0.9	2.1 ± 1.2	1.6 ± 1.2
10	0.8 ± 0.4	1.4 ± 0.6	2.7 ± 1.7	2.3 ± 2.2
11	0.7 ± 0.2	0.4 ± 0.5	2.3 ± 1.1	1.2 ± 1.3
12	0.6 ± 0.2	1.2 ± 0.8	2.3 ± 1.4	1.4 ± 0.9
24	0.7 ± 0.2	0.8 ± 0.8	1.8 ± 1.1	1.0 ± 1.2

1. LOD: limit of detection (0.2 ng/mL for 8-PN, and 6-PN, IX; 0.1 ng/mL for XN)

TABLE XV. UNCONJUGATED SERUM CONCENTRATIONS OF HOP PRENYLFLAVONOIDS FOR EACH INDIVIDUAL SUBJECT AT EACH TIME POINT UP TO 24 H AFTER ORAL ADMINISTRATION OF HOP EXTRACT CONTAINING 1 MG 8-PN, 5.2 MG 6-PN, 3.2 MG IX, AND 85.2 MG XN (HIGH DOSE GROUP).

Time(h)	3001	3002	3003	3004	3006	3001	3002	3003	3004	3006
	8-PN (ng/mL)					6-PN (ng/mL)				
0	<LOD[1]	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
3	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
4	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
5	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
6	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
7	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
8	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
9	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
10	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
11	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
12	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
24	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	XN (ng/mL)					IX (ng/mL)				
0	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
1	<LOD	0.5	<LOD	4.2	1.0	<LOD	0.9	0.5	1.9	1.1
2	<LOD	<LOD	<LOD	1.4	0.4	<LOD	0.3	0.2	0.3	0.8
3	<LOD	<LOD	<LOD	0.8	0.5	<LOD	0.2	<LOD	0.5	0.8
4	<LOD	<LOD	<LOD	0.5	0.2	<LOD	<LOD	<LOD	1.5	0.5
5	<LOD	<LOD	<LOD	0.5	<LOD	<LOD	1.0	<LOD	1.3	<LOD
6	<LOD	<LOD	<LOD	0.3	<LOD	<LOD	0.8	0.2	0.8	<LOD
7	<LOD	<LOD	<LOD	0.3	<LOD	<LOD	0.4	<LOD	0.7	<LOD
8	<LOD	<LOD	<LOD	0.2	0.2	<LOD	0.3	0.2	0.3	<LOD
9	<LOD	<LOD	<LOD	0.2	0.2	<LOD	<LOD	0.5	0.3	<LOD
10	<LOD	<LOD	0.3	0.2	0.2	<LOD	<LOD	0.4	0.2	<LOD
11	<LOD	<LOD	0.1	0.2	0.1	<LOD	<LOD	0.2	0.3	0.1
12	<LOD	<LOD	0.4	0.1	0.1	0.8	<LOD	1.7	0.4	0.1
24	<LOD	<LOD	<LOD	<LOD	0.2	<LOD	<LOD	0.4	<LOD	<LOD

1. LOD: limit of detection (0.2 ng/mL for 8-PN, and 6-PN, IX; 0.1 ng/mL for XN)

TABLE XVI. UNCONJUGATED SERUM CONCENTRATIONS OF HOP PRENYLFLAVONOIDS FOR EACH INDIVIDUAL SUBJECT AT EACH TIME POINT UP TO 24 H AFTER ORAL ADMINISTRATION OF HOP EXTRACT CONTAINING 0.5 MG 8-PN, 2.6 MG 6-PN, 1.6 MG IX, AND 42.6 MG XN (MEDIUM DOSE GROUP).

Time(h)	2001	2002	2003	2004	2006	2001	2002	2003	2004	2006
	8-PN (ng/mL)					6-PN (ng/mL)				
0	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
3	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
4	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
5	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
6	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
7	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
8	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
9	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
10	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
11	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
12	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
24	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	XN (ng/mL)					IX (ng/mL)				
0	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
1	<LOD	<LOD	0.5	1.0	1.3	<LOD	<LOD	<LOD	0.3	<LOD
2	<LOD	<LOD	<LOD	0.4	0.5	<LOD	<LOD	<LOD	<LOD	<LOD
3	<LOD	<LOD	<LOD	0.3	0.1	<LOD	<LOD	<LOD	<LOD	<LOD
4	<LOD	<LOD	<LOD	0.2	0.2	<LOD	<LOD	<LOD	<LOD	<LOD
5	<LOD	<LOD	<LOD	0.2	<LOD	<LOD	<LOD	0.2	<LOD	<LOD
6	<LOD	<LOD	1.3	0.2	<LOD	<LOD	<LOD	1.8	1.4	<LOD
7	<LOD	<LOD	1.3	0.2	<LOD	<LOD	<LOD	1.8	2.4	<LOD
8	<LOD	<LOD	0.8	0.3	<LOD	<LOD	<LOD	1.3	2.1	<LOD
9	<LOD	<LOD	<LOD	0.2	<LOD	<LOD	<LOD	<LOD	1.4	<LOD
10	<LOD	<LOD	0.8	0.2	<LOD	<LOD	<LOD	0.6	1.0	<LOD
11	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.5	<LOD
12	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.7	<LOD	<LOD
24	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

1. LOD: limit of detection (0.2 ng/mL for 8-PN, and 6-PN, IX; 0.1 ng/mL for XN)

The time required to reach the maximum concentrations of 8-PN, 6-PN, IX, and XN (Tmax) in human serum (high dose treatment group) were 2.8, 4.0, 1.8, and 1.8 h, respectively (Table XVII through Table XX). The values of Tmax were larger in medium dose group for 8-PN, 6-PN and XN which were 7.0, 5.6 and 3.6 h, respectively, indicating a delay of absorption. 8-PN has been shown to passively diffuse in to the Caco-2 cells (48) and an increasing dose might lead to higher concentration gradient thus facilitating the diffusion into the cells. Tmax was consistent for IX in all dosage groups which was ~ 4 h. The maximum concentration (Cmax) of 8-PN was dose dependent and ranged from 1.4 ng/mL to 6.7 ng/mL (Table XVII). Similar results were observed for XN, with Cmax values ranging from 4.4 ng/mL to 27.6 ng/mL (Table XVIII) and for IX, with Cmax values ranging from 5.7 ng/mL to 37.6 ng/mL (Table XIX), respectively. Cmax values for 6-PN were ranging from 1.6 ng/mL to 7.3 ng/mL The unexpectedly high Cmax at the medium dose used resulted from one subject (2002) whose serum level was higher than for the high dose.

The half-life of XN after oral administration was approximately 19 h for all dosages with a range from 18.3 h at the low dose of 21.3 mg to a 20.7 h at the highest dose of 85.2 mg. For IX, the half-life decreased from 27.5 h at the low dose of 0.8 mg to 19.9 h at the high dose of 3.2 mg. Apparently, the half-life values for IX across 3 doses were not consistent. Since the serum concentration of 8-PN was increasing from 12 h post-dose to 24 h post-dose (Figure 4.4), the slope of the elimination phase and therefore the half-life could not be determined. The accumulation of 8-PN beginning at 12 h post-dose and extended half-life indicated in vivo formation of 8-PN due to *O*-demethylation by human CYP1A2 as reported by Guo, et al. (44) or due to metabolism by gut flora during enterohepatic recirculation (46, 47, 53). Unlike 8-PN, the half-life was consistent for 6-PN across all doses, which ranges from 21 h to 24 h.

Based on the pharmacokinetics data in Table XVII and Figure 4.4, the area under the

concentration-time curve was determined for each dosage using a log-linear trapezoidal rule for each dosage of the hop extract from time zero to the last measureable time point (AUC_{0-24}). The AUC_{0-24} ratio of 8-PN after normalization by dose was 1: 1.2: 0.8 which is in agreement with Rad et al. who reported a linear relationship between AUC and dosage of pure 8-PN up to 750 mg (25). The mean value of the AUC_{0-24} (n=5) was 50.4 h·ng/mL for high dose group (1 mg 8-PN). Assuming that the AUC for 8-PN administered in a hop extract would remain linear up to a dosage of 50 mg 8-PN as used by Rad et al. (25), then the AUC_{0-24} of 8-PN for the hop extract would be 2520 h.ng/mL. This is significantly higher than the value of 453 h.ng/mL (25) obtained after a single oral dose of 50 mg 8-PN alone (25). This indicated that the hop extract provided another source of 8-PN, and confirmed that metabolic conversion of IX to 8-PN must occur to a significant extent in the liver and/or by gut flora.

The conversion of IX to 8-PN has been reported both in vitro and in vivo (17, 44, 46, 47). The *O*-demethylation of IX could occur by human CYP1A2 and/or by gut flora. The enzyme CYP1A2 is known to have polymorphisms in humans (143). Some variants of CYP1A2 showed reduced protein expression levels to 30-60% compared with wild type. Enzymatic activity was also decreased dramatically to less than 1-3% of the wild type (143). Moreover, CYP1A2 can be induced or inhibited by various environmental factors, for examples, prescription drugs, alcohol consumption, and smoking (144, 145). Such polymorphism and individual factors could have a direct impact on the conversion of IX to 8-PN resulting in different levels of exposure of 8-PN. Besides liver CYP enzymes, gut flora also play an important role in the pro-estrogenic activity of IX in that it contributes to the conversion of IX to 8-PN. Several studies have been shown that the composition of gut microbiota community could strongly affect the conversion of IX into 8-PN (46, 47). For example, in an incubation of IX with 12 fecal cultures, one third of the incubation samples produced 8-PN, suggesting inter

individual variability. Some strain of microbiota, such as *Eubacterium limosum,* efficiently converted 90% of IX and was classified as strong producer of 8-PN. This microbial conversion has been observed in an intervention study with 50 healthy women following oral administration of hop-derived dietary supplements (17). About 15% of the study subjects were determined to be strong producers of 8-PN based on urinary excretion or microbial bioactivation capacity.

In our PK study, one subject (2006/3006) appeared to have higher exposure to 8-PN compared with the other 4 subjects. The total serum concentration of 8-PN over IX ratio (8-PN/IX) for this subject in high dose group was 0.35 compared with a mean value of 0.14 for the rest of the subjects. Similar high ratio was also observed in medium dose (0.65 compared with a mean value of 0.17). However, this high exposure was not found at the low dose. In the urinary excretion, the ratio of total 8-PN/IX ratio was ~3 fold higher in this subject compared to the other 4 subjects in medium and high dose. The difference was not pronounced in low dose group which was in agreement with serum data. Thus, our results confirmed the previous report on the inter-individual difference of IX conversion to 8-PN. However, since the phenotyping of CYP1A2 and gut microbiota species were not determined for this subject, the major contributing factor remains unknown.

For 6-PN, the AUC_{0-24} ratio after normalization by dose was 1: 1.2 : 0.5. This lack of dose linearity in AUC_{0-24} partially resulted from an unexpectedly high AUC_{0-24} of a single subject 2002 after receiving the medium dose of hops, which was 3-fold higher than the AUC_{0-24} for the high dose (Table XX). In the mean time, the AUC_{0-24} values of high dose were similar to the AUC_{0-24} values of medium dose. No significant increase was observed for any of the other subjects.

Based on the data in Table XVIII, the AUC_{0-24} ratio of XN after normalization by dose was

1: 1.7: 1.2, which indicated that XN bioavailability decreased as the dosage increased from 21.3 mg, to 42.6 mg and then to 85.2 mg. In a rat study, Leggett et al. also reported that the bioavailability of XN decreased as the dosage increased from human equivalent levels of 20 mg to 60 mg and then to 180 mg XN (53). Since XN was the most abundant prenylflavonoid in the hop extract (35%, wt/wt), solubility of XN might be limiting its bioavailability at the highest dosage of 85.2 mg XN. Nikolic et al. reported 10 μM XN was soluble in 0.05% HCl (43) and its solubility in water has been shown to be 3 μM (146). Generally, the volume of adult human stomach is 2-4 L (147) so the minimum concentration would be ~60 μM in stomach for the high dose group which might lead to insolubility. Although saturation of uptake transporters limit the bioavailability of some compounds, Pang, et al., reported that XN entered and accumulated in Caco-2 cell monolayer (as a model of the human intestinal epithelium) rapidly in a concentration dependent and saturable manner (148). Thus, administration of high dose of XN might result in a saturation of uptake into the intestinal epithelium. A similar lack of dose linearity in AUC_{0-24} was observed for IX. The AUC_{0-24} normalized to dose for IX was 1: 1.8: 1.2. (Table XIX). A decrease of AUC_{0-24} was observed for subject 2004/3004 when doses were increased. Excluding this subject, the calculated AUC_{0-24} ratio becomes 1:1:1 which indicated dose linearity of IX.

TABLE XVII. PHARMACOKINETIC PARAMETERS FOR 8-PN FOLLOWING ORAL ADMINISTRATION OF A STANDARDIZED HOP EXTRACT AT EACH OF THREE DOSAGES AS DESCRIBED IN THE TEXT. DATA EXPRESSED AS MEAN ± S.D.

		8-PN		
	Unit	Low	Medium	High
AUC_{0-24}	h x ng/mL	16.7 ± 5.1	41.0 ± 26.4	50.4 ± 26.7
AUC_{0-inf}	h x ng/mL	24.3 ± 8.9	69.2 ± 38.9	88.6 ± 52.6
Tmax	h	5.3 ± 4.5	7.0 ± 9.7	2.8 ± 4.0
Cmax	ng/mL	1.4 ± 0.3	3.9 ± 2.6	6.7 ± 3.8
Vd/F	L	118 ±75	186 ± 186	271 ± 216
CL/F	L/h	11.1 ± 3.4	10.4 ± 8.1	13.7 ± 5.5

TABLE XVIII. PHARMACOKINETIC PARAMETERS FOR XN FOLLOWING ORAL ADMINISTRATION OF A STANDARDIZED HOP EXTRACT AT EACH OF THREE DOSAGES AS DESCRIBED IN THE TEXT. DATA EXPRESSED AS MEAN ± S.D

		XN		
	Unit	Low	Medium	High
AUC_{0-24}	h x ng/mL	37.1 ± 31.9	126 ± 40	172 ± 45
AUC_{0-inf}	h x ng/mL	73.2 ± 60.1	167 ± 62	323 ± 170
$T_{1/2}$	h	18.3 ± 5.3	9.5 ± 2.2	20.7 ± 12.7
Tmax	h	3.8 ± 4.4	3.6 ± 2.6	1.8 ± 1.8
Cmax	ng/mL	4.4 ± 3.0	22.2 ± 12.1	27.6 ± 8.9
Vd/F	L	17300 ±21000	3770 ± 1390	7880 ± 1810
CL/F	L/h	657 ± 670	278 ± 87	329 ± 163

TABLE XIX. PHARMACOKINETIC PARAMETERS FOR IX FOLLOWING ORAL ADMINISTRATION OF A STANDARDIZED HOP EXTRACT AT EACH OF THREE DOSAGES AS DESCRIBED IN THE TEXT. DATA EXPRESSED AS MEAN ± S.D

	IX			
	Unit	Low	Medium	High
AUC_{0-24}	h x ng/mL	61 ± 26	229 ± 243	282 ± 115
AUC_{0-inf}	h x ng/mL	149 ± 86	418 ± 446	546 ± 301
$T_{1/2}$	h	27.5 ± 5.7	24.8 ± 24.3	19.9 ± 8.7
Tmax	h	4.2 ± 4.2	3.2 ± 3.2	4.0 ± 4.8
Cmax	ng/mL	5.7 ± 2.7	19.1 ± 15.5	37.6 ± 17.6
Vd/F	L	262 ± 135	99 ± 56	182 ± 53
CL/F	L/h	7.1 ± 4.2	5.7 ± 4.9	7.3 ± 3.5

TABLE XX. PHARMACOKINETIC PARAMETERS FOR 6-PN FOLLOWING ORAL ADMINISTRATION OF A STANDARDIZED HOP EXTRACT AT EACH OF THREE DOSAGES AS DESCRIBED IN THE TEXT. DATA EXPRESSED AS MEAN ± S.D.

	6-PN			
	Unit	Low	Medium	High
AUC_{0-24}	h x ng/mL	26.4 ± 18.6	64.6 ± 48.3	54.5 ± 35.3
AUC_{0-inf}	h x ng/mL	69.2 ± 60.0	126 ± 75	103 ± 43
$T_{1/2}$	h	23.5 ± 6.9	24.1 ± 10.7	21.4 ± 6.4
Tmax	h	7.5 ± 3.5	5.6 ± 2.8	1.8 ± 1.9
Cmax	ng/mL	1.6 ± 0.9	7.3 ± 6.2	5.8 ± 3.2
Vd/F	L	887 ± 581	1000 ± 755	1770 ± 759
CL/F	L/h	30.0 ± 26.1	28.7 ± 18.1	55.4 ± 15.4

The apparent volumes of distribution Vd/F of XN were very large, with value of 17300 L, 3770 L, and 7880 L for the low, medium and high doses of hops, respectively. For comparison, the Vd/F values for IX, 6-PN and 8-PN were much lower and ranged from 99 to 262 L for IX, 887 to 1770 L for 6-PN and 118 to 271 L for 8-PN. These high Vd/F values of XN indicated high tissue distribution and were consistent with the rapid uptake and concentration of XN by intestinal epithelial cells reported by Pang et al. and Wolff et al. (148, 149). Binding to cytosolic proteins might explain the high concentration in intestinal cells. High Vd/F values of XN in rats were also reported by Legette et al. (53). By using MALDI imaging distribution, tissue distribution of XN and XN-glucuronide has been determined in rats (54). Following daily dosing of 100 mg/kg XN by oral gavage for 5 days, a fairly diffuse distribution throughout the tissue was observed. XN accumulated in the organs along the route of absorption such as intestine, colon and the liver while XN-glucuronide was distributed along the epithelial portion of the tissue. Taking these results together, high Vd/F values for XN from the PK study were mainly due to the high tissue distribution and binding to the cytosolic proteins.

Although the amount of IX in the hop extract was 27-fold lower that the level of XN (Table VII), Cmax for IX were similar to those of XN (Table XVIII and Table XIX). For comparison, Cmax values of 6-PN and 8-PN were much lower (Tables XVII and Table XX). Also, the total amount of IX excreted in urine (including its phase 2 conjugates) was significantly larger than that of urinary XN. Several reasons could explain why the apparent concentration of XN in serum is lower than IX. First, poor absorption could result in lower bioavailability. It was reported by our group that approximately 70% of XN added to Caco-2 monolayer culture accumulated inside the cell cytosol (148). Binding of XN but not IX to cytosolic proteins in intestinal epithelial cells might contribute to the low bioavailability of XN observed in the study. Second, perhaps even more significant, XN is a chalcone that can cyclize to form IX, and this

intramolecular reaction is shown in Figure 1.2. Conversion of XN to IX has been reported to occur in the presence of gastric acid and in intestine (43, 47), but the extent of conversion within cells is unknown. However, the conversion of XN to IX in human serum was found to be negligible based on our validation data in Chapter 2. That suggests XN conversion to IX did not happen in human serum during sample storage and sample preparation and the possibility that artifact of IX can come from XN during sample analysis could be ruled out. Third, XN has a good tissue distribution based on the rats study (54). The fair diffusion to various tissues along the route of absorption could contribute to the apparent low serum concentration. Last but not least, solubility of XN may play an important role in the low bioavailability. XN can be dissolved in aqueous solution up to 20 μM. Such a huge amount of XN in high dose group (85.2 mg) could not be completed dissolved in stomach acid. The insoluble XN might be excreted unchanged into feces. However, since no fecal sample was collected in our study, the extent of excretion remains unknown. In vivo rodent studies indicate that orally administered XN is not detected in plasma and that a majority of this dose (up to ~90%) is excreted unchanged with the feces (150). In summary, all the above reasons could lead to the low bioavailability of XN in humans.

Few data exist in the literature concerning the bioavailability of 8-PN in humans. Human hepatic and intestinal metabolism greatly contributed to the inactivation of 8-PN by forming glucuronide conjugates. Since no bile or fecal samples were collected during the initial study, the extent of biliary excretion in our study remains uncertain. 8-PN has been reported to have 3.5 fold induction for ERE-luciferase in Ishikawa cells compared with negative control at concentration of 100 nM (41). In this PK study, the Cmax of total 8-PN is only 6.7 ng/mL (19.7 nM) after oral administration of 1 mg 8-PN and no free 8-PN was detected in the serum for all doses. However, EC_{50} value for the alkaline phosphatase induction assay was determined to

be 13 nM for 8-PN (41) which was within the range of the serum concentration of conjugated 8-PN.

Since no bile samples were collected in the initial study, whether conjugated prenylflavonoids were secreted into the bile and then were deconjugated in the gut or if the unconjugated prenylflavonoids were secreted into the bile and then showed enterohepatic recirculation remains unknown. However, Rad et al. reported ~20% of free 8-PN was detected in human fecal samples following oral administration of pure 8-PN from 50 mg up to 750 mg (25). Enterohepatic recirculation of free 8-PN was observed in the study as a secondary peak showed up at 5-8 h post dose (25). Interestingly, we also found the secondary peak in the total concentration-time profiles (Figure 4.4). Legette et al. reported a secondary peak at 8-12 h post dose (in total concentration over time curves) in rats following oral administration of XN up to 16.9 mg/kg (53). However, no bile or fecal samples were collected in the study either. Thus, whether XN-Glucuronide was excreted into the bile and had enterohepatic recirculation remains inconclusive.

The free concentrations of the 4 prenylflavonoids in each individual subject are summarized in Table XV through Table XVI. No free 8-PN and 6-PN were detected in any of the serum samples for all 3 doses. Free IX and XN were only detected in some subjects in medium and high doses. The data points with concentrations above LLOQ were too sparse to make any valid PK estimations. The concentrations of the unconjugated prenylflavonoids were much lower than the total concentrations indicating the majority of these compounds were converted to the phase II conjugates.

Aliquots of the 24 h urine collection for all study subjects were analyzed by using a slightly modified UHPLC-MS-MS method (Figure 4.3). As observed in the UHPLC-MS-MS analyses of the serum samples (Figure 4.2), most of the prenylflavonoids were excreted in urine as glucuronides. Data for the urinary excretion of total XN, IX, 8-PN, and 6-PN during the first 24 h are summarized in Table XXI and Table XXII. Regardless of dosage, >98% of 8-PN, IX and XN were excreted in urine as glucuronides. Since no free 6-PN was detected in urine samples, it indicated glucuronide was the only form excreted into urine for 6-PN. The average total amounts of these 4 major prenylflavonoids for each dosage group are summarized in Table XXI. From low to high dosages, the subjects in the study excreted an average of 23.9 nmol to 138.6 nmol XN; 29.9 nmol to 179.9 nmol IX; 5.1 nmol to 29.5 nmol 8-PN, and 26.2 nmol to 150.3 nmol for 6-PN.

The levels of 6-PN measured in urine after enzymatic hydrolysis of all samples were ~5-fold greater than those of 8-PN (Table XXI), which was consistent with the initial ratio of 6-PN to 8-PN in the hop extract (Table VII). However, although unconjugated XN, IX and 8-PN were detected in urine, no free 6-PN was detected in any urine sample. A possible explanation for the absence of free 6-PN in urine might be that 6-PN was more rapidly and completely metabolized to glucuronides than was 8-PN.

As the dosage of the hop extract was increased, the urinary excretion of each of the 4 prenylflavonoids increased proportionally (Table XXI). Since the prenylflavonoids were excreted primarily (>98%) as glucuronides, there was no indication such as delay of urinary excretion that hepatic UDP-glucuronyltransferases were saturated at the doses used. Urinary excretion of each prenylflavonoid as a percentage of the dose is shown in Table XXII. In the first 24 h, 0.7% to 1.5% of 8-PN was recovered in urine, and the urinary excretion of the other prenylflavonoids were similar. For comparison, Rad, et al. (25) reported recovering 4.6% to

8.1% of 8-PN in 48 h after administering considerably larger doses (50 mg to 750 mg) of synthetic 8-PN to women. In the same study, 21-24% of a single 8-PN dose was excreted in feces within 48 h, indicating that biliary excretion instead of urinary excretion was the primary route of elimination of 8-PN. XN was found mainly in the feces sample in a rats study by Avula et al. and Nookandeh et al. (150, 151). After oral administration of up to 500 mg/kg XN to rats, less than 0.5% of unchanged XN was found in urine. Similar low recovery of XN in human urine was found in our study. XN appeared to have the lowest recovery among all 4 prenylflavonoids for both free and total concentrations indicating that XN has poor bioavailability compared to the other 3 prenylflavonoids.

TABLE XXI. URINARY EXCRETION (NMOL) OF TOTAL XN, IX, 8-PN, AND 6-PN DURING THE FIRST 24 H AFTER ADMINISTRATION OF SINGLE DOSES OF A STANDARDIZED HOP EXTRACT AS INDICATED IN THE TEXT. DATA EXPRESSED AS MEAN ± S.D.

Dosage	XN	IX	8-PN	6-PN
Low	23.9 ± 17.9	29.8 ± 32.3	5.1 ± 4.4	26.2 ± 24.1
Medium	37.0 ± 26.3	89.5 ± 54.5	21.3 ± 21.6	49.8 ± 39.8
High	138 ± 113	172 ± 67.4	29.4 ± 22.9	150 ± 104

TABLE XXII. URINARY EXCRETION RECOVERY % DOSE OF UNCONJUGATED AND TOTAL XN, IX, 8-PN, AND 6-PN DURING THE FIRST 24 H AFTER ADMINISTRATION OF SINGLE DOSES OF A STANDARDIZED HOP EXTRACT AS INDICATED IN THE TEXT. DATA EXPRESSED AS MEAN ± S.D.

Dosage	Urinary excretion unconjugated (% dose in 24h)				Urinary excretion total (% dose in 24h)			
	8-PN	6-PN	XN	IX	8-PN'	6-PN'	XN'	IX'
Low	0.015±0.015	<LOD[1]	0.003±0.001	0.012±0.007	0.693±0.597	0.685±0.631	0.04±0.03	1.318±1.428
Medium	0.026±0.007	<LOD	0.002±0.001	0.012±0.007	1.449±1.467	0.651±0.521	0.031±0.022	1.98±1.205
High	0.006±0.003	<LOD	0.001±0.0003	0.014±0.005	1.001±0.781	0.982±0.684	0.058±0.047	1.902±0.746

1. LOD: limit of detection (0.1 ng/mL for 6-PN)

4.3.3 DMX stability studies

DMX can isomerize via Michael addition reaction (see Figure 4.5) leading to the formation of 8-PN and 6-PN (152). Chen et al. found that stability of DMX in methanol was greatly increased after degassing the solution (152). The poor stability of DMX in solution might be the reason for few reported quantitative analysis of this compound in the literature. There is no investigation of the stability under various analytical conditions and/or in biological matrix to date. Thus it is critical to address this issue and answer the question whether this conversion can occur during the sample preparation and analysis and whether DMX was present in the urine or serum samples. DMX wasn't observed in any of the serum samples or urine samples for the previous study, but it is present in the hop extract (see Figure 3.2).

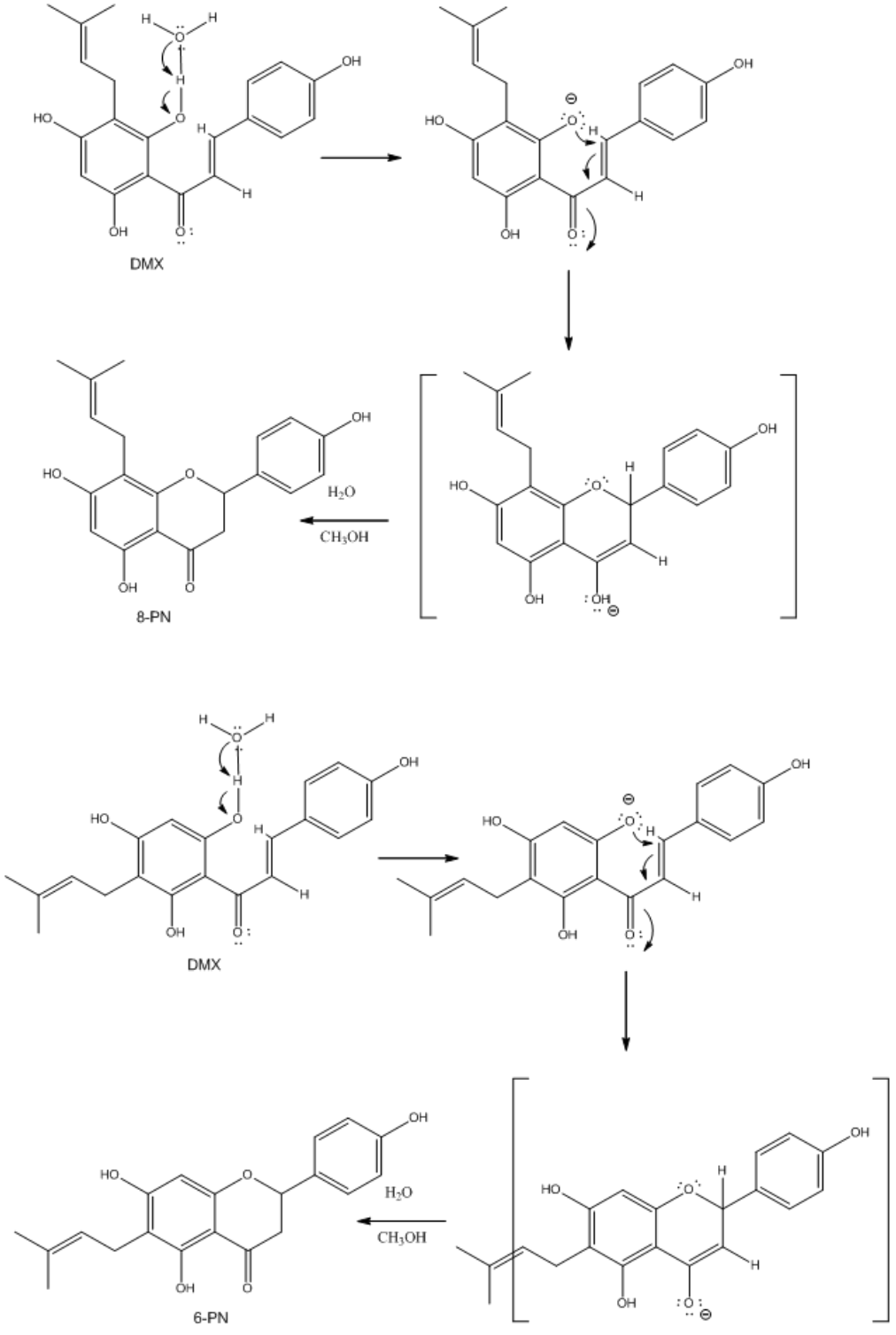


Figure 4.5. Intra-molecular Michael addition ring closure of DMX leading to the formation of 8-PN and 6-PN.

The Incubation of DMX in water/methanol (50:50) at 37°C for 1 h resulted in considerable isomerization of DMX to form a considerable amount of 6-PN and some 8-PN in a ratio of ~ 5:1 (Figure 4.6). At 25°C for 1 h, less isomerization of DMX was observed, and at 4°C for 1 h, isomerization of DMX to form 6-PN and 8-PN was negligible (Figure 4.6). At 4°C (autosampler temperature) for 2 h, DMX extracted from serum or urine also did not isomerize to 8-PN and 6-PN (Figure 4.7 and Figure 4.8, respectively). The stabilities of DMX in human serum and urine were then evaluated. DMX in human urine isomerized in 1 h at 37 °C to form a large amount of 6-PN and somewhat less 8-PN in a ratio of ~ 5:1 (Figure 4.9). In contrast, DMX in human serum was nearly stable for 1 h at 37 °C (Figure 4.10).

After extraction from serum or urine at room temperature and while stored at 4°C in the autosampler of the UHPLC-MS-MS system, these data indicate that DMX should have been sufficiently stable to have been detected. However, since these stability studies indicated that DMX is stable in serum but not in urine, any urinary DMX would have isomerized during enzymatic deconjugation at 37°C to form primarily 6-PN. Nevertheless, no unconjugated 6-PN was measured in urine. Therefore, it would appear that no measureable amounts of DMX or 6-PN were excreted in urine.

Thus, we conclude that if DMX had been present in urine samples, it most likely would have converted into 8-PN and 6-PN during the sample processing. In contrast, stability data in serum suggest that artifactual conversion during sample preparation is not a reason why DMX was not detected in the clinical samples. The most likely reason is that conversion happened inside the body.

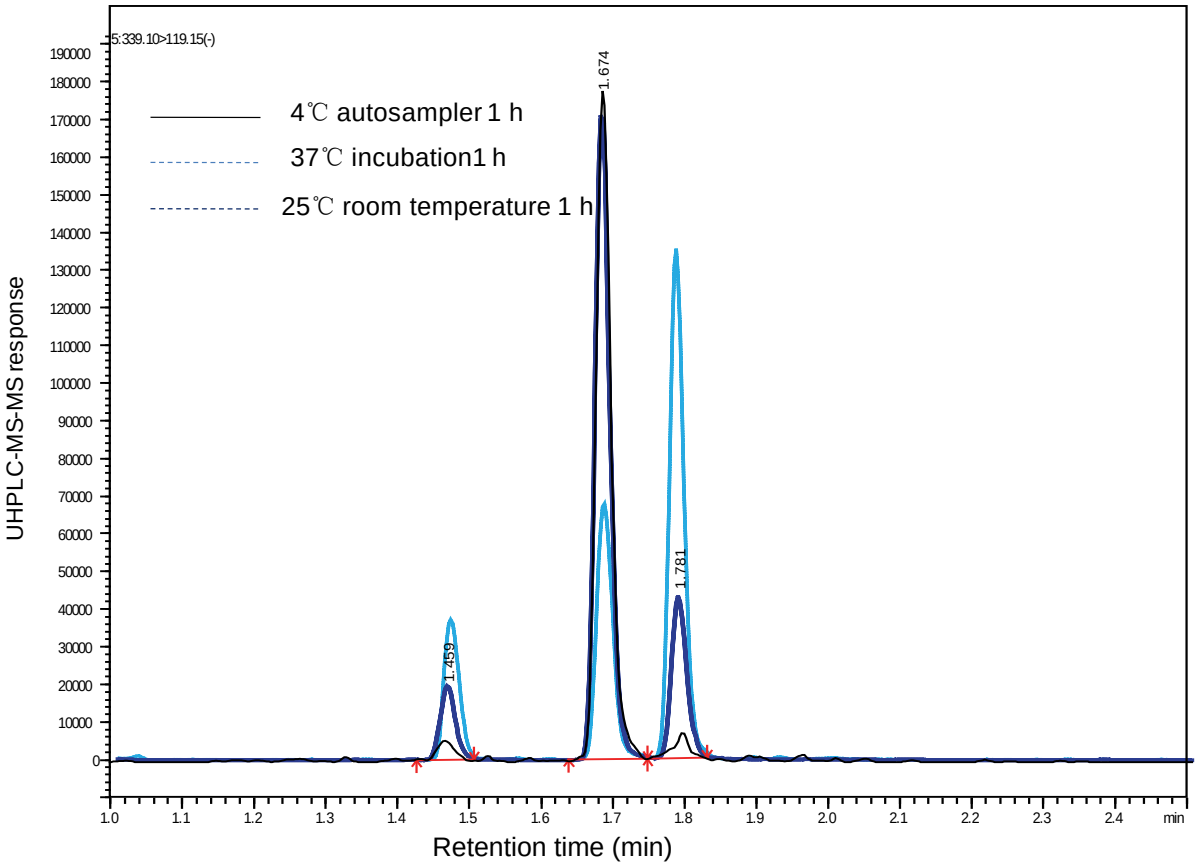


Figure 4.6. DMX in water/methanol (50:50) solution without biological matrix at 4°C for 1h (solid line), 37°C for 1h (blue line), and for 1 h (purple line). Note the significant increase of 6-PN and 8-PN at 37°C compared with less amount increase of 6-PN and 8-PN at 25°C, while no observed 6-PN and 8-PN at 4°C.

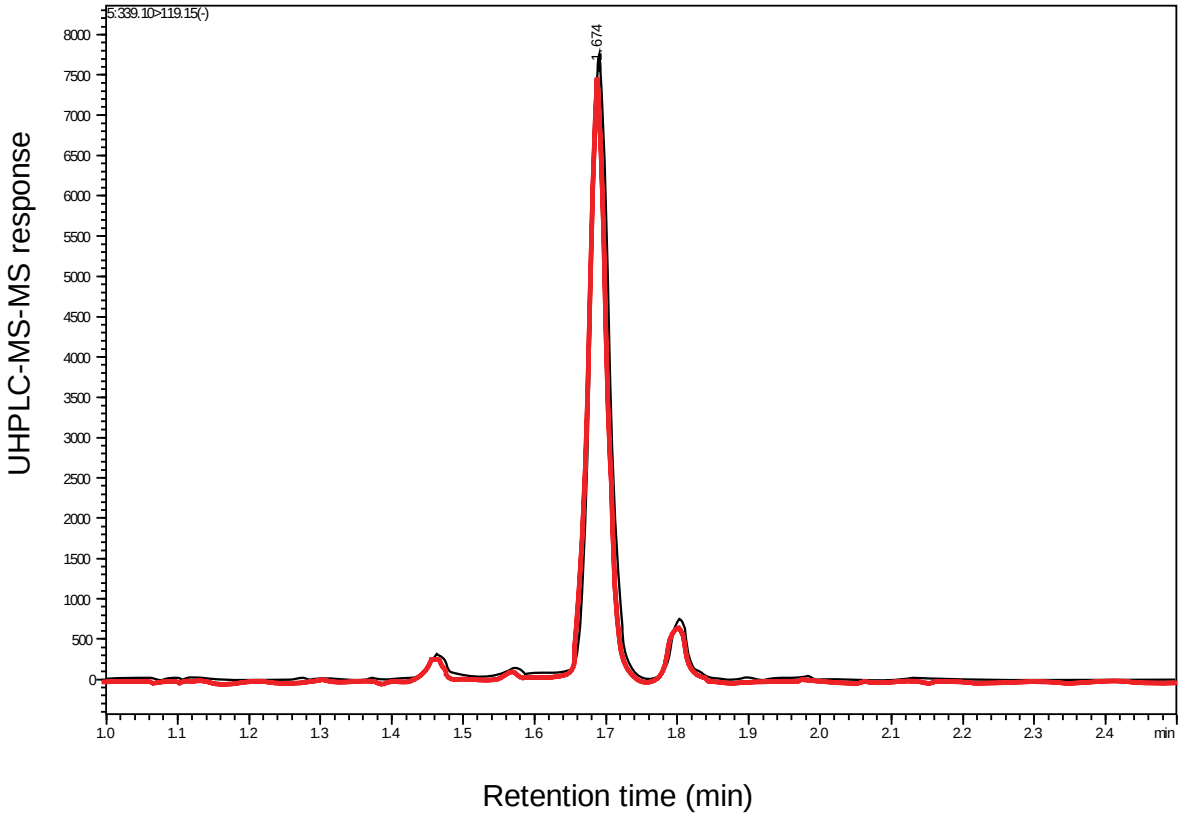


Figure 4.7. DMX spiked in serum, extracted immediately and analyzed (solid line) or the same sample after 2 h in autosampler at 4°C (red line) and analyzed using UHPLC-MS-MS. Note that no significant increase of 6-PN and 8-PN during 2 h at 4°C.

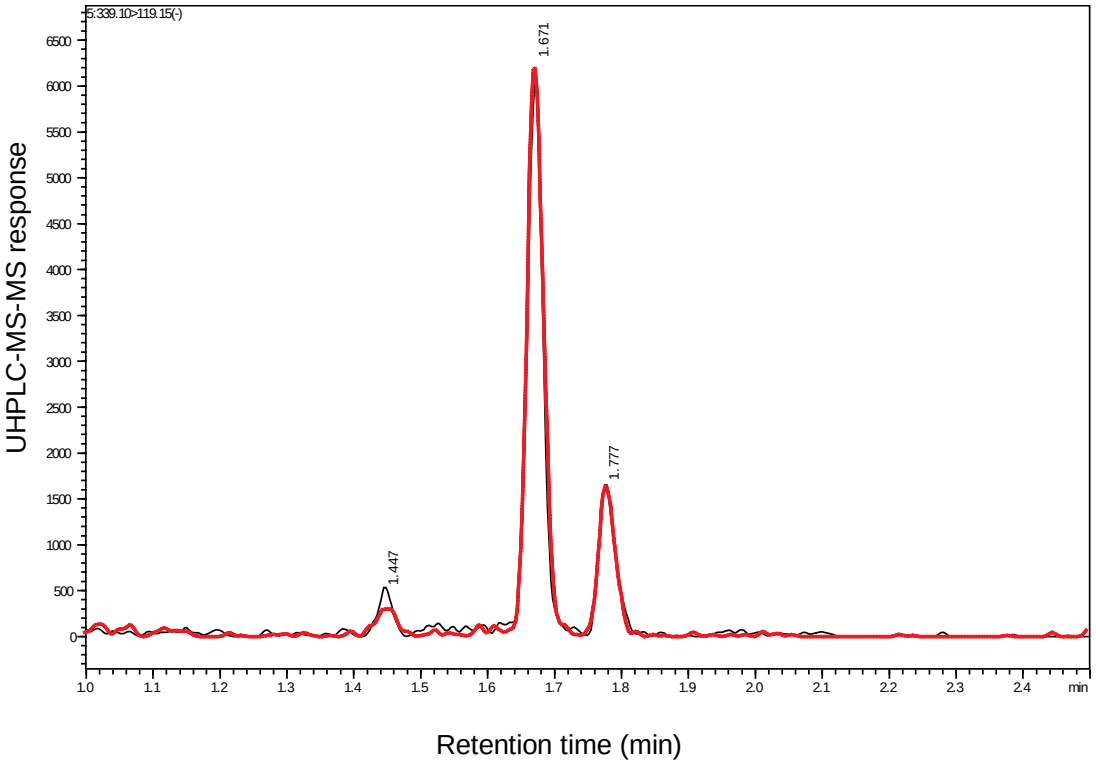


Figure 4.8. DMX spiked in urine, extracted immediately and analyzed (solid line) or the same sample after 2 h in autosampler at 4°C (red line) and analyzed using UHPLC-MS-MS. Note that no significant increase of 6-PN and 8-PN during 2 h at 4°C

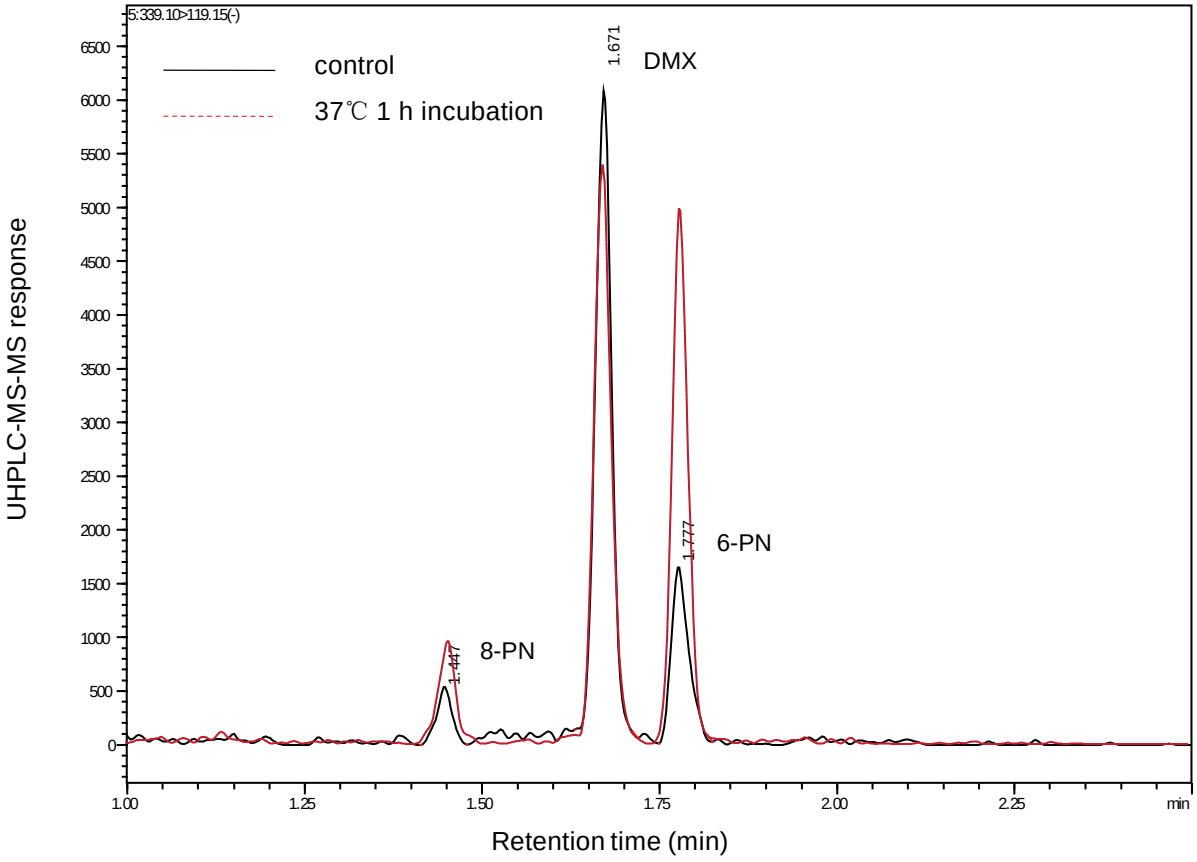


Figure 4.9. DMX spiked into urine, extracted and analyzed immediately (solid line) or spiked in urine, incubated at 37°C for 1 h (red line) and then extracted and analyzed using UHPLC-MS-MS. Note the significant increase in the amount of 6-PN during incubation.

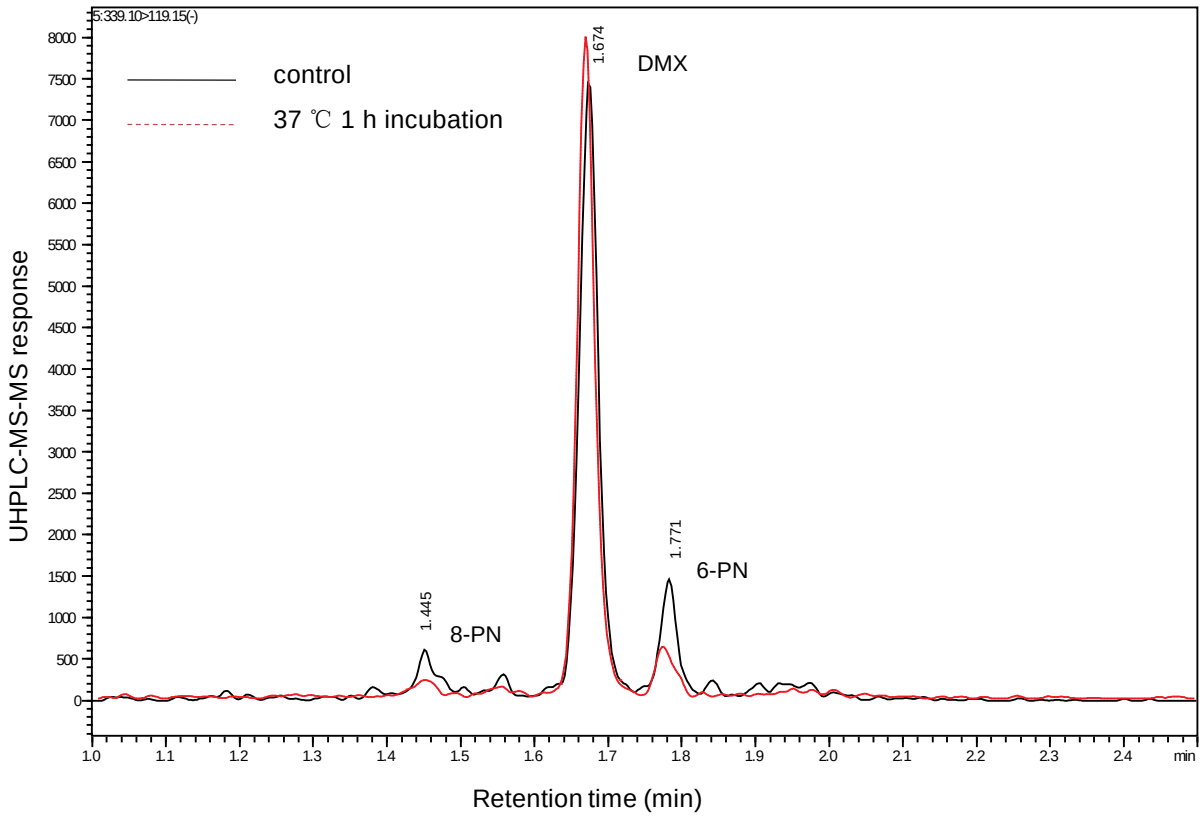


Figure 4.10. DMX spiked in serum, extracted immediately (solid line) or incubation at 37°C for 1 h (red line) and then extracted and analyzed using UHPLC-MS-MS. Note that no significant increase of 6-PN during incubation.

4.4 Conclusions

The aim of this study was to investigate the pharmacokinetic properties of all major hop flavonoids after oral administration of a standardized extract to human subjects. Serial blood samples and urine samples were analyzed using a fast and sensitive UHPLC-MS-MS method for the measurement of the prenylflavonoids. After oral administration of a standardized extract, these hop flavonoids were rapidly absorbed into the bloodstream. The predominant circulating form was glucuronide in blood and urine. Secondary peak in the concentration-time profiles was observed for all 4 major prenylflavonoids. Significant formation of estrogenic 8-PN was observed and an accumulation trend 12 h post dose was an indication of in vivo formation of 8-PN. Large inter-individual differences in the pharmacokinetic profiles of hop flavonoids were also observed. Earlier study in rats indicated that XN has limited oral bioavailability and this is probably due to the limitation of the sensitivity of the bioanalytical method (151). Our study results suggest that after oral administration of hop extract , XN is available, at least as conjugate form.

CHAPTER 5

ROUND ROBIN ANALYSIS OF BISPHENOL A AND ITS METABOLITE IN HUMAN SERUM

5.1 Introduction

BPA is used as a material for the production of epoxy resins and polycarbonate plastics (55). Epoxy resins are used as food-contact surface lacquer coatings for cans and metal jar lids. BPA can leach from can surface coatings and has been detected in canned food (56). Leaching of BPA can be facilitated when canned food is processed at high temperatures (153). Although environmental exposure (water, air and soil) can also contribute to BPA exposure to human, the primary route of human exposure to BPA is probably through intake by food (154).

As described in Chapter 1, LC-MS-MS is considered the most selective and sensitive analytical technique for the measurement of BPA and its metabolites in human serum. On the basis of several studies, the daily human intake of BPA is estimated to be <0.1 μg/kg BW/day (155). However, the levels of BPA and its major metabolites, BPA glucuronide (Figure 1.5) have been reported to vary considerably from 0.2 to 66.5 ng/mL in human serum (107, 156-159). These variations have raised concern that existing LC-MS-MS based methods for BPA analysis are perhaps unreliable or are not reproducible between laboratories. To address these concerns, a new method for the quantitative analysis of BPA, BPA-glucuronide (BPA-G) and BPA-sulfate (BPA-S) in human serum was developed and validated based on UHPLC-MS-MS. This method was compared with 3 other laboratories for the analysis of BPA and BPA-glucuronide in a round robin experiment sponsored by the National Institute of Environmental Health Sciences (NIHES) in which all 4 methods were used to measure the same set of serum samples.

5.2 Materials and Methods

5.2.1 Chemicals and Reagents

Solvents (acetonitrile, methanol and water) were LC-MS grade and were purchased from Burdick & Jackson (Honeywell, Muskegon, MI). Ammonium acetate, BPA and BPA monosulfate sodium (BPA-S) were purchased from Sigma-Aldrich (St. Louis, MO). Charcoal Dextran-stripped human serum was obtained from Innovative Research (Novi, MI). BPA glucuronide (BPA-G) and BPA disulfate were obtained from the Midwest Research Institute (Kansas City, MO). (d_6)-BPA was purchased from Cambridge Isotope Laboratories (Andover, MA). Eppendorf tubes (1.5 mL) and glass autosampler vials were purchased from Thermo Fisher (Hanover Park, IL).

5.2.2 Study Design

Verification of BPA contamination from method

To ascertain that the materials used for sample preparation and analysis did not leach BPA into the serum specimens, all solvents and materials were tested for BPA. Then, method blanks were analyzed to ascertain there was no detectable BPA contamination. In the case of any BPA detection in the method blanks, single materials were substituted until BPA contamination was eliminated.

To ensure that the collection materials and/or methods did not influence BPA concentrations (i.e. deconjugation via interactions with materials, storage or shipping conditions), positive controls were included in the validation studies. High purity BPA-free water and BPA-free serum were spiked with BPA and BPA-G at a concentration relevant to the reported human blood levels (0.5, 1.0 and 2.0 ng/mL). These spiked samples were analyzed using the same protocol as the field blanks.

Round-robin assay validation

NIEHS provided 6 spiked and blank serum samples in 10 mL tubes and two 10 mL vials of water (one spiked and one unspiked). Samples were spiked with BPA and BPA-G at concentrations relevant to the reported human blood levels. Each assay was run in at least triplicate to evaluate the precision of the assay. Method accuracy was based on the comparison of the measured values to the expected concentrations.

Analysis of clinical serum specimens

NIEHS provided 10 patient sera (6 mL each) that had been collected at the University of California San Francisco using 2 sets of phlebotomy materials. Each sample was run in triplicate to establish the precision for each measurement.

5.2.3 Preparation of Calibration Curves

Stock solutions were prepared in methanol to a final concentration of 1 mg/mL and stored in amber glass vials. Working standards were made by serial dilution from stock solutions. QC stocks were prepared from a separate weighing of the reference standards. Calibration standards and quality control (QC) samples were prepared by mixing 5 µL of each working standard or QC solution with 195 µL charcoal dextran-stripped human serum and mixed well before protein precipitation. A separate calibration curve in water was also prepared. Calibration standards and QC samples were prepared by mixing 5 µL of each working standard or QC solution with 195 µL water and mixing. Unknown serum samples (200 µL each), calibration standards or QC solutions were mixed with 800 µL acetonitrile containing 10 ng/mL (d_6)-BPA in a 1.5 mL Eppendorf tube. The mixture was vortexed for 2 min and centrifuged for 15 min at 13000 x *g* at 4 °C. 900 µL of the supernatant was transferred to a

new Eppendorf tube and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 50 µL of 50% aqueous methanol, and 10 µL was injected onto the UHPLC-MS-MS system for analysis.

Note that the BPA-sulfate standard was found to contain a significant BPA-disulfate impurity based on UHPLC-MS analysis, and its purity was estimated to be 94.6%. Thus, the BPA-S calibration curve was adjusted according to the purity of the standard and used for quantitative analysis.

5.2.4 UHPLC-MS-MS

Chromatographic separations were carried out using a Shimadzu (Kyoto, Japan) Nexera UHPLC system interfaced to a Shimadzu LCMS-8040 (for the analysis of spiked sample) or a Shimadzu LCMS-8080 triple quadrupole mass spectrometers (for the analysis of the clinical human serum specimens) (Kyoto, Japan). Analytes were separated on a Shimadzu Shim-pack XR-ODS III (2.0 x 50 mm, 1.6 µm) C_{18} column. Mobile phase A contained 1 mM ammonium acetate in water, and mobile phase B was acetonitrile. A 1.5 min linear gradient was used from 10-100% acetonitrile (B) followed by a hold at 100% for 0.5 min at a flow rate of 0.4 mL/min. The column was re-equilibrated at 10% acetonitrile (B) for 1 min before the next injection. The total run time including equilibration was 3 min. The column oven temperature was 50 °C, and the autosampler was 4 °C.

Both Shimadzu LCMS-8040 and LCMS-8080 mass spectrometer parameters were optimized with reference standards using flow injection to achieve optimum sensitivity. Negative ion electrospray mass spectrometry with collision-induced dissociation and selected reaction monitoring (SRM) was used for the measurement of each analyte. Data were acquired by monitoring two transitions (quantifier and qualifier) for each analyte as follows:

BPA *m/z* 227 to 212 and *m/z* 227 to 133; (d_6)-BPA *m/z* 233 to 215; BPA-G *m/z* 403 to 227 and *m/z* 403 to 113; and BPA-S *m/z* 307 to 227 and *m/z* 307 to 212. The SRM dwell time was 25 ms/ion. For the LCMS-8040 mass spectrometer: the DL temperature was 300 °C, the spray voltage was 4500 V, the nebulizing gas flow was 3 L/min, and drying gas flow was 20 L/min. For the LCMS-8080 mass spectrometer: The probe temperature was 350 °C, the HSID temperature was 300 °C, the spray voltage was 4500 V, the nebulizing gas flow was 3 L/min, heating gas flow was 12 L/min, and the curtain gas flow was 3.5 L/min, the probe protrusion was 0.5 mm.

Quantification and data analysis

Data acquisition was carried out using Shimadzu Labsolution software. Calibration curves were constructed by plotting the peak area ratio for BPA and its surrogate standard (d_6)-BPA versus the corresponding concentration and fitting to a linear regression equation to the data. Due to the lack of stable isotope-labeled standards for BPA-G and BPA-S, external calibration curves were used by plotting the peak area versus the corresponding concentration and fitting a linear regression equation. A weighting factor of 1/x was applied to the calibration curve. Data analysis was performed using Shimadzu Labsolution software and Microsoft Excel.

5.3 Results and Discussion

In order to obtain a faster and more sensitive alternative to LC-MS-MS, a UHPLC-MS-MS method, was developed and validated for the quantitative analysis of BPA and BPA-G using LCMS-8040 and LCMS-8080 mass spectrometers. To select product ions for use during SRM, negative ion electrospray product ion tandem mass spectra of deprotonated BPA, (d6)-BPA, BPA-G, and BPA-S were obtained and are shown in Figure 5.1 and Figure 5.2. Loss of a methyl radical from the deprotonated molecule of BPA was the base peak, this transition was

used as the SRM quantifier (Figure 5.1, top panel). Loss of phenyl from deprotonated BPA was the second most abundant fragment ion and was used as SRM qualifier. In the product ion spectrum of deprotonated molecule (d6)-BPA, similar fragmentation pathway was observed. Loss of the CD_3 radical from the deprotonated molecule (d6)-BPA was shown as the base peak and this transition was selected as the SRM (Figure 5.1, bottom panel). In the product ion tandem mass spectrum of the deprotonated molecule BPA-G (Figure 5.2, top panel), the loss of a dehydrated glucuronic acid from the deprotonated molecule BPA-G, *m/z* 227 was the base peak, and this transition was used as the SRM quantifier for BPA-G. Serial loss of BPA moiety, CO_2 and H_2O, *m/z* 113 was the second most abundant product ion and this transition was used as SRM qualifier for BPA-G. In the product ion tandem mass spectrum of the deprotonated molecule BPA-S (Figure 5.2, bottom panel), the loss of sulfate group was the most abundant fragment ion, *m/z* 227 was selected as the SRM quantifier for BPA-S. Further loss of CH_3 radical anion from ion *m/z* 227 was the second most abundant product ion and this transition was used as SRM qualifier for BPA-S.

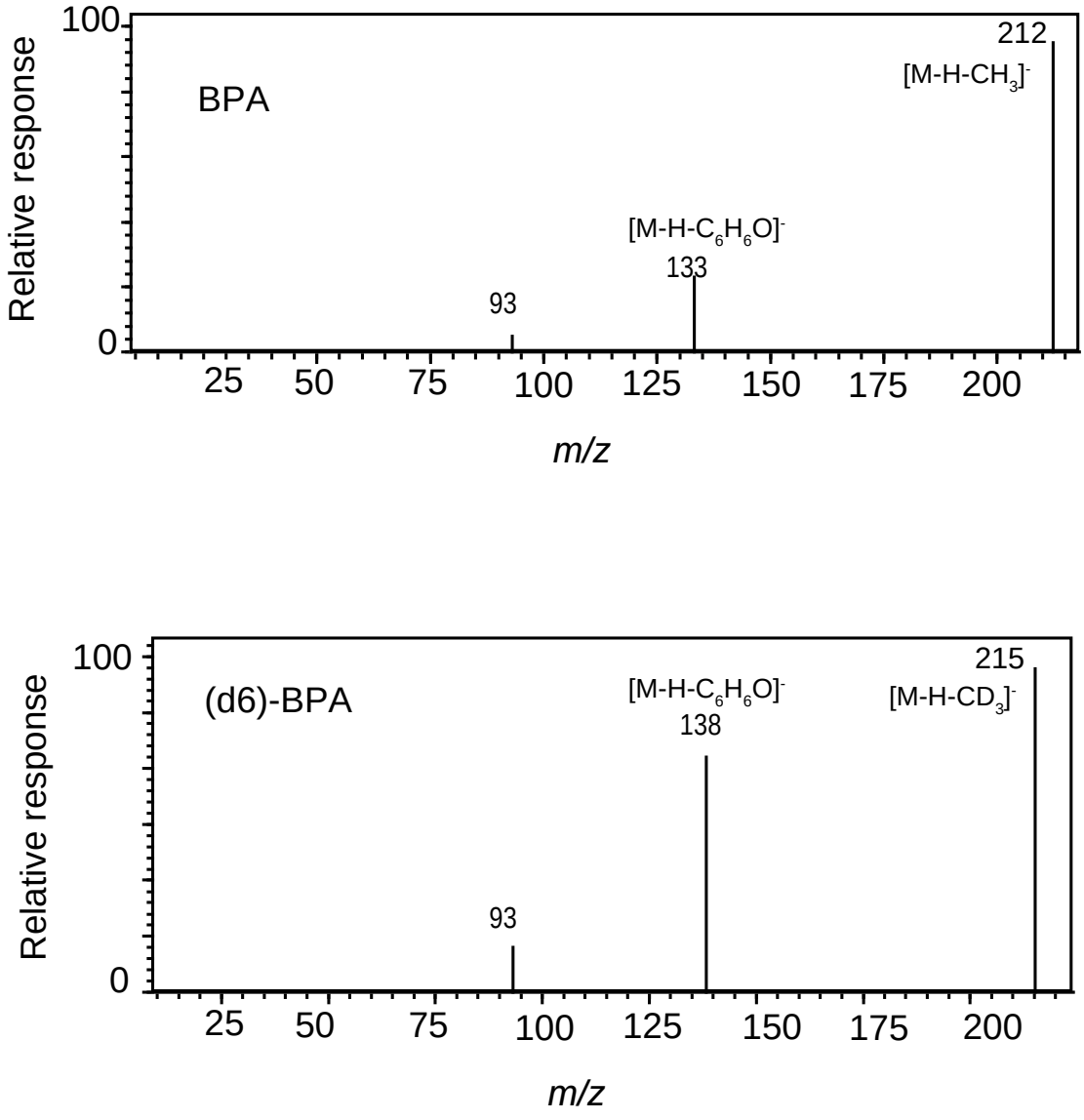


Figure 5.1. Negative ion electrospray product ion tandem mass spectra of deprotonated BPA (*m/z* 227) and (d6)-BPA (*m/z* 233).

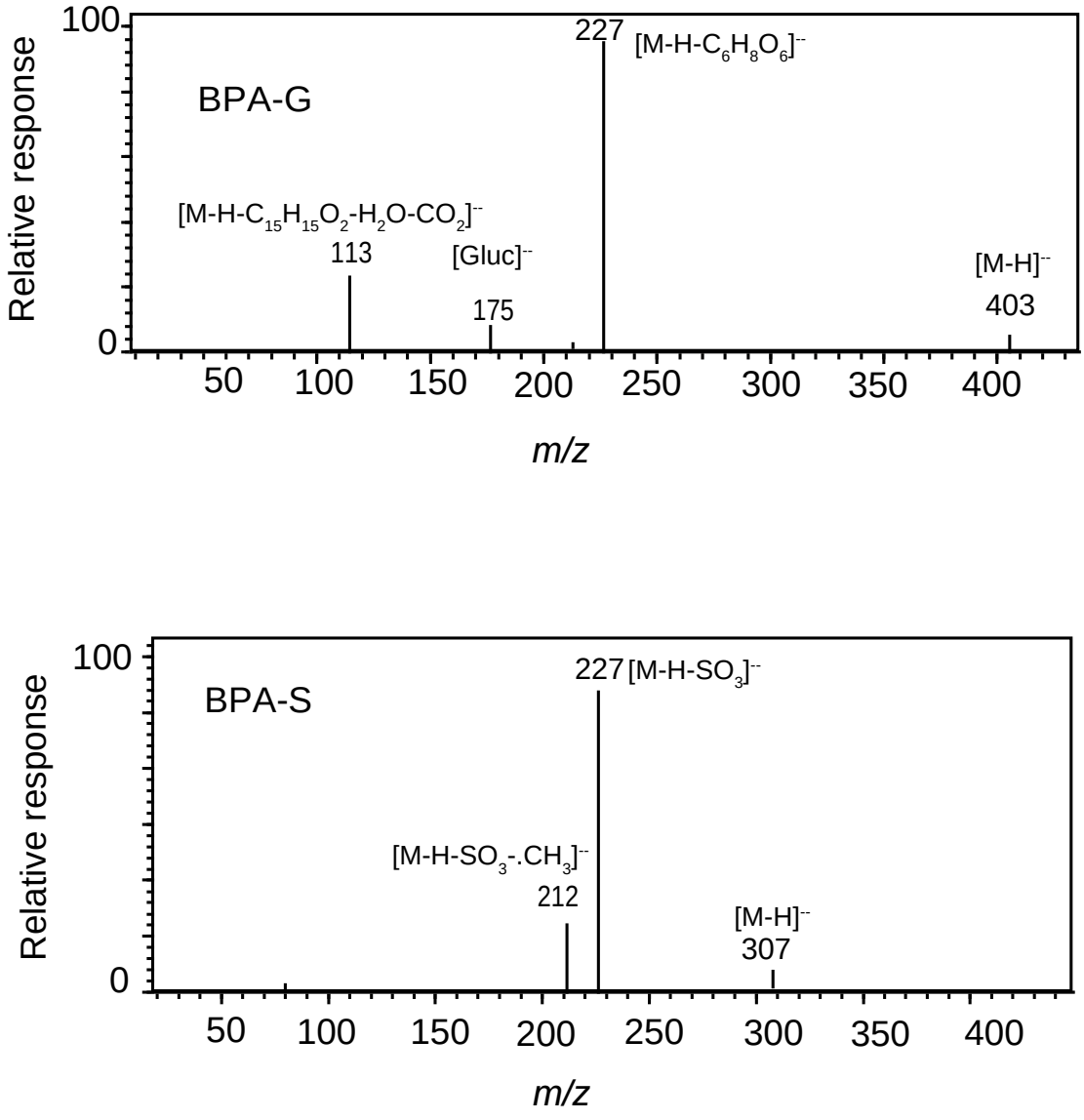


Figure 5.2. Negative ion electrospray product ion tandem mass spectra of deprotonated BPA-G (*m/z* 403) and BPA-S (*m/z* 307).

Using these SRM transitions during UHPLC-MS-MS, the retention time for BPA, BPA-G and BPA-S were 1.5 min, 1.1 min and 1.2 min, respectively. The entire analysis was complete within 3 min. (d6)-BPA co-eluted with BPA at the retention time of 1.5 min. Calibration curves (LCMS-8040) were linear over the range of 0.5 ng/mL to 50 ng/mL for BPA and BPA-G (Figure 5.3) with $R^2 > 0.99$. Calibration curves (LCMS-8080) were linear over the range of 0.1 ng/mL to 50 ng/mL for both BPA and BPA-G with $R^2 > 0.99$ (Figure 5.4).

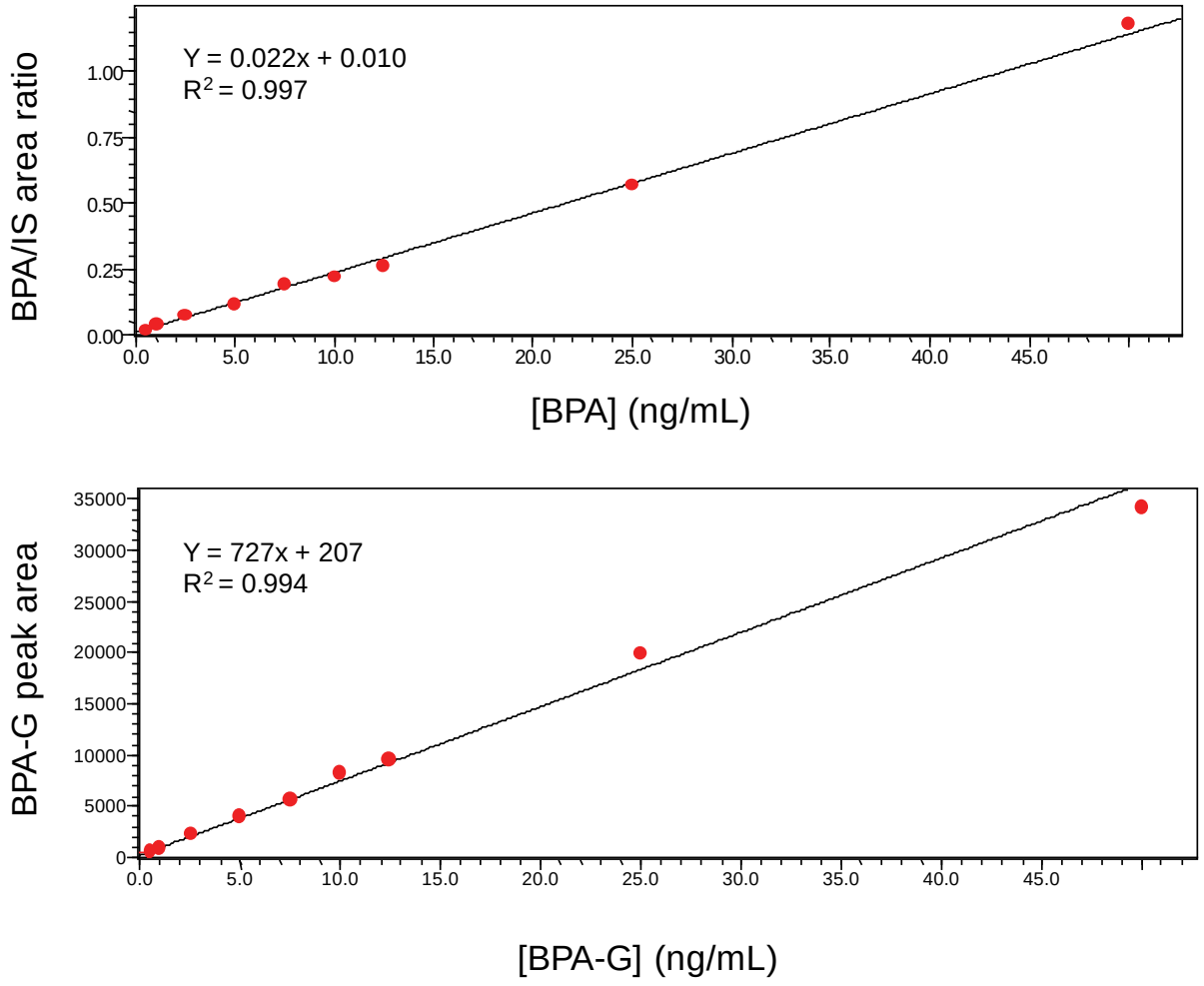


Figure 5.3. Calibration curve for BPA and BPA-G spiked into human serum (LCMS-8040).

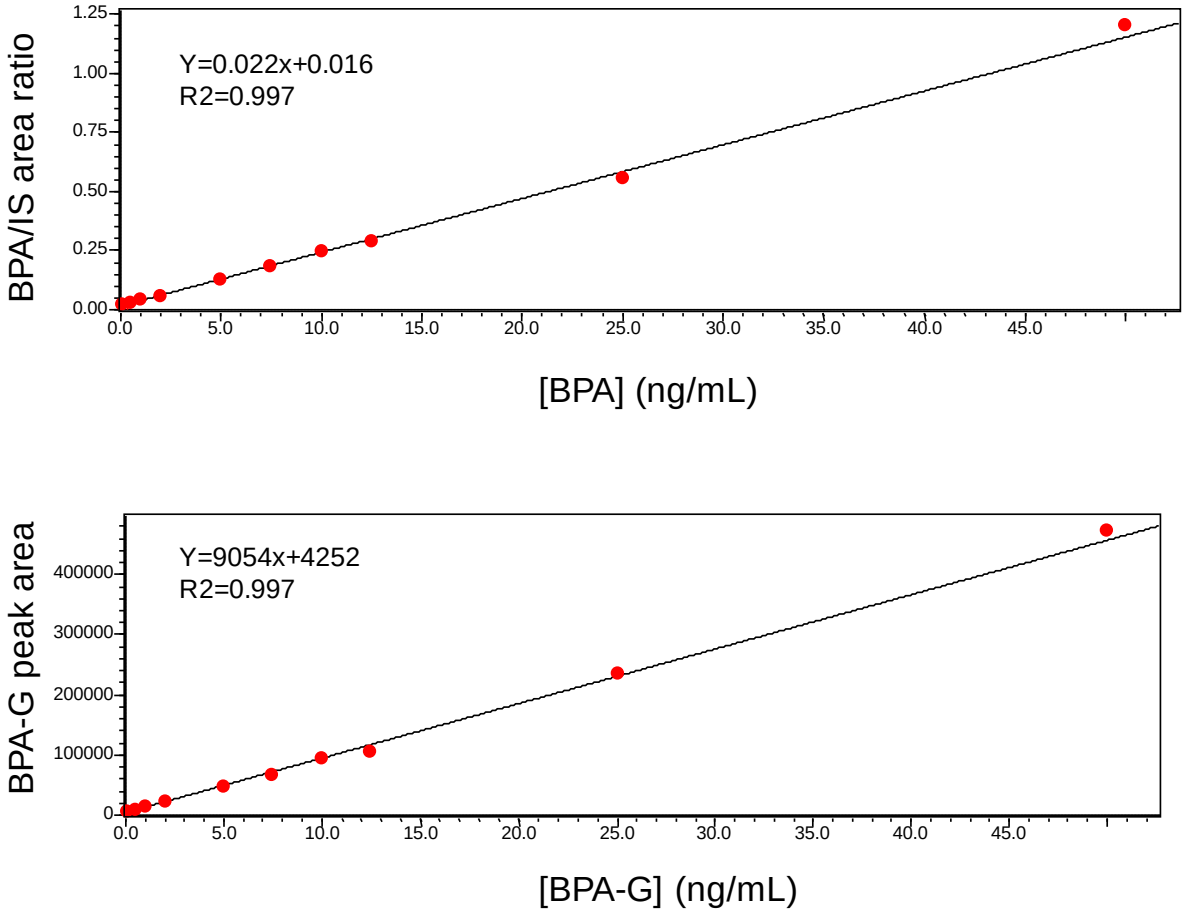


Figure 5.4. Calibration curve for BPA and BPA-G spiked into human serum (LCMS-8080).

The lower limit of quantitation (LLOQ; signal/noise ratio of 10) on LCMS-8040 was 0.5 ng/mL in serum for BPA and BPA-G (Figure 5.5). The accuracy of assay was defined as the percent of the mean of 5 replicates of QC samples at 3 different concentrations (2 ng/mL , 8 ng/mL and 15 ng/mL) compared with the true value. Measured concentration of BPA and BPA-G in human serum showed excellent accuracy with less than 10% bias (Table XXIII). The precision of the assay was defined as the coefficient of variation (CV%) calculated from 5

measurements of the QC samples at 3 different concentrations. The precision of the assay (CV%) was less than 12%.

TABLE XXIII. ACCURACY AND PRECISION OF BPA, BPA-G, AND BPA-S IN SERUM (LCMS-8040).

Analyte	Nominal value (ng/mL)	Measured value (ng/mL)	Intra day N=5		Inter day N=15	
			CV%	Accuracy	CV%	Accuracy
BPA						
Low	2	2.1	6.3	106	10.5	105
Med	8	8.6	7.1	105	8.6	107
High	15	15.1	5.8	102	9.4	100
BPA-G						
Low	2	1.9	7.5	98	8.4	95
Med	8	7.7	8.9	103	9.8	96
High	15	14.4	7.6	102	11.5	96
BPA-S						
Low	2	1.8	5.9	103	7.4	90
Med	8	7.2	10.6	94	11.9	90
High	15	14.2	8.2	102	10.1	95

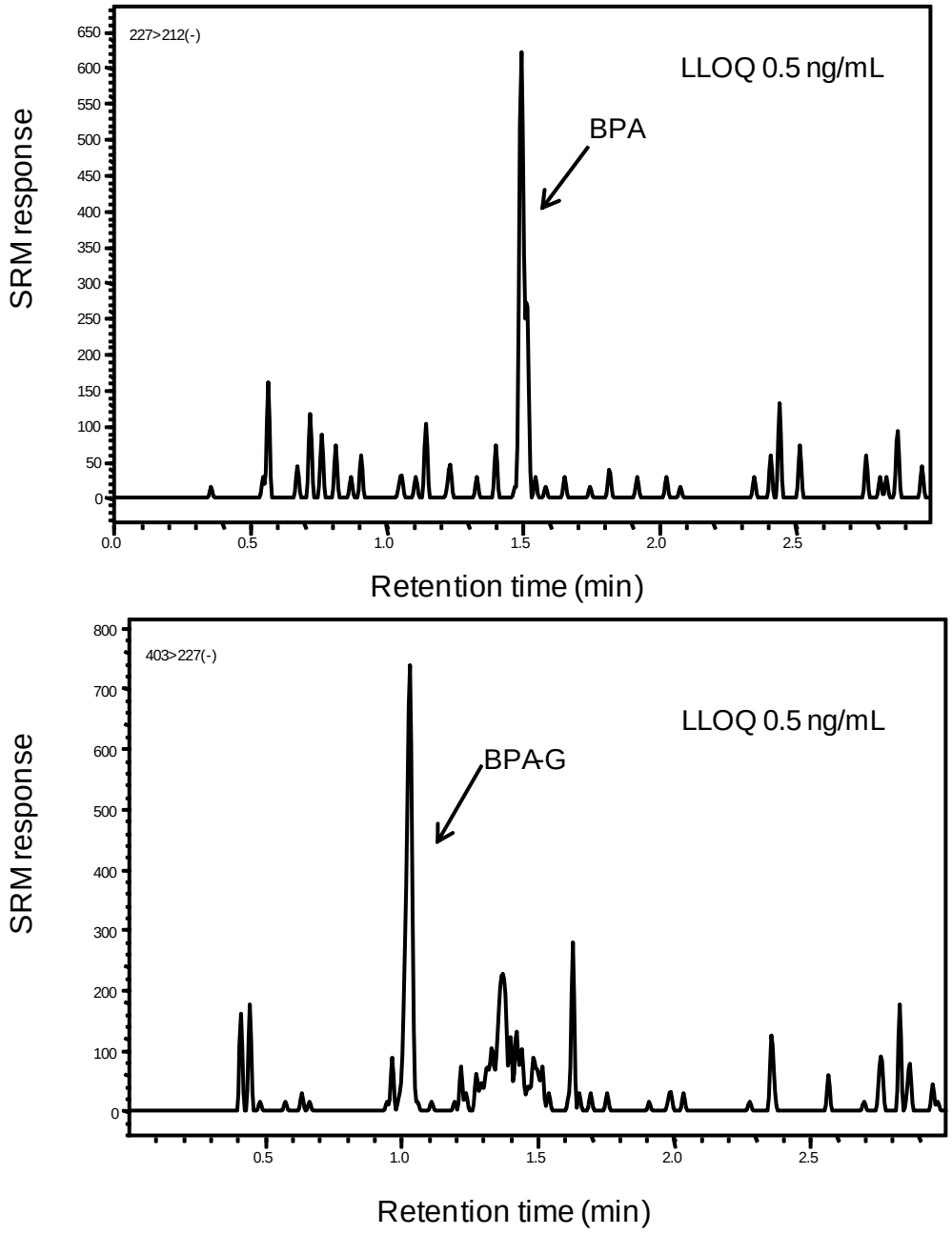


Figure 5.5. LLOQ of BPA and BPA-G in human serum (LCMS-8040)

The lower limit of quantitation (LLOQ; signal/noise ratio of 10) on LCMS-8080 was 0.1 ng/mL in serum for BPA and BPA-G (Figure 5.6). The accuracy of assay was defined as the percent of the mean of 5 replicates of QC samples at 3 different concentrations (1 ng/mL , 8 ng/mL and 15 ng/mL) compared with the true value. Measured concentration of BPA and BPA-G in human serum showed less than 15% bias (Table XXIV). The precision of the assay (CV%) was less than 13%.

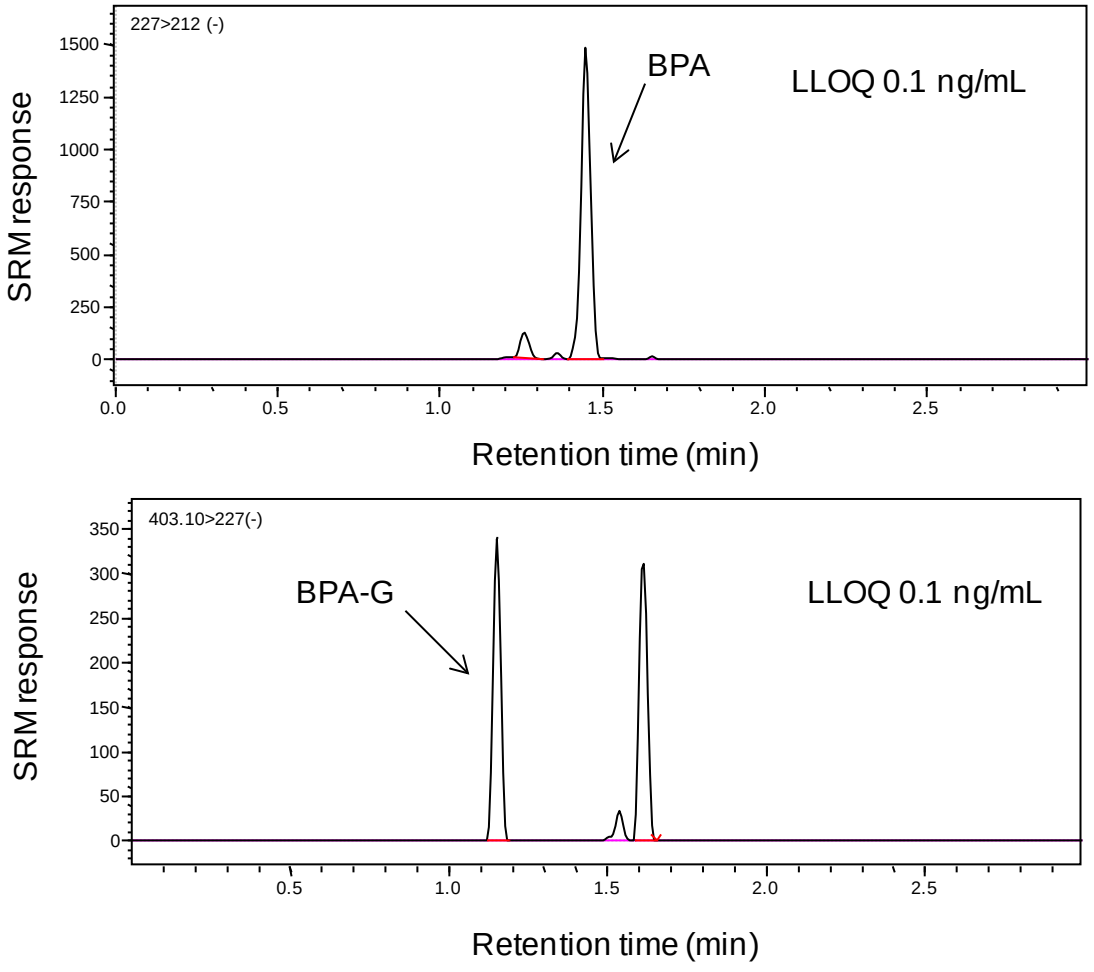


Figure 5.6. LLOQ of BPA and BPA-G spiked in human serum (LCMS-8080)

TABLE XXIV. ACCURACY AND PRECISION OF BPA AND BPA-G IN SERUM (LCMS-8080).

Analyte	Nominal value (ng/mL)	Measured value (ng/mL)	Intra day N=5		Inter day N=15	
			CV%	Accuracy	CV%	Accuracy
BPA						
Low	1	1.04	6.0	103	7.2	104
Med	8	7.9	8.3	101	10.3	99
High	15	14.4	9.5	101	9.8	96
BPA-G						
Low	1	1.05	2.5	115	10.1	105
Med	8	7.2	6.7	89	7.5	90
High	15	14.2	5.4	89	12.8	95

The recovery of BPA, BPA-G and BPA-S at low (2 ng/mL) and high (15 ng/mL) concentrations was determined 3 times and calculated by comparing the peak area ratios of the analyte/internal standard in the extracted samples with blank matrix spiked with analyte after extraction. Internal standards were post-spiked into both the extracted samples and the extracted matrix blanks. The recovery of BPA, BPA-G, and BPA-S in human serum was determined as ~90%, ~87%, and ~85%, respectively.

Matrix effect refers to ion suppression or enhancement in the measurement of analyte signal due to the endogenous components present in biological matrices. Matrix effect can directly impact the accuracy, precision, and the overall reliability of a method, thus FDA recommends the evaluation of matrix effect for bioanalytical methods (142). Two common methods are typically used to evaluate matrix interference. One is known as post-column analyte infusion (137), which is considered as a qualitative evaluation of the matrix effects. The other method compares the response of the analyte spiked into extracted blank matrix to the response of the analyte in neat solution providing a quantitative assessment (160). As an alternative to these two methods, the use of precision (%CV) of calibration line slopes

obtained from 6 different serum/plasma sources is a more rigid method for matrix effects assessments (161). The %CV values less than 3-4% would be considered free from relative matrix effect. Due to the limitation of commercially available sources for BPA free serum, the matrix effects of BPA and BPA metabolites were evaluated by the post extraction spiking experiments. Blank serum samples were extracted using protein precipitation by acetonitrile and then spiked with BPA, BPA-G and BPA-S standards to make 3 different levels of QC samples. The mean peak areas of each analyte spiked into the extracted blank matrix were compared with the neat standard solutions. The mean matrix effects for BPA, BPA-G and BPA-S was ~85%, ~81% and ~80%, respectively. A recent published LC-MS-MS method for quantitative analysis of BPA used a calibration standard dissolved in methanol without any matrix to make the calibration curves (162). This might not be appropriate given the fact that matrix effects do exist and preparing a calibration curve without blank matrix may lead to misleading concentration results.

To determine that all materials used during sample preparation and UHPLC-MS-MS analysis were free of BPA and its metabolites, solvent blank (acetonitrile) and water were analyzed using UHPLC-MS-MS. The acetonitrile solvent blanks contained no detectable BPA at a retention time of 1.5 min (Figure 5.7). To accurately quantify BPA in human serum, blank human serum was needed to prepare the calibration curve standards. Figure 5.8 shows the UHPLC-MS-MS chromatogram of the charcoal dextran-stripped human serum used for sample preparation. Although there were peaks detected in the SRM chromatogram for the *m/z* 227 to 212 transition, they were chromatographically separated from BPA (retention time 1.5 min), and it did not correspond to BPA and did not interfere with the analysis. No peaks for BPA metabolites were detected in this blank serum.

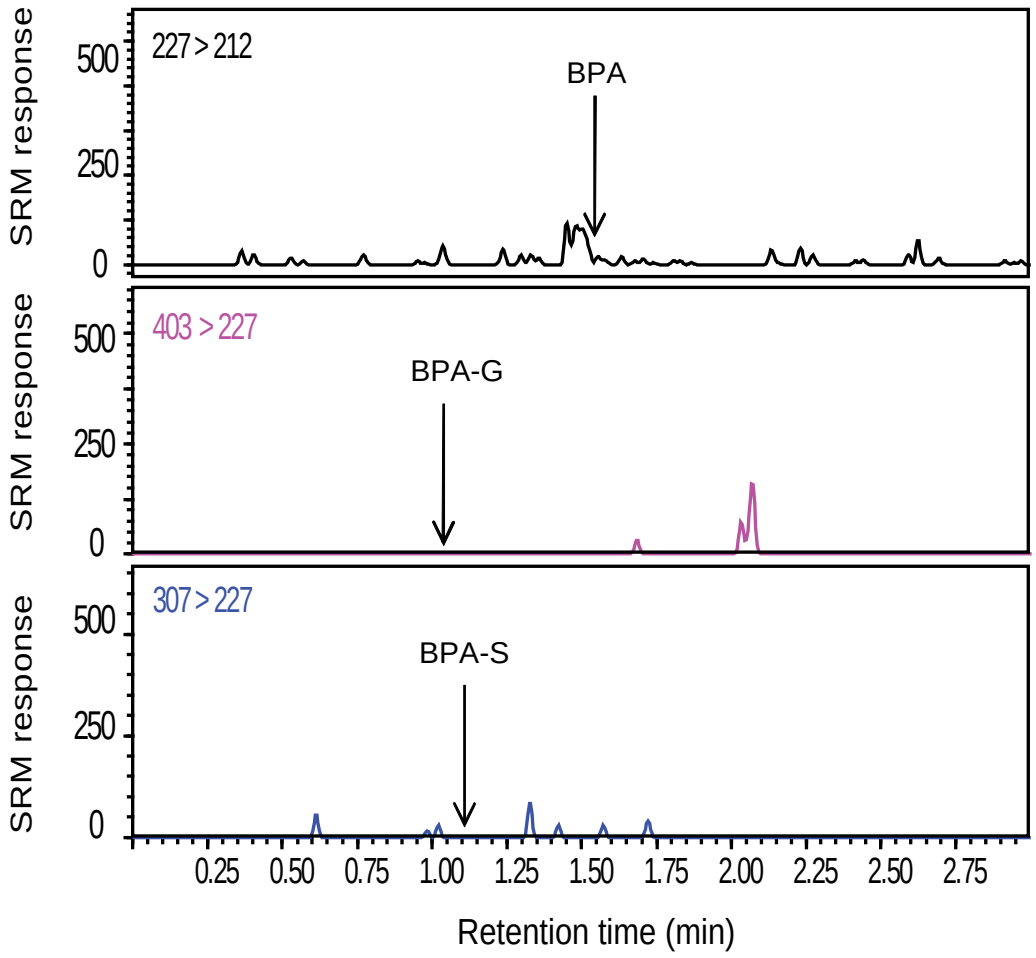


Figure 5.7. Negative ion electrospray UHPLC-MS-MS SRM of BPA, BPA-G and BPA-S in solvent blank (acetonitrile). Top: BPA ; Middle: BPA-G; Bottom: BPA-S.

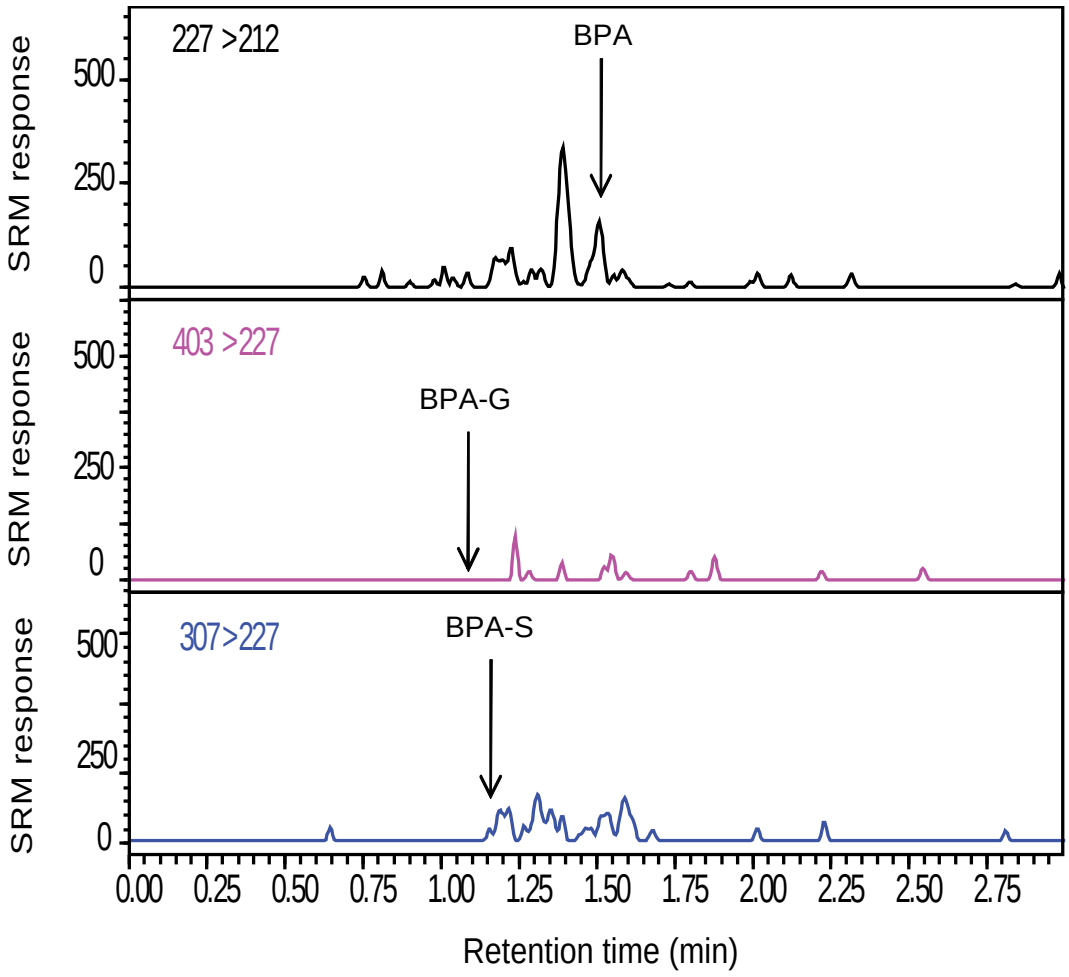


Figure 5.8. Negative ion electrospray UHPLC-MS-MS SRM of BPA, BPA-G and BPA-S in blank human serum. Top: BPA ; Middle: BPA-G; Bottom: BPA-S.

To test the positive controls in the round robin analysis, high-purity BPA-free water and BPA-free serum were spiked with BPA and BPA-G at 0.5, 1 and 2 ng/mL each. Representative chromatograms of spiked samples BPA and BPA-G at 1 ng/mL are shown in Figures 5.9.

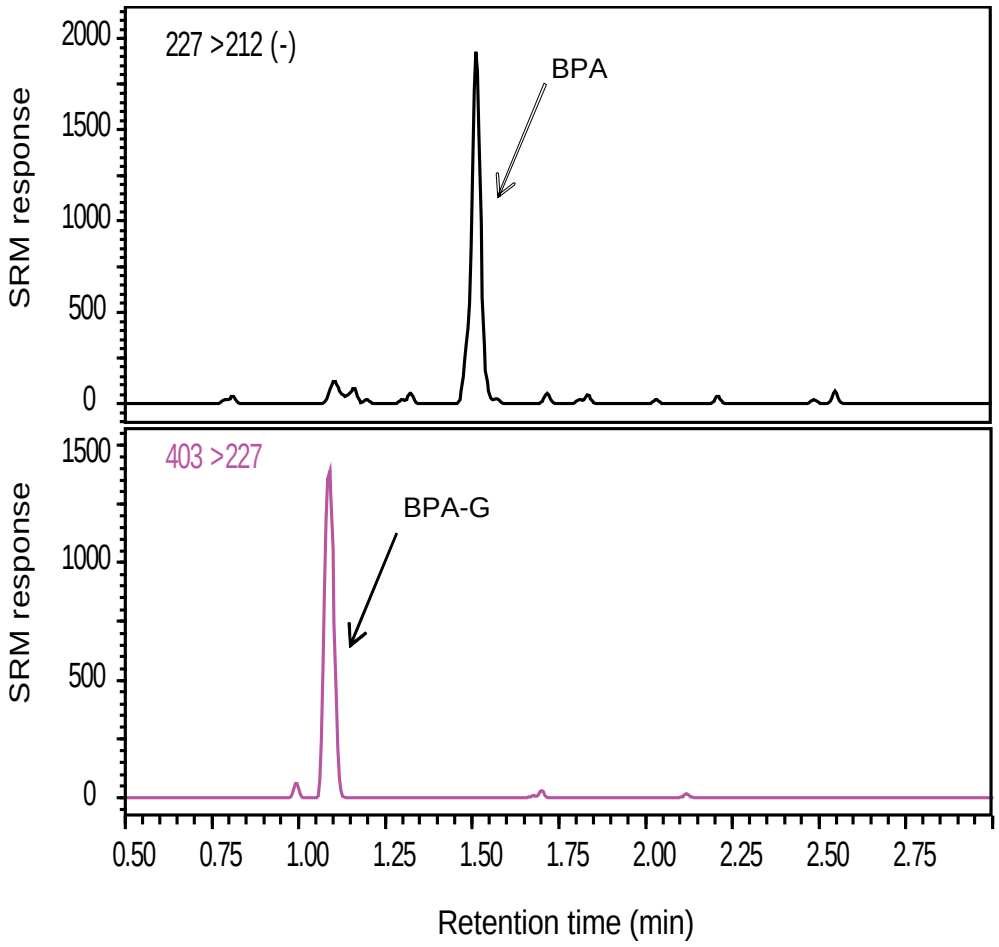


Figure 5.9. Negative ion electrospray UHPLC-MS-MS chromatograms of 1.0 ng/mL BPA and BPA-G spiked into serum. Top: BPA; and Bottom: BPA-G.

Back-calculated concentrations of BPA and BPA-G in the spiked samples are summarized in Table XXV. The accuracy of assay was defined as the percent of the mean of 3 measurements of the 3 QC samples compared with the true value. Measured concentration of BPA and BPA-G in both serum matrix and water showed excellent accuracy with less than 10% bias throughout the 0.5 ng/mL to 2 ng/mL concentration range (Table XXV). The precision of the assay was defined as the coefficient of variation (CV%) calculated from 3 measurements of the QC samples at 3 different concentrations. The precision of the assay (CV%) was less than 12%.

TABLE XXV. MEASURED CONCENTRATIONS OF BPA AND BPA-G SPIKED INTO WATER AND SERUM.

Spiked concentration (ng/mL)	Water		Serum	
	Measured BPA	Measured BPA-G	Measured BPA	Measured BPA-G
0.5	0.50 ± 0.03[1]	0.48 ± 0.03	0.49 ± 0.04	0.48 ± 0.03
1	1.05 ± 0.13	1.11 ± 0.08	1.04 ± 0.10	0.90 ± 0.02
2	2.12 ± 0.09	2.11 ± 0.08	2.13 ± 0.01	2.07 ± 0.13

[1] n=3, data expressed as Mean ± S.D.

The results for the quantitative analyses of BPA and BPA-G in spiked human serum specimens and water content from our lab together with the other 3 participating labs are summarized in Table XXVI. The values reported represent the mean ± S.D. of replicate analyses. The spiked concentration of BPA was from 0.2 ng/mL to 12.8 ng/mL and BPA-G was from 2 ng/mL to 32 ng/mL in blank human serum. To monitor method performance and determine if any of the materials used for sample preparation and analysis could be a potential source of BPA contamination for biological specimens, which may affect the accuracy of the method, 1 blank human serum and 1 blank water content were included in this

study. The results of the blank sample analyses of BPA-G demonstrated the methods from all 4 labs were free of BPA-G contamination. However, for BPA measurement in these blank samples, 3 out of 4 labs reported concentration below the limit of quantitation. Only lab 4 showed a concentration of 0.04±0.02 ng/mL in the unspiked blank serum sample and a concentration of 0.2±0.01 ng/mL in the unspiked water content. This detectable concentration of BPA in blank serum and water samples indicated that lab 4 might have background contamination during the sample analysis. For the lowest concentration of BPA spiked into serum (0.2 ng/mL), 3 out of 4 labs reported levels that were below LLOQ. For the lower concentration of the spiked sample (0.8 ng/mL), the values from our analysis were the closest to the true value while the other 3 labs showed 75%-97% bias. In high concentration range (from 3.2 ng/mL to 12.8 ng/mL), all the reported levels from our lab were very close to the spiked concentration. Overall, the results suggested the accuracy of our new UHPLC-MS-MS for the quantitative analysis of BPA in human serum was the best among all the participating labs.

A large variation was observed from all 4 independent labs analyzing replicates of the same set of samples. Although the methods used by each individual lab were similar, the difference in the results would still be considered significant. In the high concentration spiked samples (12.8 ng/mL), the reported values ranged from 2.4 ng/mL to 13.5 ng/mL. Such a huge variation was also found in the low concentration samples (0.8 ng/mL), with reported values ranging from 0.02 ng/mL to 1.0 ng/mL. The poor reproducibility of the inter-lab results was not only found in the BPA analysis, but also in BPA-G analysis. Almost 2 fold difference for the high concentration fortified samples (32 ng/mL) was observed with values ranging from 20.2 ng/mL to 39.2 ng/mL. Thus, it can be concluded that inconsistent results were found among independent labs for the measurements of BPA and BPA-G in spiked human serum

specimens and such a disparity within analytical labs may raise questions on the reliability of LC-MS-MS for the measurable levels of BPA and its metabolite in human serum.

Several factors may lead to the observed variation in different labs. First, the source of the standard and the chemical purity were unknown and might be different among various labs. This can introduce errors to the following calibration curves preparations. Second, sample preparation methods may vary among different labs. A variety of sample preparation techniques were used in the published LC-MS-MS methods. We developed a simple protein precipitation by acetonitrile which was shown to have good recoveries for all the analytes. Similar sample preparation procedures were reported by Volkel et al (82). Besides protein precipitation, SPE (C_{18}) was selected to clean up the serum samples in several research groups (118, 122). However, no recovery data was generated for the BPA metabolites since enzymatic hydrolysis was used to determine the total concentration of BPA in these methods. An alternative method was liquid liquid extraction, where various organic solvents including ethyl acetate and ethyl ether were used in the literature (118, 163). Last but not least, different LC-MS-MS instruments, mobile phase, and HPLC columns might be used among the 4 participating labs. Instrument performance was unknown for these methods.

TABLE XXVI. CONCENTRATIONS OF BPA AND BPA-G IN SPIKED HUMAN SERUM SPECIMENS AND WATER CONTENT FROM 4 PARTICIPATING LABS.

Sample type	BPA					BPA-G				
	Spiked	Measured ng/mL				Spiked	Measured ng/mL			
	ng/mL	UIC	Lab 2	Lab 3	Lab 4	ng/mL	UIC	Lab 2	Lab 3	Lab 4
serum	12.8	13.5±1.2	4.8±0.3	8.7±0.2	2.4±0.9	32	20.2±1.7	39.2±2.9	29.7±1.1	38.5±8.9
serum	6.4	9.3±0.9	3.5±0.2	5.3±0.2	2.4±0.1	16	14.2±1.6	26.2±4.8	17.9±0.2	18.8±1.3
serum	0.2	<LLOQ	<LLOQ	<LLOQ	0.3±0.08	2	1.8± 0.2	2.5±0.1	2.2±0.05	2.1±0.17
serum	0	<LLOQ	<LLOQ	<LLOQ	0.04±0.02	0	<LLOQ	<LLOQ	<LLOQ	<LLOQ
serum	0.8	1.0±0.2	0.2±0.01	0.2±0.04	0.02±0.03	4	2.4±0.2	4.6±0.4	3.5±0.06	6.2±2.0
serum	3.2	2.3±0.6	1.5±0.2	2.1±0.18	0.3±0.1	8	4.8±0.5	9.1±0.8	5.5±0.1	8.3±0.3
water	3.2	2.7±0.3	1.5±0.1	1.9±0.04	2.5±0.1	8	5.8±0.5	5.8±0.2	7.0±0.5	3.9±0.1
water	0	<LLOQ	<LLOQ	<LLOQ	0.2±0.01	0	<LLOQ	<LLOQ	<LLOQ	<LLOQ

1. Data expressed as Mean ± S.D.

The results of the quantitative analyses of BPA and BPA-G in the round robin unknown clinical samples are summarized in Table XXVII. The values represent the mean ± S.D. replicate analyses. Representative chromatograms of BPA, BPA-G, and BPA-S are shown in Figure 5.10. The back-calculated concentration for BPA in sample was 0.4 ng/mL and BPA-G in sample ID 9 was 0.5 ng/mL (Table XXVII). No detectable BPA-S was observed in any of the samples.

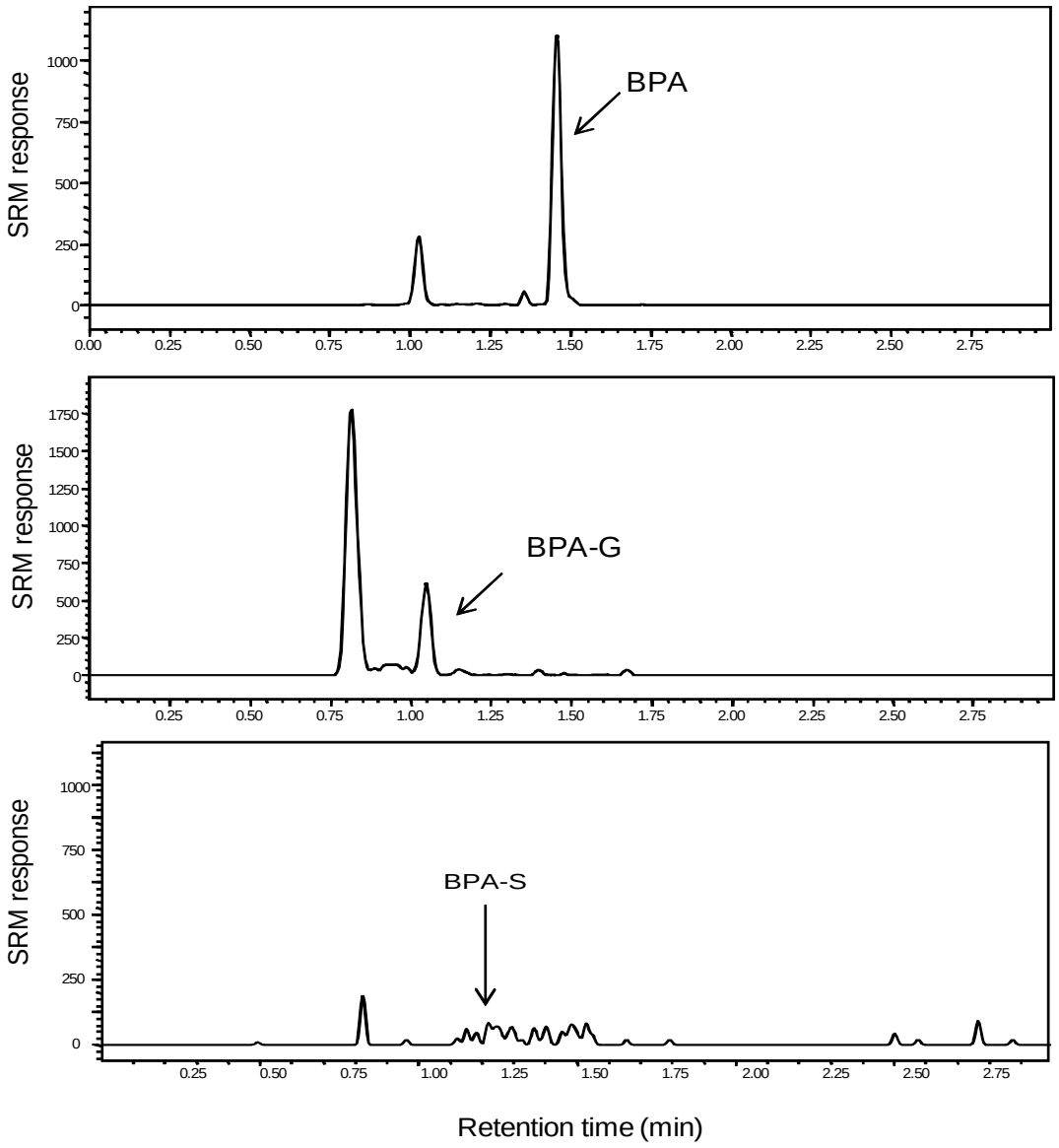


Figure 5.10. Representative chromatograms of BPA, BPA-G and BPA-S in human serum ID # 9 analyzed as part of the NIEHS round robin study. No detectable BPA-S was found in any of the serum samples.

Clinical serum specimens from human subjects were collected using 2 phlebotomy materials. Vacutainer needles and vacutainer butterfly were used to collect the first set of serum samples (Sample ID 1, 4, 5, 8 and 10). The second set of samples (Sample ID 2, 3, 6, 7 and 9) was obtained separately through a straight 21G needle. As shown in Table XXVI, our measurements of BPA concentrations in sample ID 1, 4, 5, 8, and 10 were unexpectedly high with a mean value of 18.6 ng/mL. These 5 samples were collected using vacutainer butterfly which was later found to be a potential source of the BPA contamination by UCSF researchers. Thus, the high serum concentrations were not relevant to human exposure to BPA but resulted from background contamination during sample collection. Similar results acquired from the other 3 labs also confirmed the backgroud BPA contamination from the first set of samples. Measured concentrations for BPA from our lab were very similar to lab 2 while the other 2 labs showed consistently lower level than ours.

The second set of samples contained less than 0.5 ng/mL BPA based on our measurements while Lab 2 and 3 showed concentrations below the limit of quantitation for the same set of samples probably due to the sensitivity limitation of the analytical instruments. Only Lab 4 reported BPA concentration for these 5 samples with lower values compared with our results.

Our measurement showed half of the samples had BPA-G concentrations below the limit of quantitation. For those above the limit of quantitation, the concentration ranged from 0.5 ng/mL to 4.5 ng/mL. Lab 2 and Lab 4 reported BPA-G concentrations ranging from 1.9 ng/mL to 13.7 ng/mL and 0.01 ng/mL to 4.5 ng/mL, respectively.

In summary, due to the contamination of BPA from the phlebotomy materials, half of the clinical human serum was not valid for the assessment of human BPA exposures. Clinical

relevant concentration of BPA obtained from the contamination free collection method were below 0.5 ng/mL.

TABLE XXVII. CONCENTRATIONS OF BPA AND BPA-G IN CLINICAL SERUM SPECIMENS FROM 4 PARTICIPATING LABS.

	UIC[1]		Lab 2		Lab 3		Lab 4	
ID	BPA ng/mL	BPA-G ng/mL	BPA ng/mL	BPA-G ng/mL	BPA ng/mL	BPA-G ng/mL	BPA ng/mL	BPA-G ng/mL
1	20.8±1.1[1]	<LLOQ	26.3±1.3	<LLOQ	7.7±0.1	<LLOQ	14.8±1.0	<LLOQ
2	0.3±0.06	1.4±0.07	<LLOQ	2.3±0.4	<LLOQ	<LLOQ	<LLOQ	0.8±0.01
3	0.3±0.03	1.0±0.08	<LLOQ	2.7±0.2	<LLOQ	<LLOQ	0.01±0.02	1.0±0.05
4	9.4±0.8	<LLOQ	10.9±0.8	<LLOQ	3.4±0.1	3.2±0.1	7.2±0.2	0.01±0.01
5	17.1±1.5	4.9±0.03	20.9±0.5	13.7±0.5	6.8±0.1	<LLOQ	12.9±0.2	4.5±0.25
6	0.5±0.09	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	0.24±0.06	0.03±0.00
7	0.4±0.02	2.2±0.1	<LLOQ	<LLOQ	<LLOQ	<LLOQ	0.15±0.04	3.1±0.12
8	24.3±0.7	<LLOQ	22.6±0.3	<LLOQ	9.6±0.1	<LLOQ	17.3±0.9	<LLOQ
9	0.4±0.02	0.5±0.04	<LLOQ	1.9±0.3	<LLOQ	<LLOQ	0.14±0.05	0.6±0.08
10	21.5±0.9	<LLOQ	21.8±0.6	<LLOQ	8.5±0.2	<LLOQ	16.0±0.9	0.01±0.01

1. Data expressed as Mean ± S.D.

5.4 Conclusions

We have developed a UHPLC-MS-MS based method for the quantitative analysis of BPA, BPA-G and BPA-S in human serum that is considerably faster and more sensitive than previously reported LC-MS-MS methods. The total time between UHPLC-MS-MS analyses was only 3 min. The method is at least 3 times faster than the existing LC-MS-MS methods which generally take 10 to 20 min with conventional HPLC (82, 118, 162, 164).

The limit of quantitation was 0.1 ng/mL for BPA and BPA-G on LCMS-8080 which is 5 times sensitive than LCMS-8040. The sensitivity on LCMS-8080 was sufficient for the measurement of human clinical serum specimens. The volume of human serum required per analysis was only 200 μL which is superior to the previously published methods. For example, Volkel et al reported a LOQ of 2.3 ng/mL for the measurement of BPA in serum (122) with 1 mL serum needed for the analysis. Similarly, Liao et al. recently published a LC-MS-MS based method to measure BPA in human serum by using 1 mL serum samples (162). Thus, the new UHPLC-MS-MS appears to be suitable for the biomonitoring of BPA in human serum with fast speed and high sensitivity as well as small volume of serum required.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Phytoestrogens are polyphenolic, non-steroidal plant derived compounds, which can interfere with the endogenous estrogen signaling and associated process. Hops (*Humulus lupulus* L.), the female flowers of this species, which are widely used in beers as flavor agent, are under investigation as hormone replacement therapy since they contain estrogenic prenylflavonoids. No phase I clinical trial for a standardized hop extract has been carried out in human subjects to study the pharmacokinetic profile for the major prenlyflavonoids. The experiments conducted and summarized in this dissertation provide answers to some important questions about the human exposure to hop prenylflavonoids following oral administration.

To support the phase I clinical trial, a rapid, sensitive and selective UHPLC-MS-MS method for the measurement of the major prenylflavonoids in human serum was developed, validated and reported in Chapter 2. The new method used a UHPLC coupled with a fast scanning triple quadrupole mass spectrometer which provided base line separation of all the analytes in less than 2.5 min. The new assay was 2-3 fold faster than previously published methods and retained its robustness.

A hop extract was standardized to 8-PN using LC-MS-MS with authentic reference standards as described in Chapter 3. The same hop extract was used in the following phase I clinical trial and was orally administered to 5 post-menopausal women subjects. Serial blood and 24 h urine samples were collected for analysis.

Using the new assay described in Chapter 2, the serum and urinary levels of 4 prenylflavonoids were measured in support of the phase I clinical trial as described in Chapter

4. The clinical study was a phase I study for the evaluation of the overall safety of hop extract. Three escalating doses were administered orally to the same 5 study subjects after 1 month washout period in between. It was shown that all 4 prenylflavonoids were rapidly absorbed into the bloodstream and a second peak at 5-8 h post dose indicated that enterohepatic circulation occurred. Most of the detected prenylflavonoids were in the form of glucuronic acid conjugates, and urinary excretion was found to be the minor elimination pathway for these compounds. Since no fecal or bile samples were collected, the extent of biliary excretion remains unknown.

In the future, metabolite profiling should be carried out to determine the in vivo metabolic fate of these prenylflavonoids. Reference standards of phase I hydroxylated metabolites and phase II glucuronic acid conjugate need to be synthesized, purified and characterized for qualitative and quantitative analysis. Extraction procedures for these metabolites from the biological matrix might be different from the current method for the precursor prenylflavonoids since biotransformation might change the lipophilic/hydrophilic properties of the parent compounds. Metabolite identification by LC-MS should involve both accurate mass measurement by high resolution mass spectrometry and product ion spectra acquisition for structural characterization. Important questions such as which major metabolite is in the circulation and how polymorphisms might alter human exposure to these prenylflavonoids need to be answered.

BPA, a weak estrogenic compound, has raised controversies during the past decades because it exerts detectable hormone-like properties. A 2010 report from the US FDA raised further concerns regarding exposure to fetuses, infants and young children. Biomonitoring of BPA plays a crucial role in the exposure evaluation and risk assessment. The bioanalytical methods for the measurement of BPA concentration in human biological samples are still

under discussion. We have developed and validated a new UHPLC-MS-MS method for the detection of BPA and BPA metabolites in human serum samples and applied the method to a round robin analysis sponsored by NIEHS in which all 4 methods were used to measure the same set of serum samples. Our method was the fastest and among the most sensitive and accurate of those tested in the round robin analysis. However, a large inter lab variation of the measured concentrations of BPA and BPA-G was observed for the spiked human serum samples. Also, a large discrepancy in human clinical serum samples was detected. Given the inconsistent results in the measurement of BPA and its metabolite in human serum by different labs, future studies should be focused on establishing a preferred protocol and identification of the key contributing steps and factors that might impact the accuracy and precision of the assay.

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PUBLICATIONS

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