Anthocyanins are absorbed in glycated forms in elderly women: a pharmacokinetic study[1–4]

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ABSTRACT

Background: Anthocyanins are potent antioxidants that are widely distributed in fruit, vegetables, and red wines. Anthocyanin products are also prescribed as medicines in many countries for treating various diseases. However, the pharmacokinetics of dietary anthocyanins are not known in humans because these glycosides were long considered nonabsorbable.

Objective: The objective of this study was to determine whether anthocyanins can be absorbed as glycosides and to evaluate their pharmacokinetics in humans.

Design: Four healthy elderly women consumed 720 mg anthocyanins. A series of blood and urine samples were collected before and after consumption of the anthocyanins. Anthocyanins were measured in plasma and urine by combining an octadecylsilane solid-phase extraction for sample preparation and an HPLC system with diode array for anthocyanin separation and detection. The structures of anthocyanins as glycosides in plasma and urine were further confirmed by using liquid chromatography–mass spectrometry.

Results: Anthocyanins were detected as glycosides in plasma and urine. The maximum plasma concentration of total anthocyanins varied from 55.3 to 168.3 nmol/L, with an average of 97.4 nmol/L, and was reached within 71.3 min. The elimination of plasma anthocyanins appeared to follow first-order kinetics. The elimination half-life of plasma total anthocyanins was calculated to be 132.6 min. Most anthocyanin compounds were excreted in urine during the first 4 h. The excretion rate of total anthocyanins was 77 μg/h during the first 4 h and 13 μg/h during the second 4 h.

Conclusion: Anthocyanins are absorbed in their unchanged glycated forms in elderly women. *Am J Clin Nutr* 2001; 73:920–6.

KEY WORDS Anthocyanins, flavonoids, antioxidants, pharmacokinetics, absorption, elderly, women

INTRODUCTION

Results from our laboratory showed that many fruit and vegetables have strong antioxidant capacities, mainly because of non–vitamin C phytochemicals (1–4). Of these phytochemicals, anthocyanins are an important group of natural antioxidants (5–10). Anthocyanins are water-soluble glycosides and acylglycosides of anthocyanidins, which are polyhydroxyl and polymethoxyl derivatives of 2-phenylbenzopyrylium (flavylium cation). Anthocyanins belong to a large and widespread group of plant constituents known collectively as flavonoids. The most common naturally occurring anthocyanins are 3-*O*-glycosides or 3,5-di-*O*-glycosides. Anthocyanins are potent antioxidants in vitro. Anthocyanins scavenge $O_2^{\bullet -}$ (5, 6), $OH^{\bullet}$ (6), $ROO^{\bullet}$ (peroxyl radical; 7), and nitric oxide (8) and inhibit lipid peroxidation induced by Cu^{2+} (9), ascorbic acid plus Fe^{2+} (10), doxorubicin (10), and ultraviolet light radiation (6). Anthocyanins protect human LDL against copper or peroxyl radical–induced oxidation (11, 12).

Natural anthocyanins are prescribed as medicines in many countries. They have been reported to have positive effects in the treatment of various microcirculation diseases resulting from capillary fragility (13–16), such as preventing cholesterol-induced atherosclerosis (17), inhibiting platelet aggregation, and improving visual function (18, 19). There have been no reported adverse effects from ingestion of grape anthocyanins in humans since grape skin extracts were approved by the Food and Drug Administration to be used as a food colorant (20).

Anthocyanins are widely distributed in fruit and vegetables, such as blueberries, elderberries, strawberries, blackberries, cranberries, raspberries, cherries, currants, grapes, plums, red onions, red cabbage, sweet potatoes, and corn. Red wine is also rich in anthocyanins (21). The intake of anthocyanins in humans has been estimated to be 180–215 mg/d in the United States (22), which is much higher than the intake (23 mg/d) of other flavonoids, including quercetin, kaempferol, myricetin, apigenin, and luteolin (23).

However, anthocyanins exist in plants as glycosides, which were long considered nonabsorbable in humans. Therefore, the pharmacokinetics of dietary anthocyanins are not known in humans. Recently, we developed an HPLC procedure for measuring the anthocyanins in plasma and showed clearly, for the first

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[2] Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee by the US Department of Agriculture and does not imply approval to the exclusion of other products that may be suitable.

[3] Supported by the Comunidad de Madrid (CS-M).

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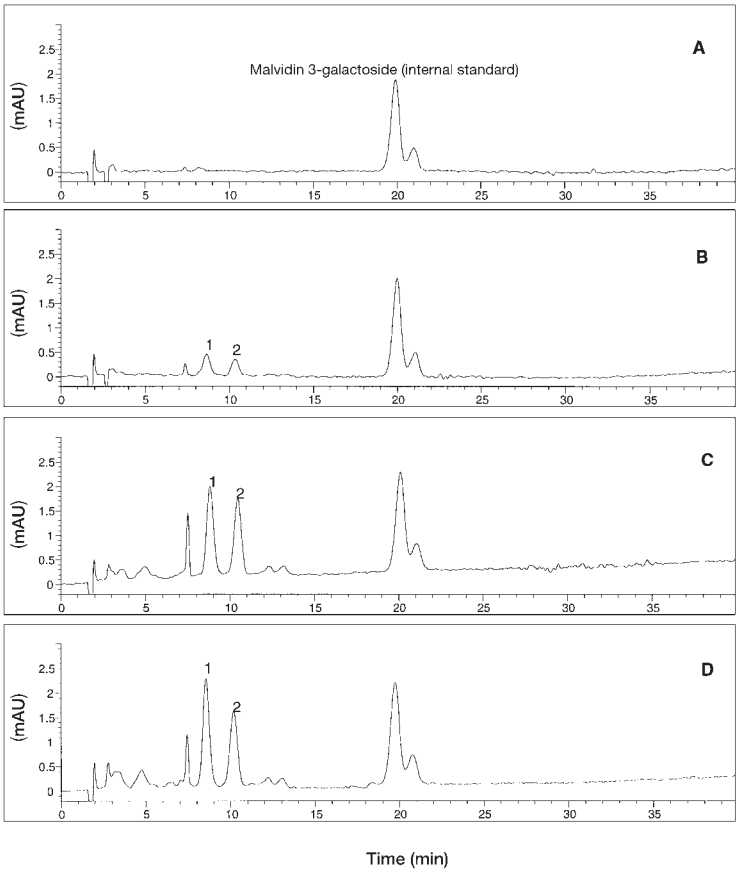

FIGURE 1. Representative HPLC chromatograms of plasma samples collected from the elderly women before (A) and 10 (B), 20 (C), and 45 (D) min after consumption of 720 mg anthocyanins. Peak 1 and peak 2 had the elution time and spectrum of cyanidin 3-sambubioside and cyanidin 3-glucoside, respectively. All peaks other than the internal standard (malvidin 3-galactoside) decreased after 1 h and disappeared after 24 h. A binary linear gradient method was used for the HPLC analysis. Mobile phase A was an aqueous solution of 25 mmol sodium acetate/L and mobile phase B was methanol containing 25 mmol sodium acetate/L. Both mobile phases were adjusted to pH 1.5 with trichloroacetic acid. The flow rate was 1 mL/min. mAU, milliabsorbance units.

time in one human subject, that anthocyanins are absorbed in the blood (24). In the present study we investigated further whether anthocyanins are absorbed in their original glycated forms in 4 elderly women. The pharmacokinetics of these dietary components were also determined.

SUBJECTS AND METHODS

Subjects

Four healthy elderly women aged 67 ± 4 y were recruited to participate in this pharmacokinetic study. All study participants were in good health on the basis of a medical history questionnaire, a physical examination, and normal results of clinical laboratory tests. All of the subjects fulfilled the following eligibility criteria: *1*) no history of cardiovascular, hepatic, gastrointestinal, or renal disease; *2*) no alcoholism; *3*) no antibiotic or supplemental vitamin or mineral use ≥4 wk before the start of the study; and *4*) no smoking. The study protocol was approved by the Human Investigation Review Committee of Tufts University and the New England Medical Center and written, informed consent was obtained from each study participant.

Study design

Each subject was admitted to the Metabolic Research Unit (MRU) at the Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging at Tufts University 48 h before the day of sampling. Subjects fasted overnight in the evening before the day of sampling. In the morning of the sampling day, an intravenous catheter was inserted into one forearm. A 15-mL heparin-treated blood sample (zero baseline sample) was obtained from each fasting subject, after which the subjects were given 720 mg anthocyanins contained in 12 g elderberry extract dissolved in 500 mL water before the study. The elderberry extract contained mainly cyanidin 3-sambubioside, cyanidin 3-glucoside, and maltodextrin. Blood samples (15 mL) were collected again at 10, 20, 30, and 45 min and 1, 2, 4, 6, and 24 h after consumption of the elderberry anthocyanins. Urine samples were collected from these subjects before the consumption of the elderberry anthocyanins and between 0 and 2, 2 and 4, 4 and 6, 6 and 8, 8 and 12, and 12 and 24 h after consumption of the elderberry anthocyanins.

Lunch was provided 4 h after the blood sampling and dinner was provided 11 h after consumption of the drink. The diets provided to these subjects during their residency in the MRU were designed to contain no anthocyanins, to be low in other

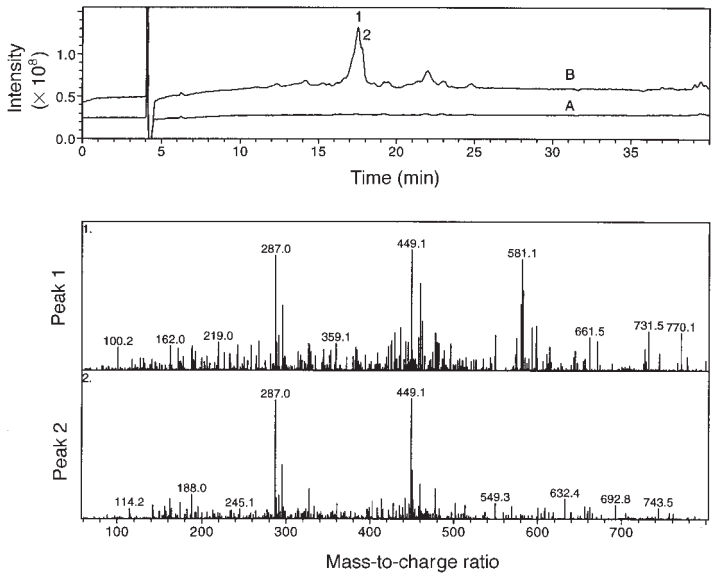

FIGURE 2. Liquid chromatography–mass spectrometry analysis of anthocyanins in the plasma samples collected before (A) and 60 min after (B) consumption of 720 mg anthocyanins by the elderly women. A binary linear gradient method was used for the HPLC analysis. Mobile phase A was 0.5% formic acid and mobile phase B was methanol. The flow rate was 0.4 mL/min. Peaks 1 (17.44–17.55 min) and 2 (17.67–17.79 min) labeled in this analysis correspond to peaks 1 and 2 in Figure 1 (B–D).

flavonoids, and to meet the recommended dietary allowances for protein and energy (25). All meals were prepared under the supervision of a dietitian in the MRU. The consumption of water was not limited but food and other beverages were not allowed during the residency.

Anthocyanin analysis in plasma and urine

The blood samples were centrifuged at 500 × *g* for 10 min at 4°C. The plasma samples were quickly removed and immediately treated with an aqueous solution of 0.44 mol trifluoroacetic acid/L (1:0.2, by vol) as previously described (24). These trifluoroacetic acid–treated plasma samples were then stored at −80°C before HPLC and HPLC–mass spectrometric analyses for anthocyanins. The urine samples were also treated with 0.44 mol trifluoroacetic acid/L (1:0.2, by vol) and then stored at −80°C before analyses for anthocyanins.

Malvidin 3-galactoside was added to the plasma samples (2 mL) to a final concentration of 1 μg/L. Malvidin 3-galactoside was not detected in the elderberry anthocyanins and was used as an internal standard to correct for the possible loss of anthocyanins during the sample preparation. The plasma sample was then extracted by using the octadecylsilane solid-phase extraction cartridge (Sep-Pak C_{18}) as previously described (24). Water-soluble compounds, polar lipids, and neutral lipids in the plasma samples were eluted with an aqueous solution of 25 mmol sodium acetate/L (pH 1.5), dichloromethane, and benzene, respectively. Anthocyanins were recovered finally with methanol containing 25 mmol sodium acetate/L (pH 1.5) for HPLC analysis or with methanol containing 5% formic acid for analysis by liquid chromatography–mass spectrometry (LC-MS). The anthocyanins in the methanol phase were evaporated to dryness, redissolved in the methanol containing 25 mmol sodium acetate/L, and mixed with an aqueous solution of 25 mmol sodium acetate/L (1:3, by vol). After being centrifuged to remove any possible undissolved materials, the sample was analyzed for anthocyanins by HPLC with an HP series 1100 including an autosampler, a binary pump, a Zorbax SB-C_{18} column (4.6 × 250 nm), and a diode array detector (Agilent Technologies, Palo Alto, CA). For LC-MS, the anthocyanins in the methanol phase were evaporated and redissolved in the methanol containing 5% formic acid. Low-resolution electrospray mass spectrometry was performed with an Esquire-LC Mass Spectrometer (Bruker Daltonik, Bremen, Germany), an ion trap instrument equipped with an electrospray interface. The mass spectrometer was operated in the positive-ion mode (electrospray voltage, 4000 V; capillary exit, 95.6 V; capillary offset, 69.8 V; skim 1, 25.8 V; dry gas, 9 L/min; temperature, 300°C).

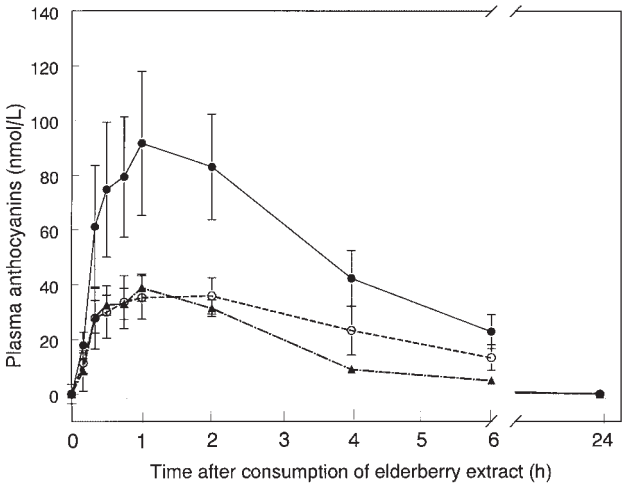

FIGURE 3. Mean (±SE) plasma total anthocyanin (●), cyanidin 3 sambubioside (○), and cyanidin 3-glucoside (▲) concentrations after consumption of 720 mg anthocyanins by 4 elderly women. The point at 24 h represents all 3 lines.

TABLE 1
Main pharmacokinetics of anthocyanins in the elderly women after a single oral dose of 720 mg elderberry anthocyanins[1]

	C_{max}	t_{max}	k	$t_{1/2}$
	nmol/L	*min*		*min*
Total anthocyanins	97.4 ± 24.5[2]	71.3 ± 16.6	0.005355 ± 0.000496	132.6 ± 11.2
Cyanidin 3-sambubioside	38.9 ± 7.4[2]	71.3 ± 16.6	0.004534 ± 0.00083	168.9 ± 30.6
Cyanidin 3-glucoside	42.5 ± 4.5	65.0 ± 20.6	0.007230 ± 0.000504	97.3 ± 7.0

[1] $\bar{x}$ ± SEM; n = 4. C_{max}, maximum plasma concentration; t_{max}, time to reach the maximum plasma concentration; k, terminal elimination rate constant [slope × (−2.303); the slope was derived from linear regression of the terminal portion of the log plasma concentration versus time profile]; $t_{1/2}$, elimination half-life (ln2/k).

[2] Cyanidin 3-glucoside equivalent.

The urine samples were also treated with the Sep-Pak C_{18} extraction cartridge; 10 mL urine was loaded on the cartridge and washed with an aqueous solution of 15 mL of 25 mmol sodium acetate/L (pH 1.5). Anthocyanins were then recovered with 2 mL methanol containing 25 mmol sodium acetate/L (pH 1.5) or 5% formic acid, which was directly used for HPLC or LC-MS after filtration. The recovery of anthocyanins in urine samples was >90%. Therefore, the internal standard was not routinely used for the analysis of anthocyanin in urine.

RESULTS

No compounds, other than malvidin 3-galactoside, were detected at 520 nm in the plasma samples collected before consumption of the elderberry anthocyanins (**Figure 1**A). At least 5 compounds in addition to malvidin 3-galactoside were detected at 520 nm in the plasma samples collected after consumption of the elderberry anthocyanins (Figure 1, B–D); 2 of the main compounds (peaks 1 and 2) showed the elution time and spectrum of cyanidin 3-sambubioside and cyanidin 3-glucoside, respectively.

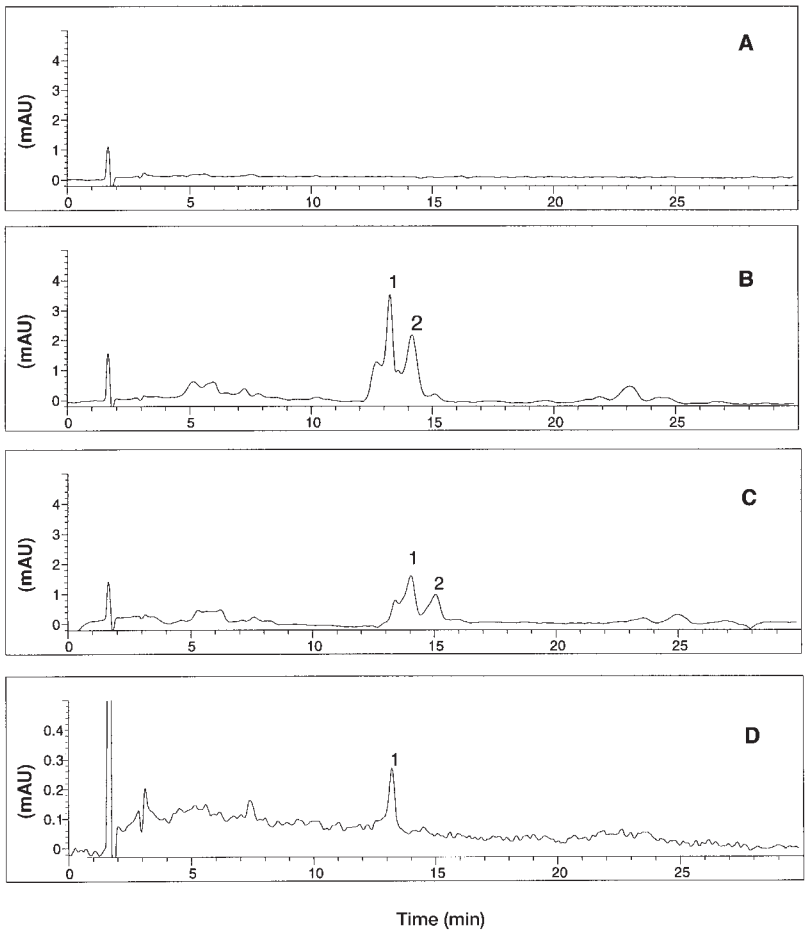

FIGURE 4. Representative HPLC chromatograms of urine samples collected from the elderly women before (A) and 0–2 (B), 2–4 (C), and 12–24 (D) h after consumption of 720 mg anthocyanins. Peaks 1 and 2 had the elution time and spectrum of cyanidin 3-sambubioside and cyanidin 3-glucoside, respectively. A binary linear gradient method modified from that for plasma samples was used for the HPLC analysis. Mobile phase A was an aqueous solution of 25 mmol sodium acetate/L and mobile phase B was methanol containing 25 mmol sodium acetate/L. Both mobile phases were adjusted to pH 1.5 with trichloroacetic acid. The flow rate was 1 mL/min. mAU, milliabsorbance units.

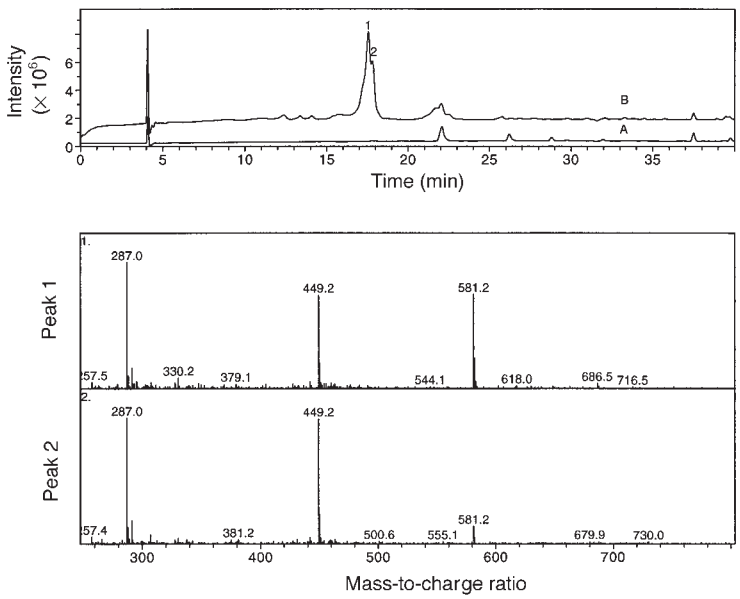

FIGURE 5. Liquid chromatography–mass spectrometry analysis of anthocyanins in the urine samples collected before (A) and 0–2 h after (B) consumption of 720 mg anthocyanins by the elderly women. A binary linear gradient method was used for the HPLC analysis. Mobile phase A was 0.5% formic acid and mobile phase B was methanol. The flow rate was 0.4 mL/min. Peaks 1 (17.51–17.61 min) and 2 (17.71–17.82 min) labeled in this HPLC mass spectrometric analysis correspond to peaks 1 and 2 in Figure 4 (B–D).

Confirmation of the identity of these 2 major peaks was provided by LC-MS (**Figure 2**). Cyanidin 3-sambubioside and cyanidin 3-glucoside accounted for 92.5% of the total anthocyanins detected in the elderberry concentrate (26). The molecular ion of cyanidin 3-sambubioside [mass-to charge ratio (*m/z*)*:* 581.1], which contains one xylose and one glucose molecule, and its fragment, cyanidin aglycone (*m/z:* 287.0), were detected in the mass spectrometric spectrum of the suspected cyanidin 3-sambubioside in plasma (Figure 2). The molecular ion of cyanidin 3-glucoside (*m/z:* 449.1) and its aglycone (*m/z:* 287.0) were detected in the mass spectrometric spectrum of the suspected cyanidin 3-glucoside in plasma (Figure 2). The mass spectrometric spectra of these 2 suspected anthocyanin peaks detected in plasma were the same as those of cyanidin 3-sambubioside and cyanidin 3-glucoside extracted from elderberries. Cyanidin aglycone and a malvidin hexoside were also detected, by LC-MS, in the plasma sample collected after consumption of the elderberry anthocyanins, although their concentrations were low. Interestingly, neither glucuronides nor sulfates of these anthocyanins were detected in the plasma samples.

At an absorption of 520 nm, the maximum plasma concentration (C_{max}) of total anthocyanins, including cyanidin 3-sambubioside and cyanidin 3-glucoside, in the 4 elderly women varied from 55.3 to 168.3 nmol/L, with an average of 97.4 nmol/L, and was reached within 71.3 min (t_{max}). The elimination of plasma anthocyanins appeared to follow first-order kinetics (**Figure 3**). The elimination half-life ($t_{1/2}$) of plasma total anthocyanins was calculated to be 132.6 min (**Table 1**). The C_{max}, t_{max}, and $t_{1/2}$ of plasma cyanidin 3-sambubioside were 38.9 nmol/L, 71.3 min, and 168.9 min, respectively. The C_{max}, t_{max}, and $t_{1/2}$ of plasma cyanidin 3-glucoside were 42.5 nmol/L, 65.0 min, and 97.3 min, respectively (Table 1).

The urine samples collected from the elderly women after consumption of the elderberry anthocyanins became pink after treatment with trifluoroacetic acid/L. No peaks were detected at 520 nm in the urine samples collected before consumption of the elderberry anthocyanins (**Figure 4**A), but ≥6 peaks were detected at 520 nm in the urine samples collected after consumption of the elderberry anthocyanins (Figure 4, B–D). Two main peaks detected in the urine collected after consumption of the elderberry anthocyanins were cyanidin 3-sambubioside and cyanidin 3-glucoside, respectively. The molecular ion of cyanidin 3-sambubioside (*m/z:* 581.1) and its aglycone (*m/z:* 287.0) were detected in the mass spectrometric spectrum of the suspected cyanidin 3-sambubioside in urine (**Figure 5**). The molecular ion of cyanidin 3-glucoside (*m/z:* 449.1) and its aglycone (*m/z:* 287.0) were detected in the mass spectrometric spectrum of the suspected cyanidin 3-glucoside in urine (Figure 5). The mass spectrometric spectra of these 2 suspected anthocyanin peaks detected in urine were also the same as those of cyanidin 3-sambubioside and cyanidin 3-glucoside extracted from elderberries.

Several peaks in the urine samples did not show the anthocyanin spectra and thus may have been anthocyanin metabolites. On the basis of the peaks' having a typical anthocyanin absorption spectra, we concluded that most anthocyanin compounds were excreted in urine during the first 4 h. The total amount of the anthocyanins excreted during the 24 h after consumption of elderberry anthocyanins was calculated to be 397.0 ± 45.1 μg (cyanidin 3-glucoside equivalents). The excretion rate of the total anthocyanins was 77.2 ± 10.9 μg/h during the first 4 h and 13.4 ± 1.6 μg/h during the second 4 h (**Figure 6**).

DISCUSSION

As early as 1933, Horwitt (27) observed that the urine of rabbits fed 500 mg anthocyanin pigment from grapes became highly pigmented. He concluded that small quantities of the grape anthocyanins or anthocyanidins were absorbed and did pass through to the circulation. This conclusion was supported by the HPLC analysis of plasma and urine samples of rats that consumed *Vaccinium myrtillus* anthocyanins (28). However, it was long believed that only anthocyanidins, ie, the aglycones of

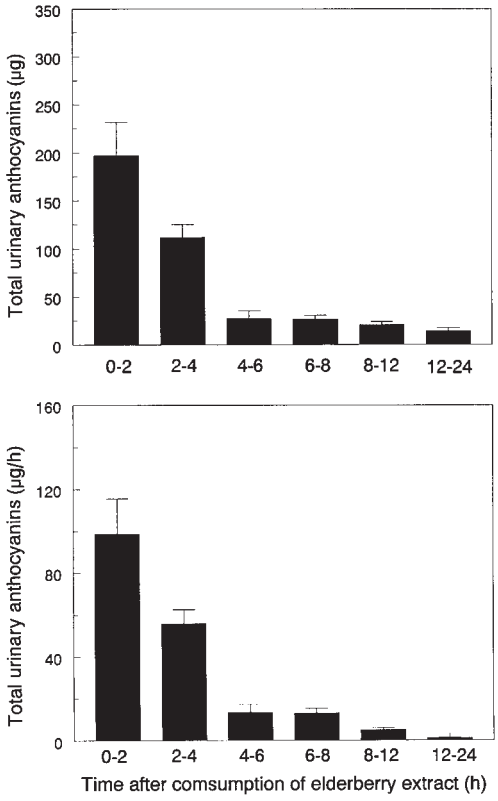

FIGURE 6. Mean (±SE) urinary excretion of total anthocyanins after the consumption of 720 mg anthocyanins by 4 elderly women.

anthocyanins, could be absorbed. In other words, anthocyanins were considered nonabsorbable without prior hydrolysis by microorganisms. The absorption of anthocyanins or anthocyanidins in humans was not well established until we detected in human plasma, by using HPLC, the compounds that have the same elution times and spectra as do elderberry anthocyanins 30 and 60 min after their consumption (24). The anthocyanins reported by Lapidot et al (29) in human urine after consumption of red wine appeared to be anthocyanin metabolites because they had ≥3 absorption peaks (at 280, 430, and 520 or 550 nm, respectively), with the main one at 430 nm. Anthocyanins should not have an absorption peak around 430 nm. Using LC-MS in this study, we showed clearly that anthocyanins were absorbed as glycosides in the elderly women. The longer $t_{1/2}$ of cyanidin 3-sambubioside than of cyanidin 3-glucoside (168.9 compared with 97.3 min) may have been related to the number of sugars contained in their structures: cyanidin 3-sambubioside contains one xylose and one glucose molecule, whereas cyanidin 3-glucoside contains only one glucose molecule.

The mechanisms underlying the absorption of anthocyanins as glycosides are not clear. The results of one study suggest that quercetin glucosides can interact with the sodium-dependent glucose transport receptors in the mucosal epithelium (30). Quercetin and anthocyanidins (aglycones of anthocyanins) belong to the same flavonoid family and share a similar basic structure. Therefore, the detection of the anthocyanins in their unchanged glycated forms may indicate the involvement of the glucose transport receptors in the absorption of these compounds in vivo.

In this study, neither glucuronides nor sulfates of the anthocyanins were detected in the plasma or urine samples. It is possible that these anthocyanin conjugates were not extracted during the sample preparation nor detected by the diode array detector. Unfortunately, we did not use β-D-glucuronidase and sulfatase digestion during the sample preparation.

In summary, for the first time, data were presented that describe the pharmacokinetics of dietary anthocyanins in humans. Anthocyanins were clearly absorbed in their unchanged glycated forms in humans.

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