High-Performance Liquid Chromatographic Determination of Xanthohumol in Rat Plasma, Urine, and Fecal Samples

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Abstract

Xanthohumol (XN) is the major prenylated flavonoid in hop plants and as such a constituent of beer. Pharmacological studies have shown that XN possesses marked antioxidant and antiproliferative effects. In order to study the resorption and metabolism of this compound, reversed-phase high-performance liquid chromatography is used for the determination of XN in rat plasma, urine, and feces. In session one, rats receive either oral or intravenous (iv) administration (20 mg/kg body weight) of XN. In session two, rats receive oral administration of 50, 100, 200, 400, and 500 mg/kg body weight XN for bioavailability studies at various dose levels. Plasma, urine, and feces are collected at varying time points and assayed for their XN content. Plasma levels of XN fell rapidly within 60 min after iv administration; no XN is detected in plasma after oral administration in either session. XN and its metabolites are excreted mainly in feces within 24 h of administration. The method is a reliable tool for performing studies of XN in different biological material.

Introduction

Hop, consisting of the dried strobiles of *Humulus lupulus* L. (Cannabaceae), is a climbing dioecian perennial herb native to Europe, Asia, and North America, extensively cultivated in temperate zones worldwide. The material of use comes exclusively from female plants that are cultivated primarily in the United States, Germany, Great Britain, the Czech Republic, and China (1–5). XN is the principal flavonoid present in hop cone extracts and has a prenylated chalcone structure (Figure 1) (6). Flavonoids, including chalcones, have been shown to inhibit the proliferation of cancer cells and inhibit tumor growth (7,8).

XN has been shown to be more active at protecting LDL against oxygen damage than the antioxidants in green tea and red wine, which has each been touted for its protective powers (7). Pharmacological studies showed that XN is 6 times more powerful as an antioxidant than those found in citrus fruits, and 4 times stronger than those in soy foods (7). It is an effective antiproliferative agent in human-breast, colon, and ovarian cancer cells (8). Pharmacological studies indicated that flavonoids have a beneficiary effect preventing cancerous cell growth by inhibiting cytochrome P450 enzymes (9–12). XN protected against arteriosclerosis and osteoporosis (13).

The mechanisms of XN absorption, bioavailability, and tissue distribution in humans are still undefined. *In vitro* studies have been performed on the biotransformation of XN in rat liver microsomes (11), but no *in vivo* studies have been investigated so far. Thus, a simple, accurate, and sensitive high-performance liquid chromatographic (HPLC) method for identifying and quantitating XN in rat plasma, urine, and feces was developed. The performance of the method was assessed by intravenous (iv) route and oral administration of XN to male Wistar rats and followed by its measurement in plasma, urine, and feces. For the HPLC analysis of blood, urine, and fecal samples, the removal of proteins is the most important clean-up step because proteins can precipitate when getting in contact with solvents or buffer salts commonly used for mobile phases. To avoid this problem, a simple methanolic precipitation of proteins has been performed prior to injection of the sample.

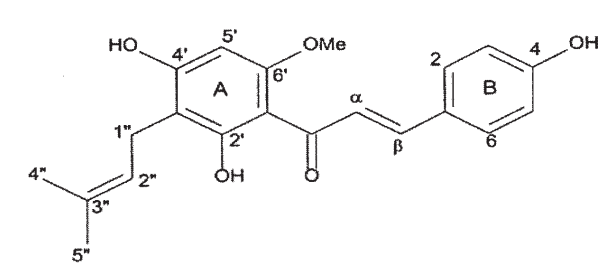

Figure 1. Chemical structure of the chalcone flavonoid XN.

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Experimental

Instrumentation

The HPLC system consisted of a model 6000A pump, model U6K injector, model 680 automated gradient controller, model 996 photodiode array detector operated at 372 nm, and computerized data station equipped with Waters Millennium software (all from Waters, Milford, MA).

Chemicals

HPLC-grade acetonitrile was obtained from Fisher Scientific (Fair Lawn, NJ). Water for HPLC use was purified in a Milli-Q system (Millipore, Bedford, MA). Trifluoroacetic acid (TFA), sodium pentobarbital, and ketamine were purchased from Sigma (St Louis, MO). XN was provided through the Hop Research Council.

HPLC procedure

A 20-µL aliquot of the sample was injected on a Synergi MAX-RP (Phenomenex, Torrance, CA) reversed-phase column (150 × 4.6 mm) with 4-µm particle size operating at 40°C. The column was equipped with a 2-cm LC-18 guard column (Supelco, Bellefonte, PA). The mobile phase comprised of solvent A (0.025% TFA in water), and solvent B (0.025% TFA in acetonitrile). The flow rate was adjusted to 1 mL/min. Separation was performed using gradient elution from 65% A and 35% B in 25.0 min to 25% A and 75% B. Each run was followed by a 10-min wash with 5% A–95% B and an equilibration period of 15 min.

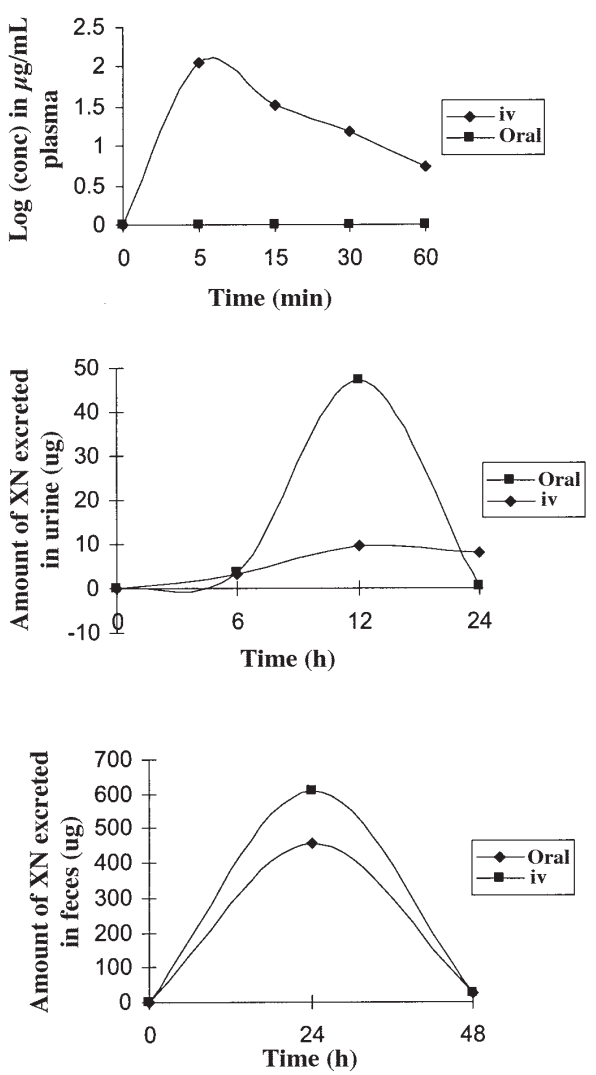

Figure 2. Time course effects of XN in plasma, urine, and feces of iv and oral routes of administration of XN (20 mg/kg body weight).

Standards and stock solutions

A standard stock solution was prepared by dissolving 1 mg substance in 10 mL methanol, and further concentrations of the standard solutions were prepared by appropriate dilution from the stock solution yielding concentrations from 250 to 0.34 µg/mL.

Animal studies and sample collection

Male Wistar rats (Harlan, Indianapolis, IN) were individually housed in suspended stainless steel caging in a climate-controlled vivarium with free access to food and water under 12-h light/dark cycle. For iv administration, rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) and ketamine (50 mg/kg), and a catheter was surgically implanted into the right jugular vein 2 days before testing. Oral administration was accomplished using stainless steel feeding tubes (Model # N-007, Popper & Sons, Inc., New Hyde Park, NY). All rats were acclimated to the metabolism cages (Model # 650-0100, Nalgene, Rochester, NY) for 24 h prior to XN administration. Prior to experimental sessions, one rat served as a reference animal, receiving 20 mg/kg body weight XN via iv administration to ensure bioavailability and absorption of XN (Figures 2 and 3). In session one, rats received either oral or iv administration (20 mg/kg body weight); under the oral administration procedure one rat received 20 mg /kg body weight pure XN and the second received an XN-rich hops extract containing 20 mg/kg body weight XN to determine if other compounds in hop affected the bioavailability of XN. In session two, a single oral administration of XN was given to each rat at the dose of 50, 100, 200, 400, and 500 mg/kg body weight. In the 50 mg/kg body weight XN condition, one rat received 50 mg/kg body weight pure XN and the

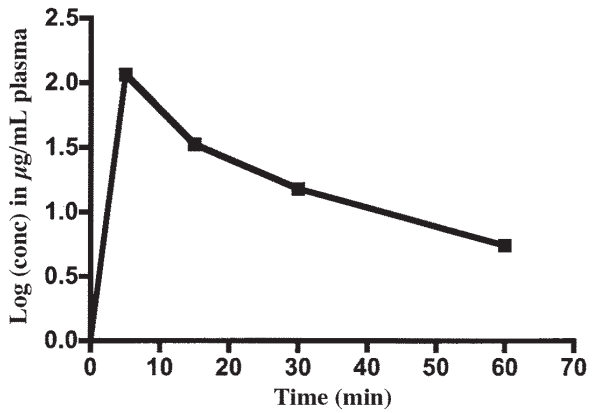

Figure 3. XN concentrations in rat blood following iv administration (20 mg/kg body weight, 250 gm at test). Data are presented as mean ± standard deviation.

second received a hop extract containing 50 mg XN.

Blood was collected via the tail vein using 50 µL heparinized microhematocrit capillary tubes immediately prior to (0 time point) and at 15, 30, 60, 120, and 240 min post XN administration. All samples were centrifuged (9000 rpm; 10 min) within 1 h of final collection and stored at approximately –20°C until analysis. Excreta (urine and feces) were collected from the metabolic chamber immediately prior to (0 time point) and at 0–6, 6–12, 12–24, 24–48, and 48–72 h (urine) and 0–24, 24–48, 48–72, and 72–96 h (feces) after XN administration. Urine and feces were stored in plastic screw capped test tubes at 4°C immediately after collection and frozen at –20°C until further analysis. Animals were euthanized via CO_2 asphyxiation at the termination of the experiment. These protocols were approved by the University of Mississippi IACUC (protocol #03-008) (University, MS)

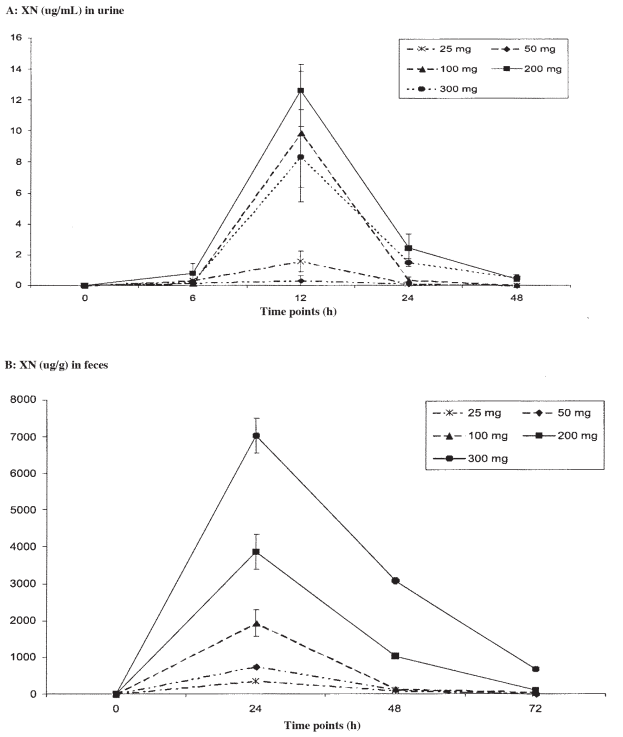

Figure 4. Dose-dependent excretion of XN via urine and feces.

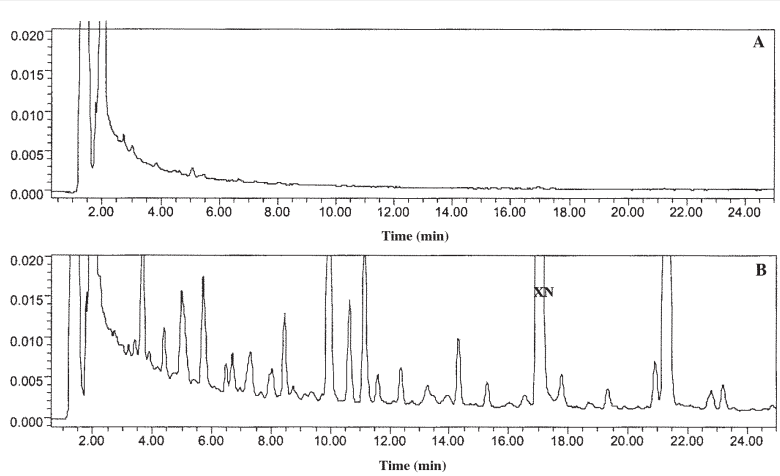

Figure 6. Typical chromatograms of a fecal sample (A) collected prior to drug administration, and (B) showing numerous metabolites collected 24 h post-XN (500 mg/kg body weight, oral, 500 gm at test) administration.

Sample preparation

To a 1.0-mL polypropylene eppendorf tube containing 200 µL cold methanol was added 100 µL of rat plasma or urine . The tubes were vortexed for 30 s. Separately, the fecal samples were dried and powdered. The dried weight of the known quantity of feces (~ 250 mg) was extracted with 2.0 mL of methanol.

All of the mentioned samples were sonicated and centrifuged at 9,000 rpm for 10 min. After centrifugation, 20 µL of the resulting supernatant was directly injected onto the HPLC system

Results and Discussion

Method validation

A calibration curve for XN (external standard) was constructed by linear regression, and the concentration–response relationship of the present method indicated linearity ($r^2 > 0.999$) over the injected concentration range from 0.34 to 250 µg/mL for XN. Precision (%relative standard deviation) of the obtained results fell well within the predefined limits of acceptability (< 5%), the accuracy of the method was confirmed by determining the recovery spiking urine sample with known amounts of the standard compound. The recovery rate was found to be 98.5%. The limit of detection was 0.013 µg/mL and the limit of quantitation was 0.1 µg/mL.

All chromatograms were recorded at 372 nm, the maximum absorbance of XN. Peak identification was carried out by comparison of the retention time and its UV spectra (from 205–400 nm) with those of the standard.

Data resulting from session 1 is presented in Figure 2. Although XN could not be detected in plasma following oral administration, it was detected following iv administration, with T_{max} at 5 min (Figure 3) in plasma and at 12 h in urine postadministration. With iv administration of XN, an extremely rapid fall in the plasma concentration of the drug within 60 min was observed in all animals tested ($n = 3$). Under iv and oral administration conditions, 6.33% and 1.72% of the XN were found to be excreted unchanged, respectively. XN was excreted primarily in the feces within 24 h with trace amounts detected in urine (iv administration condition only). These data demonstrate that only a small amount of XN is excreted unchanged, the majority is found in feces.

XN dose response data from session two are presented in Figure 4. Analyses revealed that 1.73–3.59% of administered XN is excreted unchanged in urine and feces, mainly in feces because of this total amount only 0.06–0.49% was found in urine (Figure 4A). Observing the urine

data more closely showed that at the 6-h time point, only trace amounts of XN were detected. Maximum peak levels of XN were found at the 12-h time point and following a dose-dependent concentration gradient. At the 24-h time point, XN levels were greatly reduced; however, a dose-dependent concentration gradient was still evident. After 48 h, XN levels detected were similar to that at the 6-h time point. Consistent with these observations, statistical analyses of these data revealed a significant dose [$F(4, 8) = 5.14$, $p < 0.05$], time [$F(4, 32) = 24.11$, $p < 0.0001$], and dose x time interaction [$F(16, 32) = 3.13$, $p < 0.005$]. The overwhelming majority (99.51–99.94%) of XN excreted was detected in feces (Figure 4B). Highest XN levels were detected at 24 h following a dose-dependent concentration correlation. At the 48-h time point, XN levels detected were reduced; however, a dose-dependent concentration gradient was still evident. After 72 h, only trace amounts of XN were found even at the highest dose administered. Consistent with these observations, statistical analyses of these data yielded a significant dose [$F(4, 8) = 115.48$, $p < 0.0001$], time [$F(3, 24) = 178.54$, $p < 0.0001$], and dose x time interaction [$F(12, 24) = 35.73$, $p < 0.0001$].

In plasma, urine, and fecal samples, XN was adequately resolved from adjacent peaks regardless of whether it was administered iv or orally at a relative retention time of 17.33 min (Figure 5). From the chromatograms it has been shown that in the iv administration of the drug, XN was detected in plasma, urine, and feces, and in the oral administration XN was detected in urine and feces. Controls were run simultaneously under identical conditions with out XN.

A single oral dose of XN (10, 50, 100, 200, 400, and 500 mg/kg body weight) or an equivalent dose of an XN-rich hops extract was given to each rat by gavage. The hops extract contained the equivalent of 10 or 50 mg XN/kg body weight in order to determine whether other constituents affected the bioavailability of XN. The results revealed no pharmacokinetic differences between extract and pure compound. The study was also performed to see whether an increased dose of XN has any influence on the bioavailability of the compound. Oral absorption of the drug was generally poor, but the concentration of XN in urine and fecal samples increased with the dose of the drug administered. The major metabolites found in plasma and urine were also found in the feces, but feces appear to contain a number of new peaks (Figure 6), which may indicate a number of additional metabolites of XN.

Conclusion

The method allows a rapid, sensitive, and reproducible determination of XN in rat plasma, urine, and fecal samples.

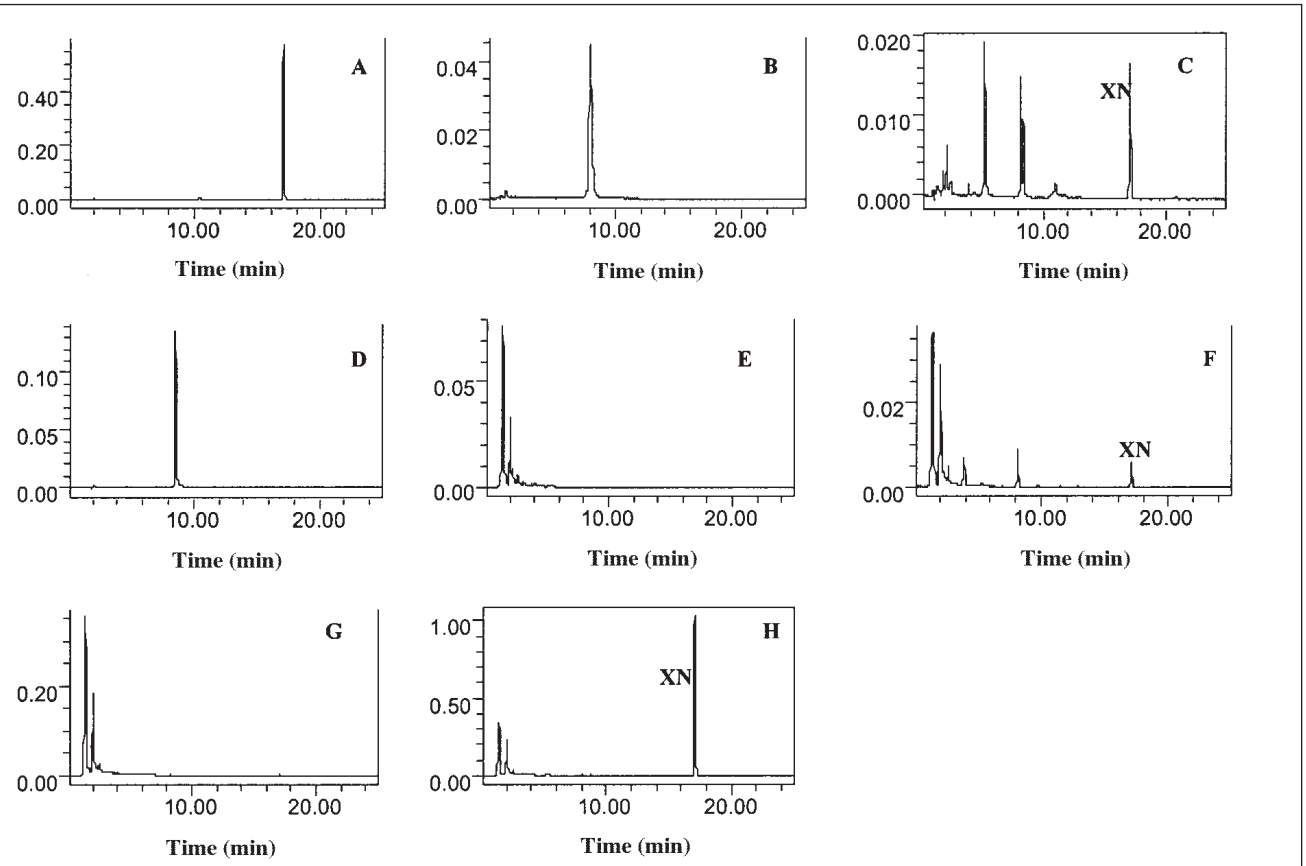

Figure 5. Typical chromatograms of an (A) XN standard (83.3 µg/mL), (B) blood sample prior to drug administration, (C) blood sample collected from rat blood 5 min post-XN administration (20 mg/kg body weight, iv, 250 gm at test), (D) blood sample collected 30 min post-XN administration (20 mg/kg body weight, oral, 250 gm at test), (E) urine sample prior to drug administration, (F) urine sample collected 6–12 h post-XN (40 mg/kg body weight, oral, 400 gm at test) administration, (G) fecal sample collected prior to drug administration, and (H) fecal sample collected 24 h post-XN (40 mg/kg body weight, oral, 400 gm at test) administration.

Absorption of XN after oral administration was generally poor, unchanged XN was basically excreted in urine and feces. After a single iv or oral dose, XN was excreted in the feces mainly within 24 h. There was no significant difference in plasma, urine, and feces samples of rats treated with XN or hops extract, which shows that the hops extract did not have an influence on the oral bioavailability of XN at the doses investigated (i.e., 10 or 50 mg/kg body weight of XN). The method was a reliable tool for performing studies of XN in different biological material and for the further study of metabolites.

Acknowledgments

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