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Pomegranate Juice Ellagitannin Metabolites Are Present in Human Plasma and Some Persist in Urine for Up to 48 Hours[1,2]

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Abstract

Ellagitannins (ETs) from pomegranate juice (PJ) are reported to have numerous biological properties, but their absorption and metabolism in humans are poorly understood. To investigate the pharmacokinetics of pomegranate ETs, 18 healthy volunteers were given 180 mL of PJ concentrate, and blood samples were obtained for 6 h afterwards. Twenty-four–hour urine collections were obtained on the day before (−1), the day of (0), and the day after (+1) the study. Ellagic acid (EA) was detected in plasma of all subjects with a maximum concentration of 0.06 ± 0.01 μmol/L, area under concentration time curve of 0.17 ± 0.02 (μmol · h) · L^{-1}, time of maximum concentration of 0.98 ± 0.06 h, and elimination half-life of 0.71 ± 0.08 h. EA metabolites, including dimethylellagic acid glucuronide (DMEAG) and hydroxy-6H-benzopyran-6-one derivatives (urolithins), were also detected in plasma and urine in conjugated and free forms. DMEAG was found in the urine obtained from 15 of 18 subjects on d 0, but was not detected on d −1 or +1, demonstrating its potential as a biomarker of intake. Urolithin A-glucuronide was found in urine samples from 11 subjects on d 0 and in the urine from 16 subjects on d +1. Urolithin B-glucuronide was found in the urine of 3 subjects on d 0 and in the urine of 5 subjects on d +1. Urolithins, formed by intestinal bacteria, may contribute to the biological effects of PJ as they may persist in plasma and tissues and account for some of the health benefits noted after chronic PJ consumption. Whether genetic polymorphisms in EA-metabolizing enzymes (e.g., catechol-O-methyl transferase and glucuronosyl transferase) are related to variations in response to PJ remains to be established. J. Nutr. 136: 2481–2485, 2006.

Introduction

Ellagitannins (ETs)[5] are bioactive polyphenols abundant in some fruits and nuts, such as pomegranates, black raspberries, raspberries, strawberries, walnuts, and almonds (1,2). Despite numerous reports of the biological properties and human health benefits of ETs, knowledge of their bioavailability, pharmacokinetics, disposition, and metabolic fate in humans is limited (1).

Pomegranate (*Punica granatum* L.) fruits are widely consumed fresh and as a beverage, such as juice (PJ). In commercial juice processing methods, ETs, which are abundant in the fruit peels, are extracted in large quantities into the juice. Punicalagin [2,3-hexahydroxy-diphenoyl-4,6-gallagylglucose], which occurs as isomers (chemical structure shown in **Fig. 1**), is the predominant ET present in PJ as a result of this process (3–5). ETs belong to the chemical class of hydrolyzable tannins, which release ellagic acid (EA) on hydrolysis (chemical structure shown in Fig. 1). In addition, PJ contains other polyphenols, such as anthocyanins that are present in the fruit arils and impart its brilliant red-purple color (3).

The potent antioxidant properties of PJ have been attributed to its high content of punicalagin isomers that can reach levels >2 g/L juice (3–5). ETs have also been identified as active antiatherogenic compounds in PJ (6,7). Our group and others have shown that pomegranate fruit extracts and its purified ETs inhibit the proliferation of human cancer cells and modulate inflammatory subcellular signaling pathways and apoptosis (8–11). Pomegranate fruit extract has also been shown to significantly reduce prostate tumor growth and prostate-specific antigen (PSA) levels in athymic nude mice implanted with CWR22Rv1 prostate cancer cells (12).

Although the absorption, metabolism, distribution, and excretion of pomegranate ETs in animals (4,5) and in humans (13) have been reported, to our knowledge their pharmacokinetics in humans have not been examined other than in a recent study from our laboratory in 1 male volunteer (14). In that single

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[2] Supplemental Figures 1 and 2 are available with the online posting of this paper at jn.nutrition.org.
[5] Abbreviations used: AUC, integrated area under the curve from t = 0 to infinity; C_{max}, maximum concentration; COMT, catechol-O-methyl transferase; DMEAG, dimethylellagic acid glucuronide; EA, ellagic acid; ET, ellagitannin; m/z, mass to charge ratio; PJ, pomegranate juice; PSA, prostate specific antigen; T_{max}, time point at which maximum plasma concentration occurs; $T_{1/2E}$, elimination half-life.
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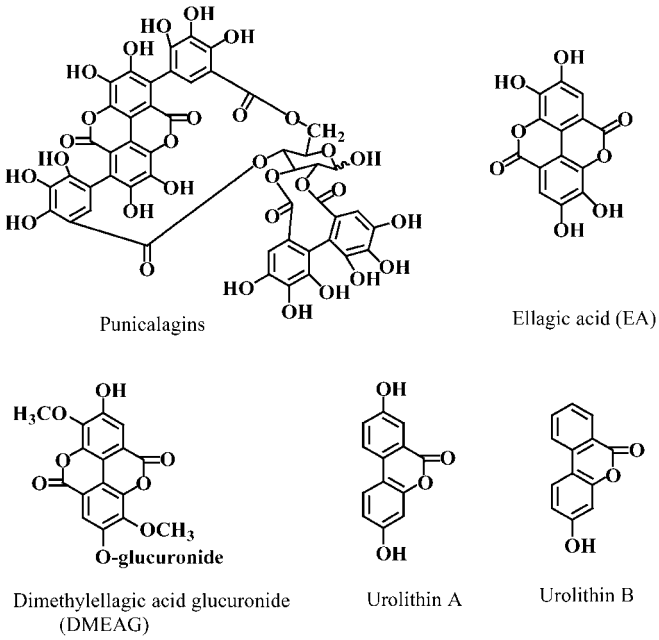

Figure 1 Chemical structures of punicalagin isomers, the major ETs present in pomegranate juice, and its metabolites, EA, DMEAG, urolithin A, and urolithin B.

individual we observed no ET in the blood, but we noted its conversion to EA, which was rapidly cleared from the circulation in 5 h. Once absorbed, polyphenols undergo metabolism by phase II enzymes, such as glucuronosyl transferases and sulfotransferases. These processes increase excretion and detoxification by increasing their water solubility. In addition, catechol polyphenols can undergo methylation by catechol-O-methyl transferase (COMT) enzyme, which, in turn, decreases water solubility and may likely alter biological activity (15). Because we suspected that the pomegranate polyphenol metabolism, like the metabolism of other phytochemicals, might vary among individuals depending on their genotype of these metabolizing enzymes (16–18), we decided to investigate both ET pharmacokinetics and metabolism in 18 normal healthy volunteers (10 males and 8 females). We also conducted tandem liquid chromatography mass spectrometry (LC-MS/MS) analyses of urine, collected on day −1 (prestudy day), 0 (study day) and +1 (poststudy day) to identify potential biomarkers for PJ intake for currently planned human studies of PJ in prostate cancer patients. Recent studies from our group demonstrated a reduced rate of increase in PSA by 50% in advanced prostate cancer patients (19) and the ability of PJ to inhibit human prostate cancer xenograft growth in mice (A. J. Pantuck and A. Belldegrun, Department of Medicine, University of California, Los Angeles, personal communication). To our knowledge, this is the first study to examine the pharmacokinetic parameters of pomegranate ETs in humans.

Materials and Methods

Reagents and instruments

All solvents were HPLC grade from Fisher Scientific. Ellagic, formic, and phosphoric acids, and β-D-glucuronidase (type X-A from *Escherichia coli*) and arylsulfatase (type VIII from abalone entrails) enzymes were purchased from Sigma-Aldrich. PJ concentrate was provided by POM Wonderful. The HPLC-UV analyses were carried out on a Waters Alliance 2690 system equipped with a photo diode array detector (Waters) and data handling was with Waters Millenium, version 3.02 software. The HPLC-MS system consisted of the LCQ Classic Finnigan system (ThermoFinnigan), equipped with a HP 1100 series HPLC system consisting of an autosampler/injector, quaternary pump, column heater, and diode array detector with Xcalibur 1.2 software (Finnigan).

Human study design

Eighteen normal healthy human subjects (10 males and 8 females) with a mean age of 32.6 ± 10.2 y and a mean BMI of 21.3 ± 1.4 kg/m^2 were recruited for the study. Subjects had no clinical disease and were not on any weight-reducing regimen, which was determined using a medical-history questionnaire. Subjects were asked to consume a "polyphenol-free" diet (no fruits, vegetables, wine, tea etc.) and to avoid antioxidant and herbal supplements for 4 d prior to the study day. Female subjects were neither pregnant nor lactating. On the study day, participants, after fasting overnight, were provided with a light breakfast of toast with butter or cereal and low-fat milk. After 20 min, baseline blood was drawn and participants ingested a single dose of PJ concentrate (180 mL; used in concentrate form for ease of consumption). Participants were allowed water ad libitum throughout the intervention. EDTA blood samples were collected before and at 0.5, 1, 2, 3, 4, and 6 h after ingestion of PJ. Participants self-collected 24-h batch urine on prestudy (−1), study (0) and poststudy (+1) days and kept the samples refrigerated until delivery. Approval for the study was obtained from the UCLA Institutional Review Board, which complied with the Helsinki Declaration of 1975 as revised in 1983. The protocol was fully explained to all subjects and informed consent was obtained prior to participation.

Composition of pomegranate juice

A serving of PJ available for human consumption in single strength form (240 mL) contains 34 g sugars, 35 g total carbohydrates, 30 mg sodium, 430 mg potassium, 0% of RDA of vitamin C and vitamin A, 4% calcium, and 2% iron (Pom Wonderful). PJ contains the following polyphenols: anthocyanins, 387 mg/L; punicalagins, 1561 mg/L; ellagic acids, 121 mg/L; and other hydrolyzable tannins, 417 mg/L.

Preparation of plasma samples

The EDTA blood samples were centrifuged at 250 × *g* for 10 min at 4°C, and the plasma was quickly removed and stored at −80°C until analyses. In order to detect "free" and "bound" metabolites, plasma samples were analyzed by HPLC and LC-MS/MS both before and after hydrolysis with glucuronidase and sulfatase enzymes as discussed below.

Plasma without glucuronidase and sulfatase enzymes. Analyses were done as previously reported (20). Briefly, plasma was adjusted to pH 2.5 with 1 mol/L potassium dihydrogen phosphate solution and 50% phosphoric acid. Each sample was vortexed with acetonitrile for 1 min and centrifuged at 250 × *g* for 15 min at 5°C. After being completely evaporated at 35°C in a SpeedVac (Savant), the supernatant liquor was reconstituted in methanol and injected onto the HPLC-MS/MS system to determine the presence and levels of ETs and ET metabolites.

Plasma treated with glucuronidase and sulfatase enzymes. Briefly, plasma was mixed with 10% ascorbic acid, 40 mmol/L NaH_2PO_4, 0.1% EDTA, 50 mmol/L sodium phosphate (pH 7.4), 500 units of β-D-glucuronidase, and 4 units of sulfatase. The mixture was incubated at 37°C for 45 min then the reaction was stopped by the addition of 1 mol/L potassium dihydrogen phosphate solution and 50% phosphoric acid. Each sample was then processed and injected as described above.

Preparation of urine samples

Aliquots (10 mL) of 24-h batch urine collected on −1, 0, and +1 d, were immediately frozen at −20°C. Samples (1 mL) were diluted with H_2O (2% formic acid):MeOH (9:1 v:v), vortexed for 30 s and centrifuged at 250 × *g* for 15 min at 5°C. The supernatant was filtered and directly analyzed by LC-MS/MS.

Analytical HPLC-UV analyses

Conditions for analyses were as previously reported (14). Briefly, pure punicalagins, isolated as previously reported (21), EA (purchased from Sigma), and urolithins A and B [synthesized in our laboratory as previously described (22)], were individually serially diluted to afford 5 different concentrations that were used for the construction of calibration curves. Each standard was injected in triplicate and concentrations

were determined from the peak area by using the equation for linear regression obtained from the calibration curve. Control plasma was spiked with individual solutions and extracted and analyzed as previously reported (14). The calibration curve was linear ($R^2 = 0.9975$) over a concentration range of 3.3–0.05 μmol/L and the calculated lower limit of quantitation of EA and urolithins A and B was 0.01μmol/L. The recoveries of EA from human plasma were 103, 120, 113, and 117% for concentrations of 1.66, 0.83, 0.42, and 0.21 μmol/L, respectively.

High performance LC-electrospray ionization MS/MS analyses

Conditions for detection of EA and EA metabolites were as follows: the column symmetery was C-18, 100 mm × 2.1 i.d., 3.5 μm (Waters). The solvent consisted of *A*) 2% formic acid in H_2O and *B*) 2% formic acid in MeOH. The gradient was determined by the percentage of A in B (initial, 99%; 30 min, 80%; 45 min, 60%; 60 min, 5%). Run time was 60 min, flow rate was 0.15 mL/min, and injection volume was 20 μL. The MS parameters were: ionization mode, electron spray ionization (ESI) in both positive and negative modes; scan range: 120–1500 amu; scan rate: 1 scan/s; cone voltage: 17 eV. Peak identities of EA metabolites were obtained by matching their molecular ions ($M - H^+$) or ($M + H^+$) obtained by ESI/MS and MS/MS with the expected theoretical molecular weights from literature data as follows: M1 (urolithin B = M − H m/z 211), M2 (urolithin A = M − H m/z 227), M3 (methyl-urolithin A = M − H m/z 241), M4 (hydroxyl-urolithin A = M − H m/z 243), M5 (dimethylellagic acid = M − H m/z 329), M6 (urolithin A-glucuronide = M − H m/z 403), M7 (urolithin B-glucuronide = M + H m/z 389), M8 (DMEAG, dimethylellagic acid glucuronide = M − H m/z 505) (4,5,13,16,20).

Statistical analysis

Pharmacokinetic curves were fitted using a pharmacokinetic mixed effects model (23). The model assumed one compartment with first-order processes. The use of a mixed effects approach allowed for the simultaneous fit of individual subject curves and the estimation of population-averaged responses. This response is characterized by the area under the plasma concentration time curve (AUC), the peak plasma concentration (C_{max}) and the time to peak concentration (T_{max}). Models were constructed using WinBUGS (23). Values in the text are means ± SD.

Results

Pharmacokinetics of plasma EA. After a single oral dose of PJ concentrate (180 mL containing 318 mg punicalagins and 12 mg of free EA), EA increased rapidly and was cleared from plasma samples of all volunteers by 5 h. The population estimate of the maximum concentration (C_{max}) was 0.06 ± 0.01 μmol/L, and the corresponding AUC was 0.17 ± 0.02 (μmol · h) · L^{-1}. The population estimate of the time to reach the peak concentration or T_{max} was 0.98 ± 0.06 h and the EA elimination half-life ($T_{1/2E}$) was 0.71 ± 0.08 h. The pharmacokinetic parameters and profile of EA are shown in **Table 1** and **Figure 2**, respectively.

Plasma EA metabolites. In the 7 subjects tested for the presence of EA metabolites in plasma, 4 had detectable urolithin A glucuronide (Supplemental Fig. 1). Three of 7 had detectable hydroxy-urolithin A, and 2 of 7 had detectable urolithin A, and only 1 of 7 subjects had the presence of either urolithin B or methyl urolithin A. After treatment with glucuronidase and sulfatase enzymes, total urolithin A (from 3 subjects) and total urolithin B (from 2 subjects) concentrations were quantified in the 0.5 and 6 h plasma samples as follows: urolithin A = 0.04 μmol/L at 0.5 h and 0.11 μmol/L at 6 h; urolithin B = 0.02 μmol/L at 0.5 h and 0.05 μmol/L at 6 h. Therefore, an increase in both urolithin A and urolithin B synthesis from ETs by intestinal bacteria began to appear as conjugates in plasma samples between 0.5 and 6 h due to the enterohepatic recirculation of these metabolites.

TABLE 1 Pharmacokinetic parameter estimates for EA detected in human plasma after the consumption of a single dose of PJ containing 318 mg ETs as punicalagins

Parameter	Means ± SD[1]	2.5% quantile[2]	97.5% quantile[2]
Rate constant of elimination, *μmol/h*	0.98 ± 0.10	0.70	1.15
Rate constant of absorption, *μmol/h*	1.08 ± 0.14	0.92	1.51
T_{max}, *h*	0.98 ± 0.06	0.86	1.09
C_{max}, *μmol/L*	0.06 ± 0.01	0.05	0.07
AUC, *(μmol · h) · L^{-1}*	0.17 ± 0.02	0.13	0.21
$T_{1/2E}$, *h*	0.71 ± 0.09	0.60	0.98

[1] Values are means ± SD, $n = 18$ over 6 h as quantified by HPLC-UV.
[2] 95% Bayesian credible intervals.

Urinary EA metabolites. EA metabolites were not detected in the baseline urine samples on d −1. On d 0, 15 of 18 subjects had detectable DMEAG in their urine. On d +1 DMEAG was not detected in any of the subjects. On d 0 but not d −1 or +1, 5 of 18 subjects had detectable EA in their urine. As expected, the urolithins formed by intestinal bacteria from EA (13,16), began to appear in the second 12 h of urine collection on d 0 and were also found on d +1. These included urolithin A-glucuronide (M6), urolithin B-glucuronide (M7) and DMEAG (M8) (Supplemental Fig. 2). The number of subjects with detectable EA and EA metabolites found in the urine are summarized in **Table 2**.

Discussion

Pomegranate ETs comprise 70% of the polyphenols in commercial PJ (3), but are assumed to be nonabsorbable due to the large size of the ET molecule (13,14). In our prior study of a single subject (14), we demonstrated that what appeared in the plasma after ET ingestion was EA. However, EA disappeared from the circulation after a few hours, making it difficult to explain the observation that PJ consumed daily can have significant effects on cardiovascular biomarkers (6,7) and on circulating PSA in prostate cancer patients (19). The present study confirmed the rapid absorption of ET and demonstrated that urolithin metabolites persist and are excreted in the urine for 48 h after administration of PJ providing a potential explanation of the effects of chronic PJ administration.

Prior work by other investigators on ET absorption yielded variable results. Cerda et al. (13) reported that neither punicalagin nor EA were detected in plasma or urine following daily intake

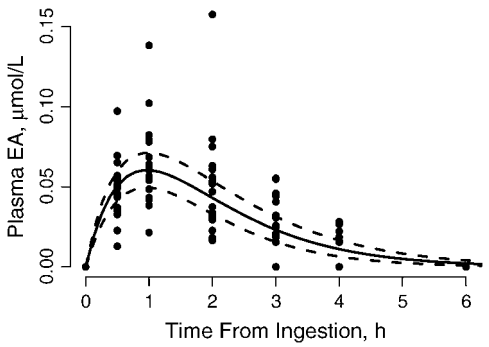

Figure 2 Pharmacokinetic profile of EA after consumption of 180 mL PJ (containing 318 mg punicalagins and 12 mg free EA). Values are means ± SD, $n = 18$.

TABLE 2 Numbers of subjects with EA and EA metabolites detected in urine samples[1]

Sample	EA	M6	M7	M8
Day −1	0/18	0/18	0/18	0/18
Day 0	5/18[2]	11/18[3]	3/18	15/18
Day +1	0/18	16/18	5/18	0/18

[1] M6, urolithin A-glucuronide; M7, urolithin B-glucuronide; M8, dimethylellagic acid glucuronide (DMEAG).
[2] In 4 of 5 subjects where 12 h split urine collection was done, EA appeared in the first 12-h collection (Supplemental Fig. 2).
[3] In 7 of 7 subjects where 12-h split urine collection was done, urolithin A-glucuronide (M6) appeared in the last 12-h collection (Supplemental Fig. 2).

of 1 L of PJ containing 4.37 g punicalagins over 5 d, but urolithins were found in fasting plasma. Cerda et al. (4,5) found intact punicalagin in the plasma of rats following administration of 6% punicalagin in their diet for 37 d. PJ anthocyanins and their respective aglycons (anthocyanidins) were not detected in blood in previous studies (13,14) making it unlikely that these components of PJ account for its bioactivity. Stoner et al. (24) found EA in human plasma after administration of ETs from 45 g of freeze-dried black raspberries during the first 2 h after ingestion.

It has been reported that ETs release EA on hydrolysis in vivo (25), and pure EA has poor bioavailability compared with ETs (26). The poor absorption of EA directly from PJ can be explained by its poor solubility in water, its ionization at physiological pH to form poorly soluble complexes with calcium and magnesium ions in the intestine, and its ability to bind to intestinal epithelium (14,27).

Urolithin glucuronides were observed on both d 0 and +1. When 12-h split urines were collected on d 0, the urolithins only appeared in the second 12-h collection consistent with intestinal bacterial formation of urolithins and enterohepatic recirculation of EA (13). Urinary EA metabolites, such as urolithins, arise from biotransformation by the intestinal microflora on EA (28). Following absorption, EA and urolithins undergo conjugation, and conjugated forms with methyl, glucuronyl, and sulfate groups are found in plasma and excreted in the urine (13,16).

Plasma EA, derived from ETs by hydrolysis, contains 2 ortho-dihydroxyl groups (catechol structure) and may be transformed via the activity of COMT (15) to dimethylellagic acid, which is further glucuronidated to form DMEAG. The finding of DMEAG in the urine from 15 of 18 subjects only on the day of PJ administration provides a useful nutritional biomarker for determining compliance with PJ consumption in clinical studies.

The finding that 3 of 18 subjects did not excrete DMEAG, despite having the same levels of EA in plasma as the other 15 subjects, suggests a nutrigenetic effect. This effect could be due to a polymorphism of COMT, which carries out the dimethylation of EA. A previous study by Wu et al. (29) examined the association between COMT genotype, tea intake, and breast cancer risk in women. Women homozygous for the low activity allele of COMT (L/L) had a greater benefit from green tea ingestion than women homozygous for the high activity allele (H/H) or heterozygous (H/L).

Urolithin metabolism may be dependent on the intestinal flora as defined for soy isoflavones (30), and this deserves further study. Total circulating urolithins were estimated to reach levels of 18.6 μmol/L concentrations when a larger dose of PJ (1L containing 4.3 g punicalagins) was administered to humans for 5 d (13).

In summary, we were able to demonstrate that consuming 180 mL of PJ concentrate was associated with maximum plasma concentrations of EA of 0.06 μmol/L after 1 h and the EA metabolites, total urolithin A of 0.14 μmol/L and total urolithin B of 0.01 at 6 h. Therefore, it is possible that these metabolites contribute to the bioactivity of PJ beyond the effects exerted by ETs and EA. Not all subjects had urolithins in their urine after PJ administration. Therefore, further research is warranted to determine the stability of the urolithin-producer phenotype and the nature of the interindividual differences in bacteria that are responsible for production of urolithins. Finally, polymorphisms of COMT and UGTs might be associated with differences in excretion of DMEAG and tissue concentrations of EA metabolites that affect the impact of PJ on the progression of diseases such as advanced prostate cancer.

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