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Bioavailability of ellagic acid in human plasma after consumption of ellagitannins from pomegranate (*Punica granatum* L.) juice

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Abstract

Background: Ellagic acid (EA) and hydrolyzable ellagitannins (ETs) are dietary polyphenols found in fruits and nuts and implicated with potent antioxidant, anticancer and antiatherosclerotic biological properties. Unfortunately, there are no reports on the bioavailability studies of EA or ETs in the human body. We conducted in vivo studies whereby a human subject consumed pomegranate juice (PJ) (180 ml) containing EA (25 mg) and ETs (318 mg, as punicalagins, the major fruit ellagitannin). *Methods*: A rapid plasma extraction procedure utilizing acidic precipitation of proteins, followed by HPLC-UV analyses, was employed. *Results*: EA was detected in human plasma at a maximum concentration (31.9 ng/ml) after 1 h post-ingestion but was rapidly eliminated by 4 h. The calibration curve for quantification of EA was linear ($r^2 = 0.9975$) over the concentration range from 1000 to 15.6 ng/ml. *Conclusions*: Since EA has reportedly strong affinity for proteins and poor absorption in small animals, further studies to investigate whether the presence of free EA in human plasma may be due to its release from the hydrolysis of ETs, facilitated by physiological pH and/or gut microflora action, is warranted. EA can be considered as a biomarker for future human bioavailability studies involving consumption of ETs from food sources.
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Keywords: Ellagic acid; Ellagitannins; Pomegranates; Bioavailability; Human; HPLC

1. Introduction

Plant polyphenols play an important role in human nutrition and are implicated with numerous biological properties including antioxidant, anti-inflammatory, anticancer and antiatherosclerotic activities. Among these phytochemicals, ellagic acid (EA), a dimeric derivative of gallic acid, occurs in fruits and nuts in either its free form, as EA-glycosides, or bound as ellagitannins (ETs) [1,2]. The absorption, bioavailability and pharmacokinetics of EA administered orally have not been adequately investigated [3] and to the best of our knowledge have never been studied in the human body. Data on the daily dietary intake of EA by humans are not available and there is no information on the proportion ingested in the form of ETs [2]. There are also no reports of definitive studies on the absorption and metabolism of ETs in humans [2]. Hence, human bioavailability and pharmacokinetic studies are necessary to determine the fate of these bioactive dietary polyphenols which, apart from being prevalent in foods, are also com-

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monly used as botanical ingredients in dietary and herbal supplements.

Current knowledge on the bioavailability of EA and ETs is confined to animal studies with rats and mice [2–10]. When mice were given ETs (from raspberries or pomegranates at 600 mg/kg body weight, by gavage), EA was detected in the urine (0.05% of dose) as a result of absorption and metabolism of ETs [4]. However, virtually no EA was recovered from the blood or tissues of mice fed for 1 week on a diet containing 1% EA [5]. Following oral administration of EA to rat, 10% of the dose was excreted and detected as EA metabolites in urine and feces [6]. The low concentrations of free EA in plasma have been attributed to its low solubility in water [3], and may also be due to its extensive metabolic transformation and degradation prior to absorption. In addition, EA has been reported to bind irreversibly to cellular DNA and proteins, which may also account for its limited transcellular absorption [14]. The poor absorption of EA has been reported to impact its in vivo anti-tumorigenic activity since it is possible that sufficient concentrations are not present in plasma or target cells after oral administration [2,3]. It has been proposed that the gut microflora metabolize insoluble EA. Since ETs are more soluble, their absorption or that of their transformation products is facilitated [2]. Also since ETs are easily hydrolyzed, the in vivo action of physiological pH and /or enzymatic action by gut microflora could cause them to break down to release EA units. However, this issue as well as the pharmacokinetic profiles of ETs or EA in humans is still unexplored.

Therefore, in this study we investigated the bioavailability and pharmacokinetics of ETs and EA from a food source in human. Our results show direct evidence of the absorption of EA in the human body for the first time. This was obtained after oral consumption of pomegranate juice (PJ) by combining an extraction procedure for plasma sample preparation and an HPLC-UV system.

2. Materials and methods

2.1. Subject

One male subject (one of the authors, NPS; 35; 68 kg body weight), after fasting overnight, consumed 180 ml (6 oz) of pomegranate juice (used in concentrate form for ease of consumption; obtained from POM® Wonderful LLC, Los Angeles, CA) containing 25 mg EA and 318 mg ETs (consisting of punicalagin anomers, the major fruit ellagitannin; quantification data not shown). Structures of punicalagin which exists as anomers (**1**) [9–11] and EA (**2**) are shown in Fig. 1. Blood samples were collected before and at 0.5, 1, 2, 3, 4 and 6 h after consump-

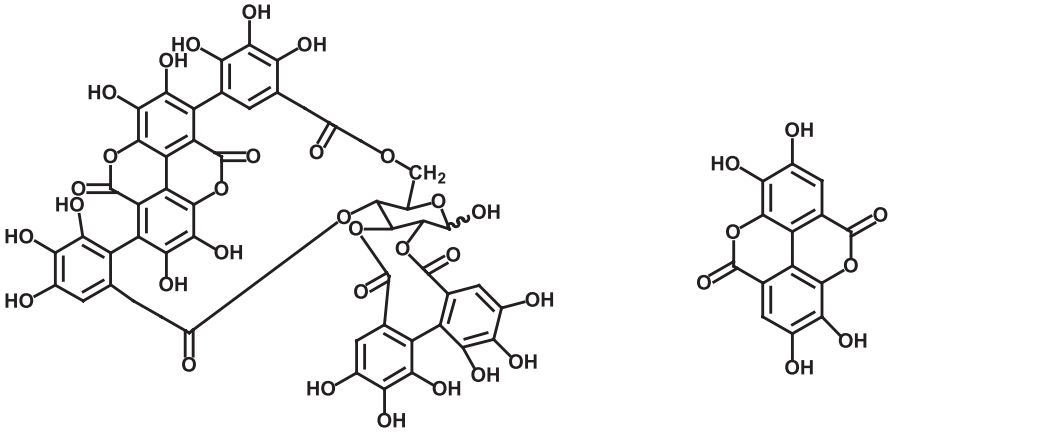

Fig. 1. Chemical structures of punicalagin (**1**), the major ET found in commercial PJ, and ellagic acid (**2**).

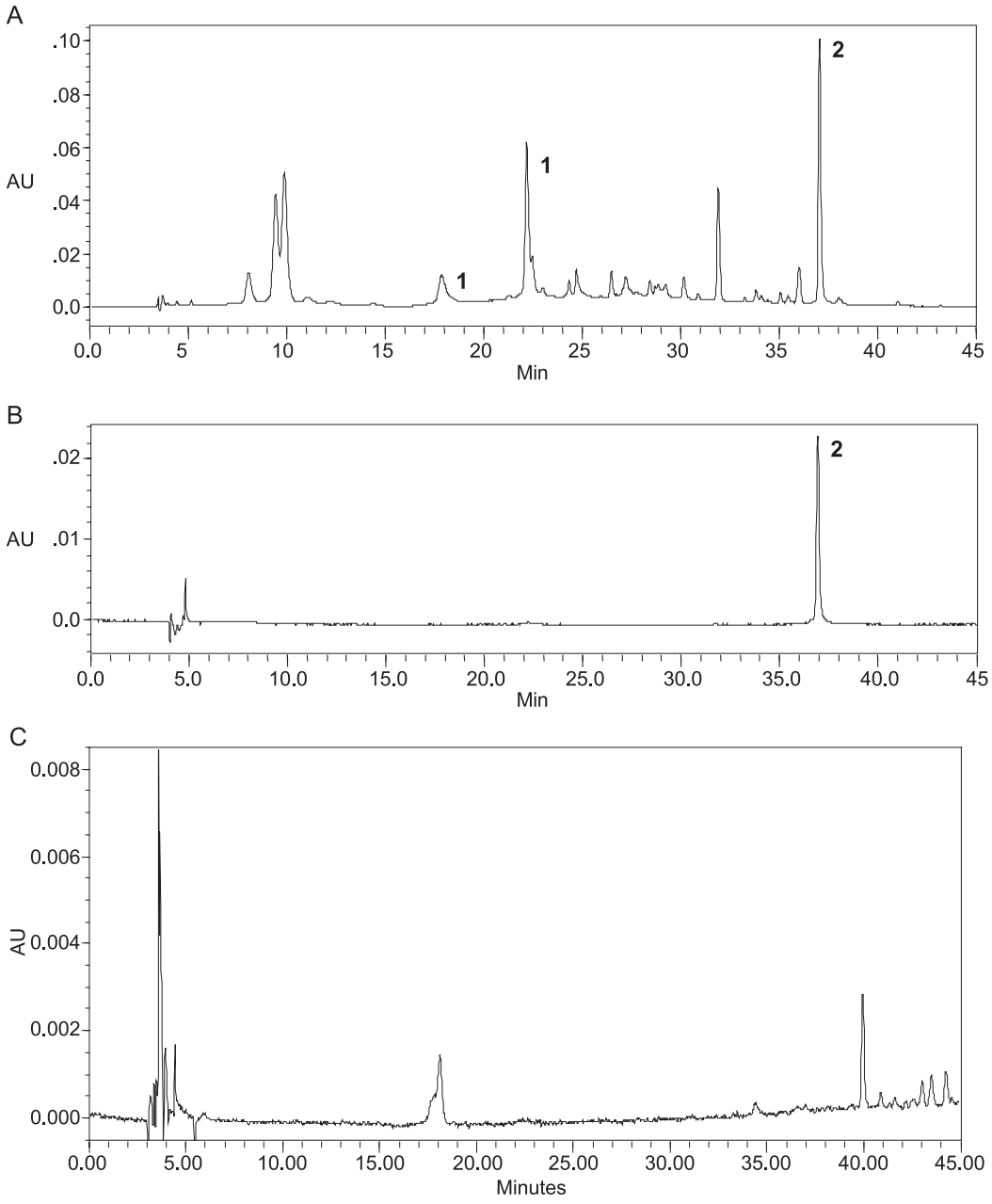

Fig. 2. HPLC chromatograms (A–I) of commercial pomegranate juice (PJ) available for human consumption; ellagic acid (EA) standard; control plasma spiked with EA standard; and plasma samples collected from a human subject before and after consumption of 180 ml of PJ containing 25 mg EA and 318 mg ETs. (A) Chromatogram of PJ showing ETs (as punicalagin anomers (**1**) at t_R 18 min and 22 min, respectively, [13]) and EA (**2**) (at t_R 37 min); (B) chromatogram of EA (**2**) standard eluting at t_R 37 min; (C) chromatogram of control plasma collected before consumption of PJ; (D) chromatogram of control plasma spiked with EA standard and processed according to extraction protocol, showing EA (**2**) eluting at t_R 37 min; (E–H) representative chromatogram* of plasma collected at 0.5 h (E), 1 h (F), 2 h (G) and 3 h (H) after consumption of PJ showing EA (**2**) eluting at t_R 37 min; (J–I) representative chromatogram* of plasma collected at 4 and 6 h after consumption of PJ showing absence of EA and ETs. (*HPLC chromatograms for these different time points were similar).

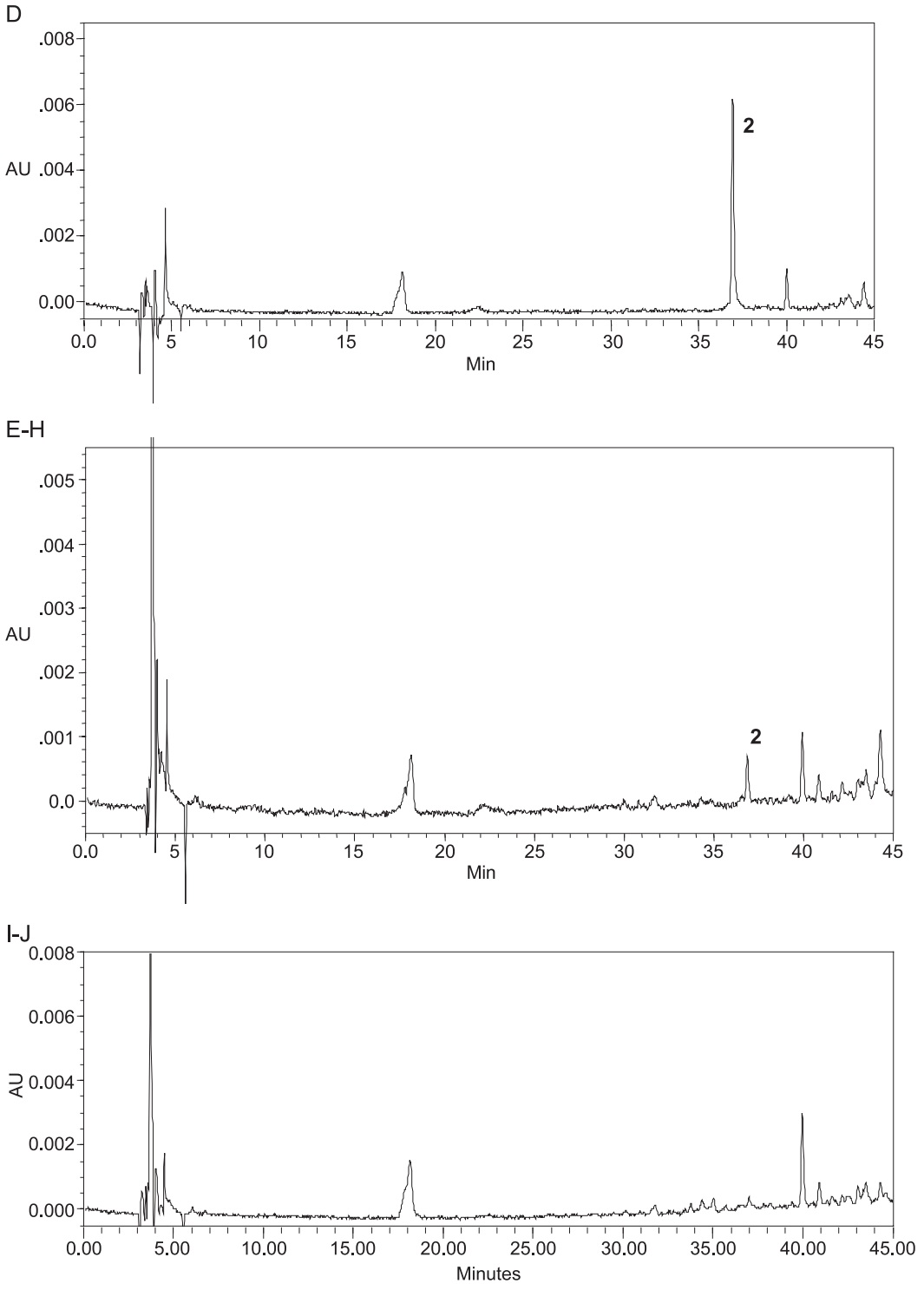

Fig. 2 (*continued*).

tion of the concentrated pomegranate juice. Pomegranate juice is commercially available (POM® Wonderful) for human consumption. Institutional Review Board (IRB) approval for studies with human subjects was obtained for this project.

2.2. Preparation of plasma samples

The EDTA blood samples were centrifuged at 3000 × *g* for 10 min at 4 °C, and the plasma was quickly removed and stored at − 80 °C until HPLC analyses. A 500-μl portion of plasma was adjusted to pH 2.5 with 150 μl of 1 mol/l potassium dihydrogen phosphate solution and 15 μl 50% phosphoric acid. Each sample was vortex mixed with 2.5 ml acetonitrile for 1 min and centrifuged at 3500 × *g* for 10 min at 5–10 °C. The supernatant liquor was evaporated to dryness at 35 °C in a SpeedVac (Savant, USA). The residue was reconstituted in 100 μl methanol and 25 μl (sample volume) was injected onto an HPLC system to determine presence and concentrations of EA and ETs.

2.3. HPLC analyses

The HPLC system included a 600 pump, 717 Autosampler, 996 Photodiode Array Detector (PDA) and Millenium^{32} Chromatography Software (all Waters, USA); and a Zorbax SB C-18 column (4.6 × 250 mm) (Agilent Technologies, Palo Alto, CA). The mobile phase, solvent A (2% acetic acid in water) and solvent B (2% acetic acid in methanol), was used under binary linear gradient conditions as follows: 0–5 min, 99% A in B; 5–30 min, 99–40% A in B; 31–40 min, 40–20% A in B; 41–50 min, 20–10% A in B with a flow rate of 0.75 ml/min. The wavelength was monitored at 366 and 378 nm for detection and quantification of EA (Sigma, St. Louis, MO) and ETs (as punicalagins; isolated as previously reported [13]), respectively.

EA standard (50 μg/ml) was solubilized in DMSO and serially diluted to afford 500, 250, 125, 62.5, 31.25 and 15.625 ng/ml solutions. Control plasma was spiked with individual solutions and extracted as previously outlined. Each plasma sample was separately extracted (× 3) and each sample was injected in triplicate on the HPLC. Concentrations were determined from the peak area by using the equation for linear regression obtained from the calibration curve. The calibration curve was linear ($r^2 = 0.9975$) over the concentration range from 1000 to 15.625 ng/ml. Since 500 μl of plasma was concentrated and reconstituted to a final volume of 100 μl, the calculated lower limit of quantitation (LOQ) of EA = 3 ng/ml. The recoveries of EA from human plasma were 103%, 120%, 113 and 117% for the concentrations 500, 250, 125, 62.5 ng/ml, respectively.

3. Results and discussion

The potent antioxidant and antiatherosclerotic properties of commercial pomegranate juices (PJ) have been attributed to their high content of polyphenols including ETs, as punicalagin anomers (**1**), and EA (**2**) (Fig. 1) [9–13]. The HPLC chromatogram of PJ (Fig. 2A) used in this study shows the ETs (as punicalagin anomers at t_R 18 and 22 min, respectively, as previously reported [13]) and EA (at t_R 37 min, standard shown in Fig. 2B). Control plasma showed no corresponding peaks detected in the plasma sample (Fig. 2C) collected before the consumption of PJ. The peaks at ~ t_R 18.5–19 and 40 min in control plasma were unidentified and are present in all plasma samples so were not attributed to the presence of PJ polyphenols or their metabolites.

ETs in intact forms were not detected in any of the plasma samples collected after consumption of PJ.

Table 1
Concentrations of ellagic acid (EA) (**2**) in human plasma collected before and after oral administration of pomegranate juice (at a dose containing 25 mg EA and 318 mg ETs)

Time (h)	Measured concentrations of EA[a] ng/ml (μmol/l)
0	n.d.
0.5	20.5 ± 1.7 (0.0679)
1	31.9 ± 2.4 (0.106)
2	22.4 ± 3.2 (0.0794)
3	16.2 ± 4.9 (0.0574)
4	n.d.
6	n.d.

Each value represents the mean ± S.D. ($n = 3$).
n.d. = not detected.
[a] Molecular weight EA = 302 g/mol.

However, since EA was detected, control plasma was spiked with different concentrations of EA standard and processed according to the extraction protocol for quantification purposes. Fig. 2D shows the EA peak eluting at 37 min in spiked control plasma. EA was detected and quantified in plasma samples collected at 0.5 h (20.5 ng/ml), 1 h (31.9 ng/ml), 2 h (22.4 ng/ml) and 3 h (16.2 ng/ml) post-ingestion (Table 1). EA was not detected in plasma samples collected at 4 and 6 h after consumption. It is noteworthy that peaks were not detected at 520 nm, for absorption of anthocyanins [15], polyphenols also present in PJ, using this sample extraction method (data not shown).

In conclusion, by combining an extraction procedure for plasma sample preparation and an HPLC-UV system, we have successfully obtained direct evidence of the absorption of EA in human from a food source for the first time. EA can be considered as a biomarker for future human bioavailability studies involving consumption of ETs from food sources. However, further studies should be designed to investigate whether the presence of free EA in human is due to its release from the hydrolysis of ETs, facilitated by physiological pH and/or gut microflora action. Also, since polyphenol bioavailability and pharmacokinetics would vary in human subjects, further clinical studies with $n>1$ should be investigated. Dietary interventions with ETs consumed for extended time periods would also be useful to evaluate if intact ETs bioaccumulate in humans as previously reported for small animals [9,10]. Since biologically active ETs and EA molecules have important impacts on human health, studies on the detection of their metabolites, methods to increase their bioavailability and strategies to increase their concentration in blood and target tissues are warranted.

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