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# Avenanthramides Are Bioavailable and Accumulate in Hepatic, Cardiac, and Skeletal Muscle Tissue Following Oral Gavage in Rats

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**ABSTRACT:** Avenanthramides (AVA), polyphenols found exclusively in oats (*Avena sativa* L.), may play a role in the anti-inflammatory and antiatherogenic activity of oats. The bioavailability of AVA has been demonstrated previously, but its distribution at the organ and tissue level and the extent of conjugation following ingestion have been unexplored. Synthetic AVA was administered to 24 rats by oral gavage, whereas 6 control rats received saline. AVA concentrations were measured via HPLC in plasma, liver, heart, and gastrocnemius (GAS) obtained over a 12 h period (0, 2, 4, 12 h; $n = 6$ at each time point). Samples were extracted with and without glucuronidase-sulfatase to assess the level of conjugation. We conclude that AVA are bioavailable to the blood circulation following oral ingestion in the rat and reach peripheral tissues where they can be taken up by various organs differentially. With AVA remaining in the organs for up to 12 h, it seems possible to maintain an increased level of AVA in the rat via repeated feedings.

**KEYWORDS:** *Avena sativa*, oats, bioavailability, alkaloids, polyphenols, avenanthramide

## INTRODUCTION

Epidemiologic evidence has shown the association of whole grain consumption and markers of cardiovascular health.[1−4] Oats (*Avena sativa* L.), in particular, have been identified as a food that may confer multidimensional health benefits to the consumer. Oat consumption has been shown to reduce serum cholesterol and triglycerides by increasing gut viscosity and altering bile acid metabolism.[5−8] The soluble fiber $\beta$-glucan has been identified as the main active ingredient for these functions.[9] In addition to $\beta$-glucan, oats contain an abundance of polyphenols that have potential benefits in the treatment of disease and dysfunction associated with aging.[10−13] Furthermore, the anti-inflammatory and anti-itch properties of oatmeal have been known for centuries.[14]

Avenanthramides (AVA) are a group of hydroxycinnamoylanthranilate alkaloids exclusive to oats.[15] Over 25 AVA compounds have been identified, but three classified as AVA-A, -B, and -C are the most abundant and differ only by a hydrogen, hydroxyl, or methoxy on C-3 of the cinnamic acid ring. In vitro, all three AVA display antioxidant capacity, and the order of activity is related to the constituent hydroxycinnamic acid residue of each fraction.[16,17] In cell culture experiments, AVA-C has been shown to have antiatherogenic properties with enhanced nitric oxide production and decreased proliferation of vascular smooth muscle cells.[18,19] It has also been shown to decrease pro-inflammatory cytokine production and adhesion molecule expression in human aortic endothelial cells via inhibiting nuclear factor kappa B (NF$\kappa$B) activation.[20,21] Recent cell culture research has supported the efficacy of NF$\kappa$B inhibition in the anti-inflammatory action of AVA.[22]

Dietary AVA-C supplementation has been shown to reduce exercise-induced oxidative stress in rats.[23] The effects, however, were tissue-specific, raising questions about the distribution of AVA following oral ingestion. Chen et al. showed that AVA-A and -B are bioavailable in hamsters fed a mixture containing oat phenolic compounds.[24] More recently, AVA-A, -B, and -C were found to be present in human plasma samples following an oral dose of AVA-enriched mixture.[25] However, neither of the studies has measured the concentration of AVA in body tissues besides plasma. Furthermore, all analyses of AVA concentration were performed in the presence of glucuronidase-sulfatase, an enzyme that cleaves glucuronic acid and sulfate groups conjugated to the hydroxycinnamic acids on AVA.[26] Therefore, all measurements are of total AVA, while the distribution of conjugated versus free concentrations remains unknown.

The purposes of this study were (1) to measure the concentration of different fractions of AVA and the time courses of their appearance after an oral ingestion in rats; (2) to assess the content of various AVA fractions in the liver, heart, and skeletal muscle and their time courses; and (3) to examine the extent and time course of the conjugation of AVA in the postprandial period.

## MATERIALS AND METHODS

**Animals.** All procedures were performed in compliance with the University of Wisconsin Animal Care and Use Committee. Female Sprague−Dawley rats ($n = 30$), aged 100−149 d, were housed 2 per cage on a 12 h:12 h dark:light cycle with standard rat chow pellets and tap water available ad libidum. They were fasted overnight (12 h) before the experiment and then given access to food ad libidum 2 h prior to AVA ingestion to upregulate digestive enzymes.

**AVA Synthesis.** AVA-A, -B, and -C were synthesized by previously described methods.[15,16,18] Briefly, for AVA-A, 4-acetoxycinnamic acid is converted to 4-acetoxycinnamoyl chloride by reaction with thionyl chloride. This compound is combined with 5-hydroxyanthranilic acid in 10 mL of pyridine. Solvents are removed by rotary evaporation, and

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the residue is dissolved in acetone/acetic acid/$H_2O$ (80/10/10, v/v/v) overnight. Acetyl protecting groups are removed by reaction with methanol/$H_2O$/$NH_4OH$ (50/40/10, v/v/v). For AVA-B, the same steps are followed with a starting material of 4-acetoxy-3-methoxycinnamic acid. For AVA-C, caffeic acid is reacted with acetic anhydride overnight in the presence of pyridine. The resulting precipitate is the starting material for the steps described for AVA-A.

**AVA Administration and Tissue Collection.** Synthetic AVA-A, -B, and -C at a dose of 20 mg of each AVA per kg body weight were mixed in saline and administered to the nonanesthetized rats by oral gavage (curved 2-in. needle with 2.25 mm ball, Popper & Sons, New Hyde Park, NY). Rats were killed by decapitation at 1, 2, 4, or 12 h postingestion ($n = 6$ at each time point). Controls ($n = 6$) were not gavaged and were killed immediately following the AVA administration to other animals. Mixed arteriovenous blood was collected in tubes lined with heparin and immediately centrifuged at 15 000*g* for 10 min to attain plasma, which was immediately stored at −80 °C. Heart, liver, and gastrocnemius muscle (GAS) were excised, immediately frozen in liquid nitrogen, and stored at −80 °C. Hearts were rinsed in ice-cold saline and dried on absorbent paper before freezing.

**AVA Measurement.** *Homogenization.* A piece of frozen heart, liver, or muscle tissue was weighed and quickly transported to a small amount of 0.1 M phosphate buffer (pH = 7.4) on ice. The volume of the phosphate buffer was then adjusted to 10 mL per g of tissue. The piece of tissue was then minced with scissors and homogenized with a motor-driven Potter-Elvehjem Teflon glass homogenizer at 0−4 °C.

*Extraction.* AVA was extracted from the tissue homogenate and plasma by the procedure of Chen et al.[24] with modifications. To 200 μL of plasma or homogenate was added 20 μL of vitamin C-EDTA. At this point, two separate methods were used for AVA extraction. For analysis of total (free plus conjugated) AVA, 20 μL of glucuronidase-sulfatase (Type H-2 from *Helix pomatia*) was added to cleave any glucuronide and sulfate groups that can be conjugated to AVA by liver enzymes, so that all AVA can be detected. For free AVA analysis, no enzyme was added, and therefore conjugated AVA was not detected. All samples were incubated for 45 min at 37 °C. Next, 500 μL of 100% acetonitrile (ACN) was added to the tubes. After 5 min, the samples were centrifuged at 15 000*g* for 5 min. The supernatant, which contains the AVA, was removed, and the solvent was evaporated by motorized vacuum pump (Fisher Scientific) at a pressure of approximately 200 mmHg for approximately 5 min. The residue was reconstituted in 200 μL of high-pressure liquid chromatography (HPLC) aqueous solvent. Again, the samples were centrifuged at 15 000*g* for 5 min. The supernatant was transferred to an HPLC vial with a punch-through disk cap for HPLC injection.

*HPLC Analysis.* Following the extraction procedure, all samples were analyzed for AVA concentration with a procedure based on Milbury[27] on a dual pump Shimadzu HPLC system with a UV−vis spectrophotometric detector, a Supelco C18 column with inline guard column, and a 23-min ACN gradient using two solvents: A (5% ACN in $H_2O$, 0.1% formic acid) and B (99.9% ACN, 0.1% formic acid). Total flow rate was held constant at 1 mL/min with 13% B at time 0, and increasing to 60% B at 18 min, the column was kept at room temperature, and an injection volume of 20 μL was used. Absorption at 330 nm was tracked by Shimadzu EZStart 7.2.1 software, which generates a trace of absorption over time for each sample. The software detects peaks and reports retention time (RT), which is used to identify the compound, and area under the curve (AUC). Plasma samples of control animals were spiked with various concentrations of each AVA fraction to create a standard curve for each fraction. Spiked samples were subjected to the extraction and detection procedures described above.

**Glutathione.** Liver glutathione (γ-glutamylcysteinylglycine) levels were measured by HPLC using a method based on Reed et al.[28] and modified by Ji and Fu.[29] Briefly, tissue was transferred directly to a solution containing 7% perchloric acid with 2 mmol/L phenanthroline (pH < 2.0, 1:10 w/v). After thawing, tissues were homogenized with a glass homogenizer. The homogenate was incubated on ice for 10 min and then centrifuged at 1000*g* for 1 min. An aliquot of 250 μL of supernatant was transferred to a tube containing 10 μL of 0.4 mmol/L iodoacetic acid and excess sodium bicarbonate. After the homogenate was incubated at room temperature for 1 h, 2 μL of 2,4-dinitrofluorobenzene (Sanger's reagent, Sigma Chemical, St. Louis, MO) was added, and the samples were kept in the dark for 28 h before the HPLC detection.

Following the method of Reed et al.,[28] concentrations of reduced glutathione (GSH) and oxidized glutathione disulfide (GSSG) were determined using a UV−vis detector at 365 nm wavelength and quantified with standard curves generated using GSH and GSSG standards.

**Statistical Analysis.** One-way analysis of variance followed by Tukey's honestly significant difference test was used to detect significant differences among time points ($P < 0.05$). Analysis was performed with *R* statistical software.

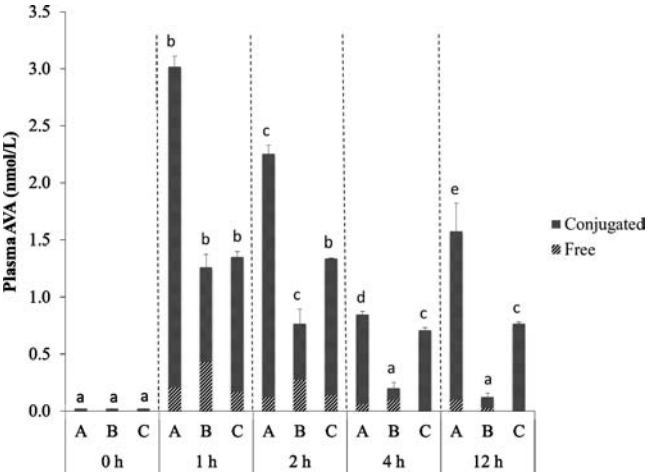


**Figure 1.** Concentrations of AVA-A, AVA-B, and AVA-C in plasma over time following oral gavage. Values are mean ± SEM, $n = 6$ for each time point. Within a single AVA type, bars not sharing a letter are significantly different ($P < 0.05$).

## RESULTS

**Plasma.** No AVA was detected in the plasma of control (C) rats not receiving AVA. Following oral gavage of a mixture containing 20 mg/kg body weight of each AVA fraction, AVA-A, -B, and -C were detected in plasma (Figure 1). Total AVA-A concentrations were 3.08 ± 0.23, 2.25 ± 0.18, 0.87 ± 0.07, and 1.57 ± 0.61 nmol/L at 1, 2, 4, and 12 h, respectively ($P < 0.05$ vs C at all times). Conjugated AVA-A concentrations were 2.81 ± 0.12, 2.13 ± 0.20, 0.79 ± 0.13, and 1.48 ± 0.68 nmol/L ($P < 0.05$ vs C at all times), which represent 91%, 95%, 91%, and 94% of the total AVA-A, at 1, 2, 4, and 12 h, respectively.

The time course of AVA-B bioavailability mirrored that of -A, although concentrations were lower at each time and did not reach significance at 4 and 12 h. Total AVA-B concentrations were 1.17 ± 0.27 ($P < 0.05$ vs C), 0.59 ± 0.31 ($P < 0.05$ vs C), 0.20 ± 0.12, and 0.13 ± 0.09 nmol/L at 1, 2, 4, and 12 h, respectively. Conjugated AVA-B concentrations were 0.84 ± 0.38 ($P < 0.05$ vs C), 0.49 ± 0.30 ($P < 0.05$ vs C), 0.10 ± 0.08, and 0.10 ± 0.10 nmol/L, representing 72%, 83%, 50%, and 77% of total AVA-B at 1, 2, 4, and 12 h, respectively.

AVA-C displayed a slightly different time course with the concentration remaining elevated at 2 h as opposed to the steep

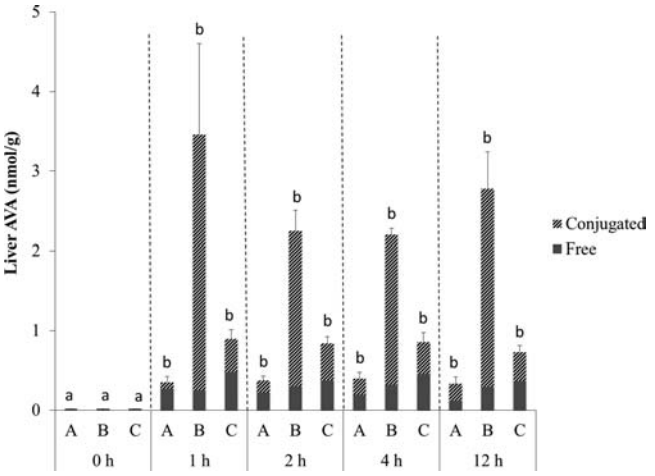


**Figure 2.** Concentrations of AVA-A, AVA-B, and AVA-C liver over time following oral gavage. Values are mean ± SEM, $n = 6$ for each time point. Within a single AVA type, bars not sharing a letter are significantly different ($P < 0.05$).

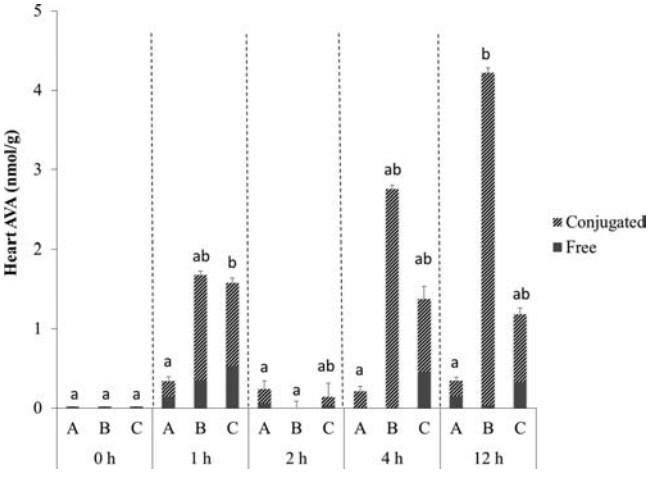


**Figure 3.** Concentrations of AVA-A, AVA-B, and AVA-C heart over time following oral gavage. Values are mean ± SEM, $n = 6$ for each time point. Within a single AVA type, bars not sharing a letter are significantly different ($P < 0.05$).

drop-off observed with -A and -B. Total AVA-C concentrations were 1.27 ± 0.38, 1.26 ± 0.30, 0.70 ± 0.08, and 0.76 ± 0.10 nmol/L at 1, 2, 4, and 12 h postgavage, respectively ($P <$ 0.05 vs C at all times). Conjugated AVA-C concentrations were 1.19 ± 0.14, 1.20 ± 0.01, 0.70 ± 0.05, and 0.76 ± 0.04 nmol/L ($P < 0.05$ vs C at all times), representing 94%, 95%, 100%, and 100% of total AVA-C at 1, 2, 4, and 12 h postgavage, respectively.

**Liver.** All three fractions of AVA were detected in the liver of AVA-fed rats but not C rats, with no AVA detected at 0 h in any rats (Figure 2). The total concentrations for AVA-A were 0.35 ± 0.07, 0.37 ± 0.06, 0.40 ± 0.04, and 0.33 ± 0.04 nmol/g ($P < 0.05$ vs C at all times), at 1, 2, 4, and 12 h, respectively. The concentrations of conjugated AVA-A were 0.08 ± 0.11, 0.15 ± 0.11, 0.21 ± 0.12 ($P < 0.05$ vs C), and 0.21 ± 0.09 ($P < 0.05$ vs C) nmol/g, representing 23%, 41%, 52%, and 64% of total AVA-A at 1, 2, 4, and 12 h, respectively.

AVA-B was measured in greater concentration than -A and -C. The total concentrations of AVA-B were 3.46 ± 2.30, 2.25 ± 0.51, 2.20 ± 0.16, and 2.78 ± 0.93 ($P < 0.05$ vs C at all times) nmol/g for AVA-B at 1, 2, 4, and 12 h, respectively. Conjugated AVA-B concentrations were 3.20 ± 2.33 ($P < 0.05$ vs C), 1.95 ± 0.52, 1.88 ± 0.18, and 2.49 ± 1.03 nmol/g, representing 92%, 87%, 85%, and 90% of total AVA-B at 1, 2, 4, and 12 h, respectively.

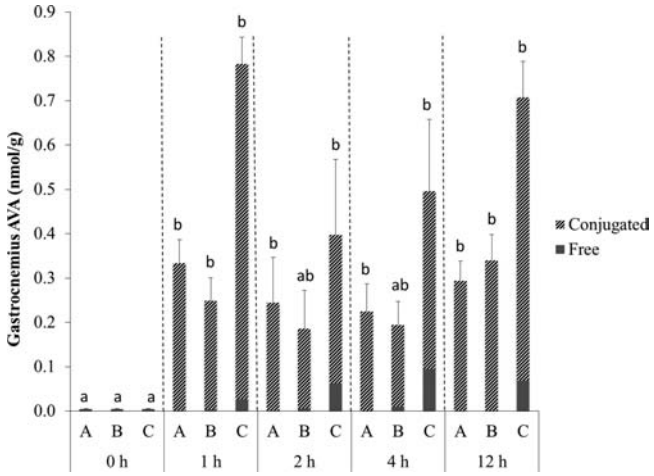


**Figure 4.** Concentrations of AVA-A, AVA-B, and AVA-C in gastrocnemius over time following oral gavage. Values are mean ± SEM, $n = 6$ for each time point. Within a single AVA type, bars not sharing a letter are significantly different ($P < 0.05$).

Total AVA-C concentrations were 0.90 ± 0.24, 0.84 ± 0.17, 0.86 ± 0.24, and 0.73 ± 0.17 nmol/g ($P < 0.05$ vs C at all times) for AVA-C at 1, 2, 4, and 12 h postgavage, respectively. Conjugated AVA-C concentrations were 0.42 ± 0.33, 0.46 ± 0.22 ($P < 0.05$ vs C), 0.40 ± 0.18, and 0.36 ± 0.18 nmol/g, representing 47%, 55%, 47%, and 49% of total AVA-C at 1, 2, 4, and 12 h postgavage, respectively.

**Heart.** Figure 3 shows the time course of AVA concentration changes in heart. At baseline and in C rats, no AVA was detected in cardiac homogenates. AVA-A was not detected at statistically significant levels at any time point.

AVA-B was not detected at 2 h postgavage, but at 1, 4, and 12 h the total concentrations were 1.68 ± 1.44, 2.75 ± 1.86, and 4.22 ± 1.92 ($P < 0.05$ vs C at each time) nmol/g, respectively. Conjugated AVA-B concentrations were 1.33 ± 1.37, 2.75 ± 1.86, and 4.19 ± 2.91 ($P < 0.05$ vs C at each time) nmol/g, representing 79%, 100%, and 99% of total AVA-B at 1, 4, and 12 h, respectively.

AVA-C accumulation in the heart was fleeting, with concentrations failing to reach significance after 1 h. Total AVA-C concentrations were 1.58 ± 0.80 ($P < 0.05$ vs C), 0.14 ± 0.29, 1.37 ± 1.04, and 1.18 ± 0.71 nmol/g, at 1, 2, 4, and 12 h postgavage, respectively. Conjugated AVA-C concentrations were 1.05 ± 0.83 ($P < 0.05$ vs C), 0.12 ± 0.23, 0.92 ± 0.75, and 0.86 ± 0.37 nmol/g, representing 66%, 86%, 67%, and 73% of total AVA-C at 1, 2, 4, and 12 h, respectively.

**Gastrocnemius.** No AVA was detected in GAS at baseline (Figure 4). GAS total AVA-A concentrations reached 0.33 ± 0.05, 0.25 ± 0.10, 0.22 ± 0.06, and 0.29 ± 0.05 nmol/g ($P < 0.05$ vs C at all times) at 1, 2, 4, and 12 h, respectively. All measured AVA-A was in the conjugated form.

Total AVA-B concentrations were 0.25 ± 0.05, 0.19 ± 0.09, 0.19 ± 0.05, and 0.34 ± 0.06 nmol/g ($P < 0.05$ vs C) at 1, 2, 4, and 12 h, respectively. No statistically significant concentration of free AVA-B was measured; all AVA-B was conjugated.

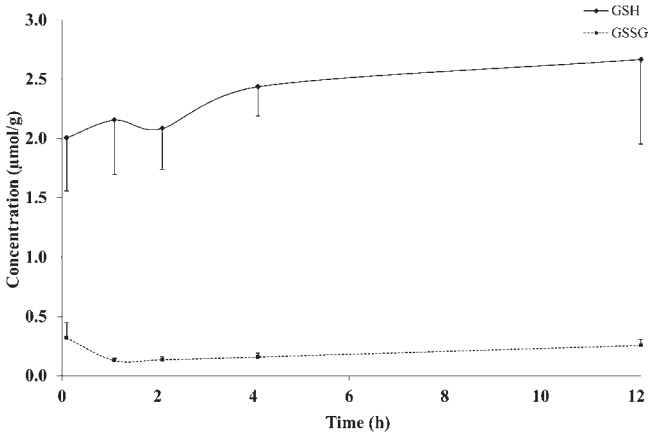


**Figure 5.** Concentrations of GSH and GSSG in liver over time following oral gavage.

Total AVA-C concentrations were 0.78 ± 0.06, 0.40 ± 0.17, 0.50 ± 0.16, and 0.71 ± 0.08 nmol/g ($P < 0.05$ vs C at all times) at 1, 2, 4, and 12 h postgavage, respectively. Conjugated AVA-C concentrations were 0.76 ± 0.06, 0.34 ± 0.17, 0.40 ± 0.16, and 0.64 ± 0.08 nmol/g ($P < 0.05$ vs C at all times), representing 97%, 85%, 80%, and 90% at 1, 2, 4, and 12 h postgavage, respectively.

**Liver Glutathione.** No significant changes in liver GSH concentration, GSSG concentration, or GSH:GSSG ratio were detected (Figure 5).

## DISCUSSION

**Plasma.** Structural differences most likely account for the differences in bioavailability of AVA.[25] AVA-A is unsubstituted at the carbon-3 position of the cinnamic acid, while -B and -C contain a methoxy and hydroxy group, respectively, at this position. Thus, AVA-A is less hydrophilic and less readily eliminated in the urine correlating with its increased plasma concentration.[25]

Chen et al.[25] reported maximal concentration values of 112.9 ± 28.2, 13.2 ± 3.9, and 41.4 ± 15.8 nmol/L for AVA-A, -B, and -C, respectively, following an oral dose of an AVA-enriched mixture (AEM) in older humans. These values far exceed the concentrations measured in this study using different animal species and dosages, but the rank order of concentration (i.e., A ≫ C > B) was preserved. In addition, peak plasma AVA concentrations were measured 1 h postgavage in this study, while they were measured 2 h after ingestion in humans[25] and 15 min after ingestion in hamsters.[24] Notably, in humans, AVA-B was the slowest fraction to be eliminated, while it was eliminated most quickly in rats. These discrepancies may be the result of differences in phase I and II metabolism between species.[30] Also, the differences in absorption via the gut and/or differences in the method of delivery (AEM versus pure compound) may play a role as glycosylated AVA may need to be cleaved to the aglycone form before it can be absorbed from the gut.[31] Furthermore, biliary excretion may differ between humans and rats due to the presence of the gall bladder in humans.[32] Finally, it is possible that plasma AVA reached a higher peak concentration before the first measurement at 1 h. This would explain the relatively low concentrations measured.

**Liver.** In contrast to plasma, liver showed increased concentrations of AVA-B, and a large percentage of AVA-B detected in liver was conjugated. On the other hand, the peak percentage of AVA-A and -C that was conjugated was lower than in plasma (64.4% vs 94.9% and 55.2% vs 95.5%, respectively). These data suggest a disconnect between plasma and liver AVA concentration, a phenomenon previously described in flavanone aglycones and glucuronides.[33]

The liver receives blood from two sources: the hepatic portal vein carrying blood from the gut and the hepatic artery carrying circulating blood from the heart. Immediately following the ingestion, the portal vein should represent the major source of AVA in the liver. As time elapses and less AVA enters circulation from the gut, hepatic AVA is likely coming from arterial blood circulation. This transition is seen clearly in the case of AVA-B (Figure 2) wherein the concentration drops dramatically between 1 and 2 h postgavage and also to a lesser degree in the case of AVA-C.

Changes in glutathione concentrations, particularly GSH depletion, are associated with disease[34] and toxicity.[35] The lack of change (Figure 5), particularly in GSH:GSSG ratio, indicates a lack of redox stress from the AVA. This is consistent with the finding of Chen et al.[25] that serum glutathione concentrations remain mostly unchanged after an oral dose of AEM with only a transient increase in GSH. High concentrations of antioxidants have been shown to have pro-oxidative effects,[36−38] but this did not appear to be the case in AVA oral ingestion.

**Heart.** As in the liver, there was no change in AVA-A concentration in the heart after oral ingestion, suggesting this AVA fraction was not actively taken up by cardiac myocytes or hepatocytes. Consistent with findings in the liver, AVA-B and -C were mostly in the conjugated state in heart. Because AVA-B concentration in the plasma was lower than those in AVA-A and -C, whereas its concentration was higher than AVA-A and -C in liver and heart, it seems that AVA-B was preferentially taken up into these tissues. Tissue-specific differences in uptake and metabolism have been shown to lead to differences in tissue distribution and concentration of phytochemicals.[32] In our previous study, AVA-B oral supplementation resulted in reduced ROS production and increased antioxidant enzyme activity.[23] We hypothesize that conjugation of AVA-B with glucuronic acid and/or sulfate may facilitate the uptake by cells and increase tissue concentration.

The time course of AVA-B and -C concentration in heart homogenates was remarkably different from those of plasma and liver. After a peak at 1 h postgavage, AVA-B and -C concentrations dropped to baseline levels at 2 h, and then rebounded to elevated levels at 4 and 12 h. We currently do not have a clear explanation for this pattern of change. We speculate that during the first couple of hours after oral ingestion, liver and skeletal muscle were more capable of taking up plasma AVA-B and -C than the myocardium; however, heart gradually enhanced AVA uptake when AVA concentration reached stable levels in other tissues. The high AVA-B level found at 12 h provides a potential explanation for the observed changes reported in our previous study, such as increased GSH peroxidase activity and decreased lipid peroxidation in the heart after chronic AVA-B supplementation.[23]

**Skeletal Muscle.** All fractions of AVA were almost exclusively conjugated in the GAS muscle. AVA concentrations were elevated at all time points, and more conjugated than free AVA was measured, which is consistent with the findings in liver and heart. However, AVA concentrations in muscle were much lower

than those in liver and heart up to 12 h after oral ingestion, indicating the uptake from the circulation was slow and modest. Skeletal muscle composes about 40% of body weight and could be a potential "sink" for AVA. At rest, skeletal muscle has a rather low metabolic rate, and muscle blood flow differs greatly among various muscle types. Thus, it is not surprising that a single oral gavage resulted only in a small elevation in muscle AVA content. In a previous study, we found that rat supplemented with AVA-B in the diet showed significantly higher SOD activity in soleus and vastus lateralis muscle, and lower ROS level in soleus.[23] These data could possibly be explained by an increased AVA uptake in the muscle.

**Conclusions.** This is the first study to our knowledge to measure AVA concentration in rat plasma, liver, heart, and skeletal muscle following oral ingestion and to determine the level of glucuronic acid and sulfate conjugation of AVA in the rat. The three AVA fractions appear to be taken up and distributed among the tissues differentially. In comparison to human and hamster, AVA bioavailability in rats is low, but the rank order of plasma concentration by AVA type (A ≫ B > C) is the same.

Because AVA is detectable in plasma and organs for up to 12 h following ingestion, the possibility exists that repeated feeding could result in increased steady-state levels of AVA in various tissues.

## ■ ABBREVIATIONS USED

ACN, acetonitrile; AEM, avenanthramide-enriched mixture; AVA, avenanthramide; GAS, gastrocnemius; GSH, reduced glutathione; GSSG, oxidized glutathione disulfide; HPLC, high-pressure liquid chromatography; NF$\kappa$B, nuclear factor kappa B.

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