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Pharmacokinetics and Bioavailability of the Isoflavone Biochanin A in Rats

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ABSTRACT

Biochanin A (BCA) is a dietary isoflavone present in legumes, most notably red clover, and in many herbal dietary supplements. BCA has been reported to have chemopreventive properties and is metabolized to the isoflavone genistein (GEN), BCA conjugates, and GEN conjugates. The metabolites may contribute to the chemopreventive effects of BCA. The absorption, metabolism, and disposition of BCA have not been determined in rats. Our objective was to evaluate the pharmacokinetics and metabolism of BCA in rats. Male Sprague-Dawley rats were administered BCA by intravenous injection (1 and 5 mg/kg), by intraperitoneal injection (5 and 50 mg/kg), and orally (5 and 50 mg/kg). Plasma and bile samples were enzymatically hydrolyzed in vitro to determine conjugate concentrations for BCA and GEN. Equilibrium dialysis was used to determine protein binding. The BCA and GEN concentrations in plasma, urine, and bile were determined by liquid chromatography-tandem mass spectrometry (LC/MS/MS). The pharmacokinetic parameters of BCA were analyzed by noncompartmental analysis. Significant levels of BCA conjugates and GEN conjugates were detected in plasma and bile. Both BCA and GEN were found to have a high clearance and a large apparent volume of distribution; the bioavailability of both was poor (<4%). Reentry peaks were evident after oral administration of both BCA and GEN, suggesting enterohepatic cycling. The free fraction of BCA in rat plasma was 1.5%. A 2-compartment model that included both linear and nonlinear clearance terms and enterohepatic recirculation best described the plasma data. This represents the first evaluation of the dose-dependent pharmacokinetics and metabolism of BCA in rats.

KEYWORDS: Biochanin A, pharmacokinetics, intraperitoneal administration, enterohepatic recirculation, rat, genistein

INTRODUCTION

High intake of isoflavones has been associated with a variety of human health benefits, including prevention of cancer,[1] cardiovascular diseases,[2] and osteoporosis.[3] The biochemical and pharmacological properties of isoflavones that may be related to these health benefits are multifaceted and may involve isoflavones' antioxidative,[4] antiestrogenic (as well as estrogenic),[5] and antiangiogenic activities,[6] as well as their inhibition of procarcinogen metabolic activation[7] and inhibition of cell growth.[8] The most studied isoflavone is genistein (GEN), a soy-derived isoflavone that has been shown to be an effective chemopreventive agent of chemical-induced carcinogenesis in vivo.[9-11] GEN, together with other soy isoflavones, may also reduce the low-density lipoprotein (LDL) cholesterol level in animal models[12,13] and in premenopausal women,[14] and a cardiovascular health claim was approved by the US Food and Drug Administration in 1999 for foods with significant amounts of soy protein. Biochanin A (BCA), a 4'-*O*-methyl derivative of GEN, is the major isoflavone in red clover (*Trifolium pratense*) but is not present in soy foods. This compound has also been shown to inhibit chemical-induced tumor carcinogenesis[15,16] and prevent tumor growth after implantation in animal models.[17] Isoflavone extracts from red clover with BCA as the major component have been shown to reduce the loss of lumbar spine bone mineral content and bone mineral density in women[18] and to lower the LDL cholesterol level in men.[19] These red clover isoflavone extracts, such as Promensil (Novogen Inc, Stamford, CT), are now also commercially available as dietary supplements for relieving postmenopausal symptoms such as hot flashes and bone loss and for maintaining men's prostate health. More recently, BCA and other flavonoids have been shown by in vitro studies to be potent inhibitors of the efflux transporters P-glycoprotein and breast cancer resistance protein,[20,21] which are important molecular mechanisms for both multidrug resistance and drug disposition, indicating the potential of this compound for drug interactions.

Although BCA can be regarded as a prodrug of GEN and is rapidly converted into the demethylated metabolite GEN in vitro and in vivo,[22,23] probably under the catalysis of cytochrome P450 (CYP) enzymes,[20,24] its biological effects observed in vivo are not identical to those of GEN. For example, BCA can significantly suppress the tumor growth of the human gastrointestinal cancer cells HSC-45M2 and HSC-41E6 transplanted in athymic nude mice, but GEN cannot,[25] suggesting that BCA or its metabolites, other than those derived from GEN, also exert significant in vivo effects.

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The metabolism of BCA is summarized in Figure 1. In addition to demethylation, which converts BCA into GEN, BCA and the metabolite GEN undergo rapid glucuronidation and sulfation.[28-30] The resultant conjugative metabolites have been shown to possess some biological activity[31,32] and may serve as an important source of cellular aglycones upon enzymatic hydrolysis at the target site. The oxidative metabolism of BCA and GEN by cytochrome P450 enzymes has been observed when BCA and GEN are incubated with human or rat liver microsomes. The metabolites are mainly hydroxylated products such as 3′-, 6-, or 8-hydroxy BCA or GEN.[26,27] However, the in vivo significance of these oxidative metabolites is unknown.

To better understand the in vivo effects of these isoflavones, knowledge of the kinetics of these compounds as well as the concentration-time profiles of their major metabolites is essential. To this end, several studies have been conducted to determine the pharmacokinetics of GEN in humans and in rats.[23,33-36] However, no pharmacokinetic data have been reported for BCA. Therefore, the objective of the present study was to evaluate the bioavailability and pharmacokinetics of BCA in rats after different routes of administration and to develop a pharmacokinetic model. The concentration-time profiles of the metabolites GEN and the conjugative products were also determined.

Materials and Methods

Animal Studies

Male jugular vein–cannulated Sprague-Dawley rats weighing 200 to 300 g were purchased from Charles River Laboratories (Wilmington, MA) and used for all the kinetic studies. All animal procedures were performed in accordance with Institutional Animal Care and Use Committee guidelines and followed approved protocols. Bile duct–cannulated rats were also purchased from Charles River Laboratories for the bile collection studies. Animals were acclimated with regular rat chow and drinking water ad libitum for 2 to 5 days before the study. Both BCA and GEN were purchased from Sigma-Aldrich (St Louis, MO). For the intravenous (IV) dose, BCA was dissolved in dimethyl sulfoxide (DMSO), diluted 20-fold in 25% hydroxypropyl-β-cyclodextrin (HPBCD), and dosed at 1 and 5 mg/kg via a jugular vein cannula. Oral doses of BCA were given by oral gavage in DMSO and olive oil at the ratio of 1:20 at 5 and 50 mg/kg. The intraperitoneal (IP) dose of BCA was prepared in 75% DMSO/water. GEN was dosed intravenously and orally in ethanol/Cremophor/25% HPBCD (final ratio of 1:1:6). Approximately 0.2 mL of blood was harvested from the jugular vein cannula at various time points and replaced with the same volume of saline. For the IV studies, the first 0.2 mL of blood was discarded to avoid contamination.

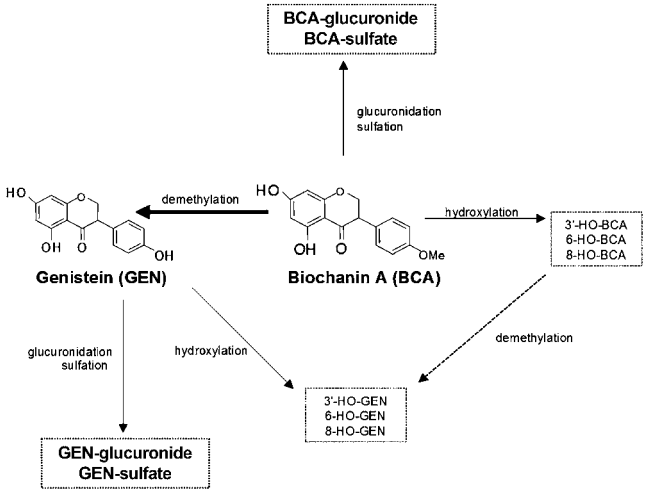

Figure 1. Proposed metabolic pathway of biochanin A (based on Roberts et al[26] and Kulling et al[27]).

Hydrolysis of Conjugates

Plasma and bile samples were enzymatically hydrolyzed in vitro to determine the amount of conjugates for BCA and GEN. The plasma or bile was precipitated in 50/50 methanol/acetonitrile and centrifuged. The supernatant was removed and was evaporated to dryness at 40°C under a stream of nitrogen. The dried product was resuspended in 0.05M phosphate buffer (pH 5.0) with 1000 units/mL of β-glucuronidase/sulfatase (Helix pomatia; Sigma Chemical Co, St Louis, MO) for 90 minutes at 37°C. The reaction was stopped by adding a 3-fold volume of 50/50 methanol/acetonitrile and then centrifuging. The supernatant was removed, dried under nitrogen, and then resuspended in the mobile phase.

Protein Binding Assay

Equilibrium dialysis (Spectrum Medical Industries Inc, Los Angeles, CA) was used to determine the protein binding for BCA and GEN. Approximately 10 mL of blood was collected from 2 male Sprague-Dawley rats weighing ~400 g on the morning of the study. The blood was centrifuged for 10 minutes and the plasma collected. BCA or GEN (1 μg/mL) was added to the plasma. The dialysis membrane was soaked in water for 20 minutes, 30% ethanol for 20 minutes, and then 100 mM sodium phosphate buffer (pH 7.4) for 20 minutes. Protein binding was determined after incubation of samples for 4 to 5 hours on a shaking water bath at 37°C. The plasma and buffer samples were precipitated with 200 μL of methanol: acetonitrile, then centrifuged; the supernatant was then removed and assayed as outlined below.

Liquid Chromatography-Tandem Mass Spectrometry Analysis

The BCA and GEN concentrations in plasma, urine, and bile were determined following the addition of 200 μL of

50/50 methanol:acetonitrile to 50-μL samples. This mixture was centrifuged, and the supernatant was removed, centrifuged, and injected onto the column. The analysis was performed on a PE Sciex API3000 triple quadropole mass spectrometer with a turbo ion spray source (Applied Biosystems/MDS SCIEX, Foster City, CA) linked to a Shimadzu LC10 liquid chromatography system equipped with an Armor column (C18, 30 mm × 4.6 mm id, 5 μm, Analytical Sales and Service Advantage, Pompton Plains, NJ). The source temperature was set at a constant 450°C. The mobile phase consisted of (A) acetonitrile, and (B) 10 mM ammonium acetate + 1% isopropyl alcohol. The sample was eluted using a gradient from 0% to 95% A at 0.75 mL/min. The ions measured for BCA were 283/239 and for GEN 269/133. The lower limit of detection was 1 ng/mL. Standards and quality controls were included with samples for every run so that intra- and interday variability was adjusted with the standards. The interday variability for the entire standard curve was ~17% for BCA and ~11% for GEN.

Noncompartmental Pharmacokinetic Analysis

The pharmacokinetic parameters of BCA were analyzed by noncompartmental analysis using WinNonlin (version 2.1, Pharsight Corporation, Palo Alto, CA). The area under the plasma concentration-time curves (AUC) was calculated by the trapezoidal method; the maximum plasma concentration (C_{max}) and the time to reach the maximum plasma concentration (t_{max}) were obtained by visual inspection of the experimental data; the terminal half-life ($t_{1/2}$) was calculated as 0.693/k, and k was the slope of the terminal regression line. The bioavailability (F) of BCA after IP and oral administration was calculated by the following equation:

$$F = \frac{oral\ AUC_{0-\infty}}{iv\ AUC_{0-\infty}} \times \frac{iv\ Dose}{oral\ Dose} \times 100, \qquad (1)$$

where $AUC_{0-\infty}$ is the AUC from time 0 to infinity.

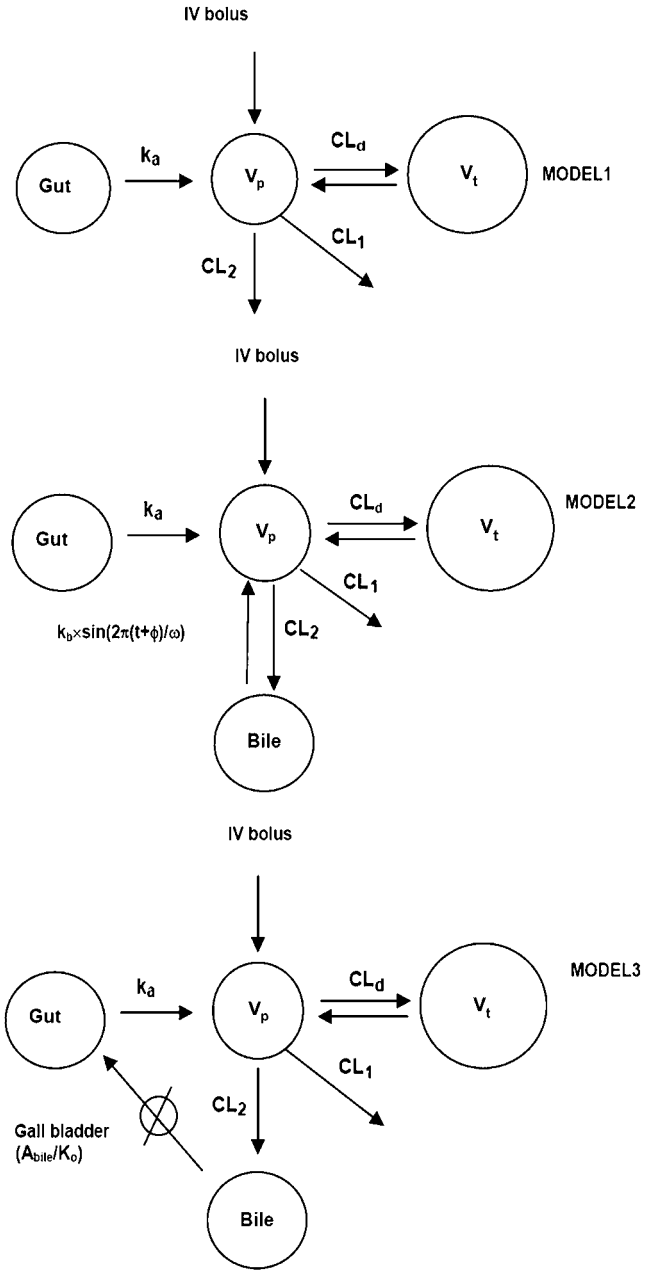

Figure 2. Schematic presentation of the pharmacokinetic models. Model parameters are described under Materials and Methods. IV indicates intravenous.

Pharmacokinetic Modeling

Several different models (Figure 2) were evaluated in fitting the IV and oral data using the Nelder-Mead method, with WinNonlin software (version 2.1). As the weighting scheme we used $1/(y_{hat})^2$. The concentration data obtained after 1 mg/kg and 5 mg/kg IV doses, and 5 mg/kg and 50 mg/kg oral doses, were simultaneously fitted into the equations. From the dose-normalized plasma BCA concentration-time profile in Figure 3, it can be seen that the plasma concentrations declined biexponentially, requiring the use of a 2-compartment model to fit the data. In addition, nonlinearity was apparent. Thus, a 2-compartment model was proposed with linear and nonlinear clearance terms. CL_1 is the linear component of the clearance, and CL_2 is the nonlinear component of the clearance, which may represent metabolism of BCA (defined as $CL_2 = V_{max}/(K_m + C)$). V_p, CL_d, V_t, and k_a are the volume of the central compartment, intercompartmental diffusion, the volume of the peripheral compartment, and the absorption-rate constant, respectively. Model 1, with 9 fitted parameters, does not include the bile compartment; that is, no enterohepatic cycling occurs. In Model 2, with 12 fitted parameters, transfer from the bile compartment to the central compartment occurs periodically, and a sine function is used to represent the periodical changes

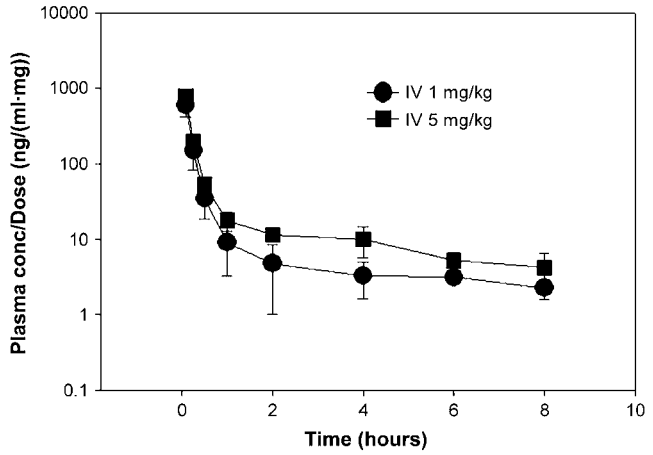

Figure 3. Dose-normalized plasma concentration-time profile after 1 mg/kg and 5 mg/kg IV doses of biochanin A. IV indicates intravenous.

($K_b \times \sin(2\pi(t + \phi)/\omega)$).[37] It is assumed that the same cycle is repeated every ω hours infinitely, with a ϕ time between cycles. To simplify the model and to minimize the number of parameters, the drug in the bile compartment is assumed to transfer directly into the plasma compartment in this model, as described by Wajima et al.[37] In Model 3, with 10 parameters, A_{bile}/K_0 is the zero-order release rate of drug from the bile compartment. A lag time (t_{lag}) was added to better describe the data. Assumptions for the models are (1) parent BCA undergoes capacity-limited nonlinear metabolism (Models 1, 2, and 3); (2) BCA glucuronide/sulfate conjugates are the major metabolites of BCA in rats (Models 2 and 3); (3) there is no elimination from the bile compartment (Models 2 and 3); (4) rates of absorption and reabsorption are equal (Model 3); (5) only 1 biliary cycle is sufficient to describe the enterohepatic cycling (Model 3); and (6) glucuronide/sulfate conjugates are combined as 1 entity (Models 2 and 3). According to the Model 3 in Figure 2, the differential equations (mass-balance) in this model can be written as follows:

IV

$$\frac{dC_p}{dt} = \frac{\left(-CL_d \times (C_p - C_t) - CL_1 \times C_p - \frac{V_{\max} \times C_p}{K_m + C_p}\right)}{V_p}, \quad C_p(0) = \frac{Dose}{V_p} \tag{2}$$

where CL_1 is the linear component of the clearance; C_p, concentration of drug in the central compartment; C_t, concentration of drug in the peripheral compartment; K_m, Michaelis-Menten constant; V_{max}, maximum metabolic capacity constant; V_p, volume of the central compartment.

$$\frac{dC_t}{dt} = \frac{CL_d \times (C_p - C_t)}{V_t} \tag{3}$$

where V_t is the volume of the peripheral compartment.

PO

$$C_p(0) = Dose1_{po} \times F1 \tag{4}$$

where F1 is the bioavailability for 5 mg/kg dose.

$$C_p(0) = Dose2_{po} \times F2 \tag{5}$$

where F2 is the bioavailability for 50 mg/kg dose.

$$\frac{dC_p}{dt} = \frac{\left(-CL_d \times (C_p - C_t) - CL_1 \times C_p - \frac{V_{\max} \times C_p}{K_m + C_p}\right)}{V_p} + \frac{K_a \times A_g}{V_p} \tag{6}$$

where A_g is the amount of drug in the gastrointestinal tract.

$$\frac{dC_t}{dt} = \frac{CL_d \times (C_p - C_t)}{V_t} \tag{7}$$

Before the lag time, the drug has not started enterohepatic circulation yet:

$$A_g = -k_a \times A_g \tag{8}$$

where k_a is the absorption rate constant of drug.

$$A_{bile} = \frac{V_{\max} \times C_p}{K_m + C_p} \tag{9}$$

where A_{bile} is the amount of drug in the bile.

After the lag time, the drug releases from the bile into the gut compartment with the rate of A_{bile}/K_0:

$$A_g = -k_a \times A_g + \frac{A_{bile}}{K_0} \tag{10}$$

where A_{bile} is the amount of drug in the bile; K_0, zero order release rate of drug from the bile compartment.

$$A_{bile} = \frac{V_{\max} \times C_p}{K_m + C_p} - \frac{A_{bile}}{K_0} \tag{11}$$

Statistical Analysis

Student's *t* test was used, with $P < .05$ set as the significance level.

RESULTS

Pharmacokinetics of BCA

The pharmacokinetic profiles of BCA and its metabolites after BCA administration are shown in Figure 4. A biexponential decline was observed in plasma BCA concentrations after IV administration. The results of the noncompartmental pharmacokinetic analysis are summarized in Table 1, Table 2, Table 3, and Table 4. The rat IV studies indicated that BCA exhibits a high clearance and a high volume of distribution in rats. The high clearance is likely due to rapid metabolism of BCA. Less than 3% of unchanged BCA was detected in the urine and bile (data are not shown). When the plasma samples were treated with the glucuronidase and sulfatase enzymes, the plasma concentrations of BCA and GEN increased ~10- and 50-fold, respectively. The GEN concentration in the plasma and bile was low. These results suggest that BCA is rapidly *O*-demethylated to GEN and that both BCA and its metabolite GEN are rapidly conjugated. When the bile samples (collected for 24 hours) were treated with the sulfatase/glucuronidase, ~18.8% of the BCA dose was recovered in the bile (average of 2 animal experiments; data not shown). The oral bioavailability of BCA was poor (2.6% at 5 mg/kg, 1.2% at 50 mg/kg).

$AUC_{0\text{-}t}$/dose increased with IV dose, but the difference was not statistically significant ($P = .070$). $AUC_{0\text{-}t}$/dose ratios were also not different after IP and oral administration of the 2 doses ($P > .16$). The ratios of $AUC_{0\text{-}t}$ of BCA conjugates to the total $AUC_{0\text{-}t}$ of total BCA (unchanged plus its conjugated metabolites), expressed as %, were 42% and 24% at IV doses 1 mg/kg and 5 mg/kg and 99.3% and 98.6% following 5 mg/kg and 50 mg/kg oral doses.

Reentry peaks of BCA in the plasma, likely due to enterohepatic recirculation, occurred at 4 to 5 hours after oral administration of the 5 and 50 mg/kg doses of BCA (Figures 4B, 4C, and 4D). Reentry peaks were also evident in the BCA and GEN conjugates profile and occurred at a similar time as for unchanged drug. The free fraction of BCA in rat plasma was 1.5%.

Pharmacokinetics of GEN

The pharmacokinetic profiles of GEN and its conjugates after GEN administration are shown in Figure 5. The results of the noncompartmental pharmacokinetic analysis are summarized in Table 1. The rat IV studies indicated that GEN exhibits a high clearance and a high volume of distribution in rats. High concentrations of GEN conjugates were observed in the plasma, indicating that GEN was rapidly conjugated (glucuronidated and/or sulfated) when GEN was dosed intravenously or orally. The oral bioavailability was also poor (3.4%) when GEN was administered at a 10 mg/

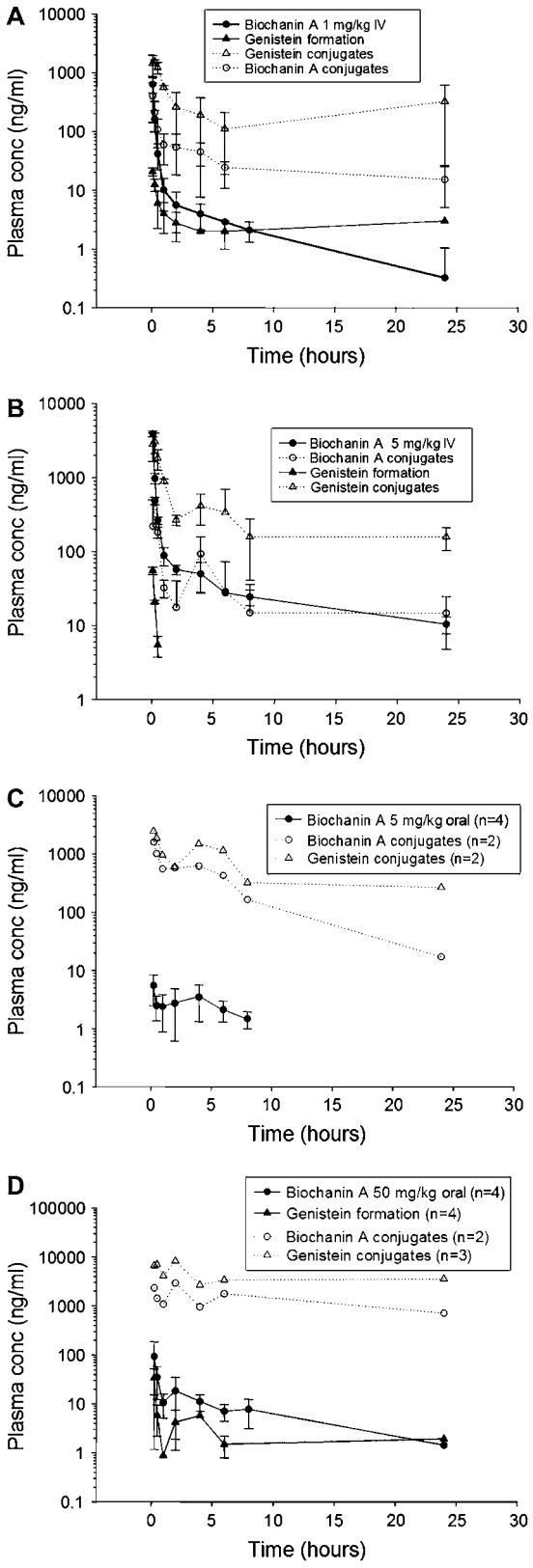

Figure 4. The pharmacokinetic profiles of BCA and its metabolites after the IV and oral administration of BCA. (A) BCA 1 mg/kg IV, (B) BCA 5 mg/kg IV, (C) BCA 5 mg/kg oral, and (D) BCA 50 mg/kg oral (mean ± SD, n = 3-4). BCA indicates biochanin A; IV, intravenous.

Table 1. Pharmacokinetic Parameters of BCA (n = 3-4) and GEN (n = 3-7) Obtained by Noncompartmental Analysis*

BCA	CL (L/h/kg)	Vd_{ss} (L/kg)	$t_{1/2}$ (h)	C_{max} (ng/mL)	AUC_{0-t} (ng·h/mL)
1 mg/kg IV	4.9 ± 2.3	10 ± 6.5	5.5 ± 3.2	651 ± 112†	258 ± 55†
5 mg/kg IV	2.8 ± 0.4	11 ± 2.5	6.8 ± 1.3	3910 ± 355†	1730 ± 204†
5 mg/kg oral			7.2 ± 4.1	13 ± 16	26 ± 22‡
50 mg/kg oral			7.1 ± 1.4	96 ± 89	151 ± 96‡
GEN	**CL (L/h/kg)**	**Vd_{ss} (L/kg)**	**$t_{1/2}$ (h)**	**C_{max} (ng/mL)**	**AUC_{0-t} (ng·h/mL)**
5 mg/kg IV	3.6 ± 1.1	8.2 ± 5.5	5.8 ± 3.0		1450 ± 417
10 mg/kg oral			7.6 ± 7.5	130 ± 47	142 ± 46

*CL indicates total systemic plasma clearance; Vd_{ss}, volume of distribution at steady state; $t_{1/2}$, elimination half-life; C_{max}, peak plasma concentration; AUC_{0-t} (ng•h/mL), area under the plasma concentration-time curve from time 0 to the last point; IV, intravenous; GEN, genistein.
†$P < .001$
‡$P < .05$

kg dose in rats. A reentry peak of GEN was observed following the 5 mg/kg IV and 10 mg/kg oral doses. The reentry peak of GEN at the dose of 5 mg/kg occurred earlier than that of BCA at the same dose. When the bile samples (collected for 24 hours) were treated with the sulfatase/glucuronidase, ~23.1% of the GEN dose was recovered in the bile (average of 2 animal experiments; data not shown). The free fraction of GEN was 1.5% in rat plasma.

IP Administration of BCA and GEN

To partially avoid the first-pass extraction, BCA and GEN were administered intraperitoneally (Figure 6). The pharmacokinetic profiles of BCA and its metabolites are shown in Table 2. The metabolite formation profile after an IP dose of BCA was similar to that after IV or oral administration of BCA (Figure 6). The C_{max} after an IP dose was ~80-fold higher than that after an oral dose when BCA was administered at 50 mg/kg (C_{max} = 7600 ng/mL after IP and 96 ng/mL after oral at 50 mg/kg; $P = .058$). A reentry peak of BCA was observed at a dose of 5 mg/kg IP but not at 50 mg/kg IP. The IP bioavailability of BCA was 40% at 5 mg/kg and 23% at 50 mg/kg IP. The ratios of AUC_{0-t} of parent BCA, BCA conjugates, metabolite GEN, and GEN conjugates to the total AUC_{0-t} of BCA + total AUC_{0-t} of metabolite GEN were 14%, 2.8%, 0.41%, and 85% at a dose of 5 mg/kg IP and 4.4%, 6.4%, 0.97%, and 88% at a dose of 50 mg/kg IP, respectively (Table 4).

Table 2. Pharmacokinetic Parameters of BCA and Its Metabolites (BCA Conjugates, GEN, and GEN Conjugates) After IP Dosing With 5 mg/kg and 50 mg/kg BCA (n = 3)*

Parameter	5 mg/kg IP	50 mg/kg IP	Difference (fold)
BCA			
C_{max} (ng/mL)	279 ± 75	7560 ± 6310	27
AUC_{0-t} (ng•h/mL)	598 ± 241	3510 ± 3000	5.9
BCA conjugates			
C_{max} (ng/mL)	176 ± 103	3450 ± 2850	20
AUC_{0-t} (ng•h/mL)	250 ± 433	4840 ± 5800	19
GEN			
C_{max} (ng/mL)	16 ± 6.0	1910 ± 1700	121
AUC_{0-t} (ng•h/mL)	23 ± 4.0	823 ± 794	36
GEN conjugates			
C_{max} (ng/mL)	974 ± 611	8090 ± 5690	8.3
AUC_{0-t} (ng•h/mL)	7750 ± 6040	60500 ± 28100	7.8†

*C_{max} (ng/mL), peak plasma concentration, was obtained by visual inspection of the experimental data; AUC_{0-t} (ng•h/mL), area under the plasma concentration-time curve from time 0 to the last point.
†$P < .05$

PK Modeling of BCA

On the basis of the Akaike information criterion (AIC) and Schwarz criteria (SC) values (Table 5), Model 3 presented in Figure 2 best described the plasma concentrations of BCA. The fit of the model to plasma concentrations is presented in Figure 7. Mean pharmacokinetic parameter estimates for the model are presented in Table 6. The rate constant for absorption was high (2.04 h^{-1}). Bioavailabilities determined for the 5 mg/kg oral dose and for the 50 mg/kg

Table 3. Pharmacokinetic Parameters of BCA Conjugates After IV Dosing With 1 mg/kg (n = 4) and 5 mg/kg (n = 3) BCA*

Parameter	1 mg/kg	5 mg/kg	Difference (fold)
C_{max}	355 ± 274	220 ± 284	0.62
AUC_{0-t}	473 ± 561	612 ± 445	1.3

*All values are mean ± SD. BCA indicates biochanin A; IV, intravenous; C_{max} (ng/mL), peak plasma concentration, was obtained by visual inspection of the experimental data; AUC_{0-t} (ng•h/mL), area under the plasma concentration-time curve from time 0 to the last point.

Table 4. Ratios of AUC_{0-t} of Parent BCA, BCA Conjugates, Metabolite GEN, and GEN Conjugates to the Total AUC_{0-t} of BCA + Total AUC_{0-t} of Metabolite GEN After IP Dosing With 5 mg/kg and 50 mg/kg BCA (n = 3)*

Parameter	5 mg/kg IP	50 mg/kg IP
BCA (%)†	14 ± 16	4.4 ± 2.2
BCA conjugates (%)‡	2.8 ± 4.9	6.4 ± 5.6
GEN (%)§	0.41 ± 0.32	0.97 ± 0.66
GEN conjugates (%)‖	85 ± 12	88 ± 5.9

*"Total" refers to unchanged isoflavone plus conjugated metabolites. All values are mean ± SD. BCA indicates biochanin A; GEN, genistein; AUC_{0-t}, area under the plasma concentration-time curve from time 0 to the last point; IP, intraperitoneal.
†BCA (%) = 100 × (AUC_{0-t} of BCA)/(AUC_{0-t} of total BCA + AUC_{0-t} of total GEN).
‡BCA conjugates (%) = 100 × (AUC_{0-t} of BCA conjugates)/(AUC_{0-t} of total BCA+ AUC_{0-t} of total GEN).
§GEN (%) = 100 × (AUC_{0-t} of metabolite GEN)/(AUC_{0-t} of total BCA + AUC_{0-t} of total GEN).
‖GEN conjugates (%) = 100 × (AUC_{0-t} of GEN conjugates)/(AUC_{0-t} of total BCA + AUC_{0-t} of total GEN).

oral dose were 0.016 and 0.008, respectively (Table 6), whereas bioavailability estimates obtained by noncompartmental analysis were 0.026 and 0.012, respectively.

DISCUSSION

As stated above, BCA is a 4'-*O*-methyl derivative of GEN. Even though BCA is converted to GEN in vivo and in vitro, the growth-inhibitory effect of BCA is not identical to that of GEN.[29,38,39] Whether the active ingredient in vivo is the parent compound or a metabolite has not been established.

At present, the pharmacokinetics of BCA have not been fully characterized. BCA was extensively metabolized to GEN in human subjects after ingestion of herbal products containing BCA.[23,40] Although *O*-demethylation of BCA has been attributed to metabolism by gut microflora,[38] hepatic microsomal enzymes can perform the same transformation.[22,41] Conversion of BCA to GEN in rat liver microsomes was found to be rapid and saturable (V_{max} of 490 pmol/min/mg, K_m of 64.5 μM).[28] Conversion of BCA into GEN also occurs in rat intestinal microsomes.[28] Multiple CYP isoforms, including CYP1A2 in liver microsomes and extrahepatic CYP1B1, participate in this demethylation reaction.[22,24] These isoforms also generate other hydroxylated metabolites,[22,41] including 3′-HO-BCA, 6-HO-BCA, and 8-HO-BCA. The main conjugation reaction of BCA in rat liver and intestinal microsome preparations was glucuronidation (K_m of 2.7-7.0 μM; V_{max}/K_m of 0.12 in liver and 0.40 in duodenum).[28] Glucuronide conjugates of BCA are likely to be 7-OH and 5-OH glucuronic acid derivatives.[28] In the human breast cancer cell line MCF-7, sulfate conjugates represent the major metabolite of BCA.[29]

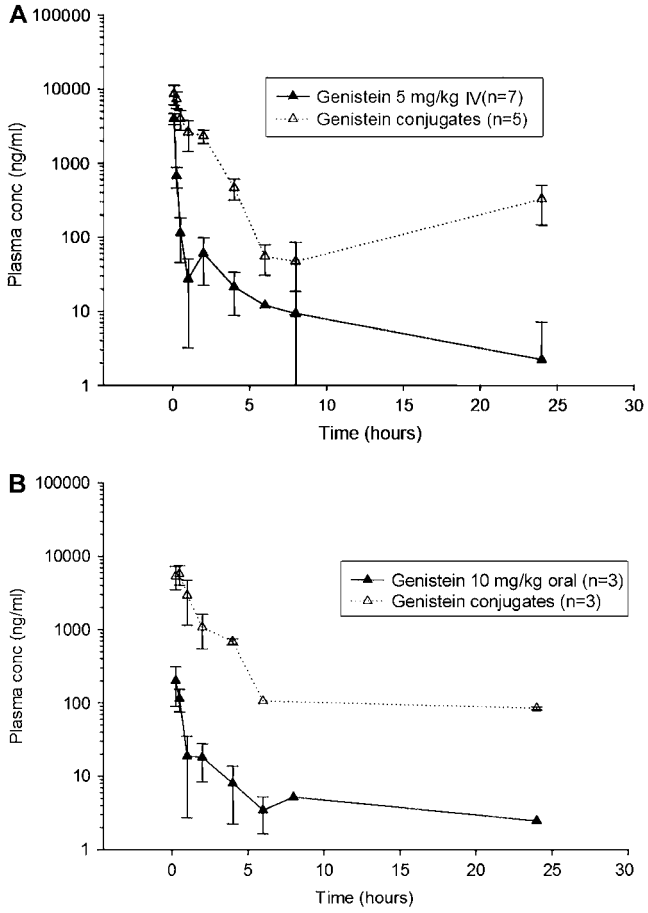

Figure 5. The pharmacokinetic profiles of GEN and its metabolites after the IV and oral administration of GEN. (A) GEN 5 mg/kg IV, and (B) GEN 10 mg/kg oral (mean ± SD, n = 3-7). GEN indicates genistein; IV, intravenous.

The plasma concentration-time profiles of oral BCA exhibited a rapid absorption phase followed by the biexponential disappearance of BCA. BCA was rapidly converted into GEN, and GEN, as well as conjugates of BCA and GEN, was detected in rat plasma. It is noteworthy that the concentration of BCA was higher than that of metabolite GEN for a long time after its IV and oral administration, especially after the 50 mg/kg oral dose of BCA, when BCA plasma concentrations were higher than those of GEN for more than 20 hours (Figure 4D). Only 2.6% after the 5 mg/kg dose and 1.2% after the 50 mg/kg dose of BCA were bioavailable after oral administration. Since the absorption of BCA was rapid because of its high permeability,[28] extensive first-pass metabolism and biliary elimination may be the main reasons for its poor bioavailability. In a previous study in rats in which BCA was administered as 1 component of a mixture of 5 flavonoids, including the isoflavones daidzein and GEN, its oral bioavailability was reported to be 21.3%.[42] The higher bioavailability in this study compared with the current investigation may be due to 2 factors. First, 5 compounds (including isoflavones, daidzein, and GEN) were administered simultaneously[42]; these other isoflavones,

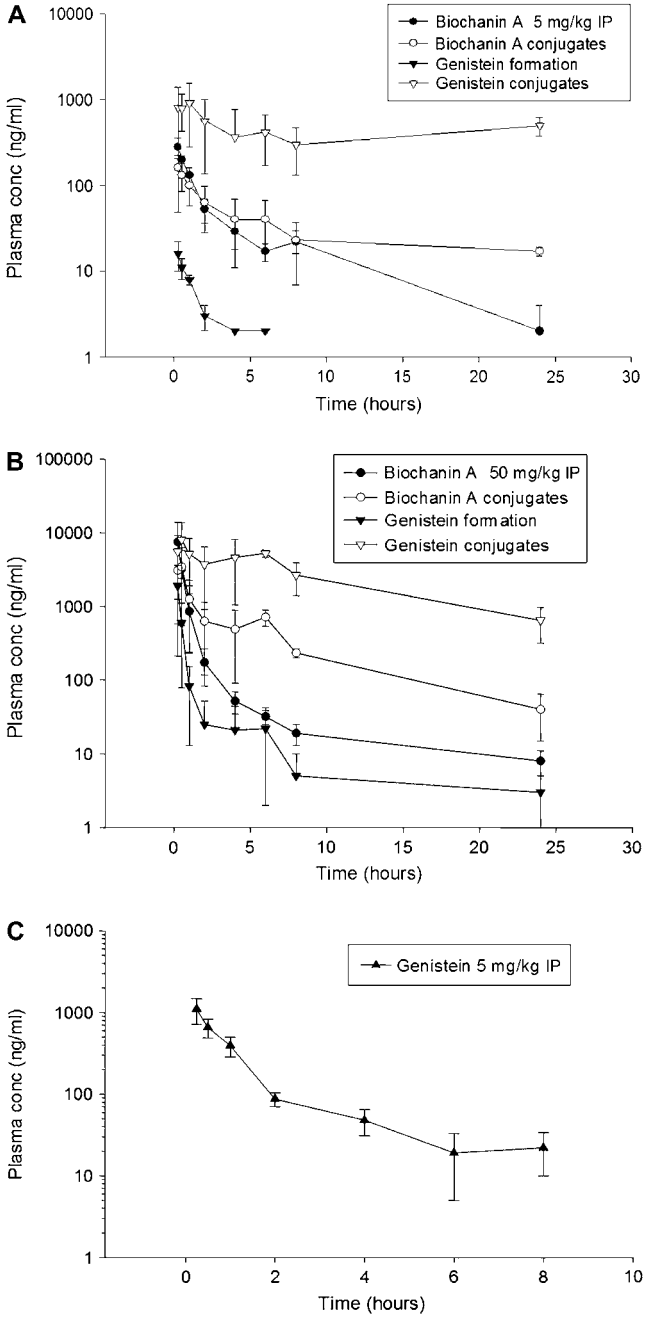

Figure 6. The pharmacokinetic profiles of BCA and its metabolites after the IP administration of BCA. (A) BCA 5 mg/kg IP, (B) BCA 50 mg/kg IP, (C) GEN 5 mg/kg IP (mean ± SD, n = 3). BCA indicates biochanin A; IP, intraperitoneal; GEN, genistein.

which are likely to have similar routes of metabolism and transport, could affect the AUC and elimination of BCA. Second, the authors used the AUC_{sc} for their bioavailability calculation, instead of AUC_{iv}. Since the absolute bioavailability of BCA after its subcutaneous administration maybe less than 100%, this may result in higher oral bioavailability estimates. In Table 4 the ratios of $AUC_{0\text{-}t}$ of BCA and its metabolites to the total $AUC_{0\text{-}t}$ of BCA + total $AUC_{0\text{-}t}$ of metabolite GEN (total refers to unchanged isoflavone plus conjugated metabolites) indicate that GEN conjugates are

Table 5. Evaluation of the 3 Pharmacokinetic Models*

	Parameters	AIC	SC	TWSSR
Model 1	9	59.7	72.3	4.01
Model 2	12	34.7	51.5	1.43
Model 3	10	30.2	44.2	1.40

*AIC indicates Akaike information criterion; SC, Schwarz criteria; TWSSR, total weighted sum of squared residuals.

the major metabolites present in plasma after IP dosing of BCA into rats. Thus, we can speculate that the 4'-*O*-demethylation reaction and conjugation reactions are the major metabolic routes of BCA.

The half-life of parent GEN found here is consistent with that found in other studies (~6-7 hours[23,33]). The half-life of GEN conjugates has been reported to be 7.9 hours in humans,[34] but in the present study it was difficult to obtain a value for the terminal slope because of the secondary and possibly tertiary peaks. Like BCA, GEN exhibits a high clearance and a high volume of distribution in rats.

Despite the extensive use of the IP route to study the effects of BCA in animal studies, the current study is the first to examine the concentrations of BCA and its metabolites after IP administration.[17,43] As would be anticipated, the mean C_{max} values for BCA after IP administration were much higher than after oral administration of the 50 mg/kg dose (80-fold; $P = .058$). The IP bioavailability of BCA was 40% at 5 mg/kg and 23% at 50 mg/kg IP—much higher values than those following oral administration (2.6% and 1.2% at 5 and 50 mg/kg oral dose, respectively). The study demonstrated that the IP route is an effective method for the administration of BCA. The reason for the decrease in bioavailability with increasing dose is unknown, but it may be due to the limited aqueous solubility of BCA and precipitation in the peritoneal fluids. A similar dose-dependent bioavailability was observed for GEN in mice[44] and quercetin in pigs.[45]

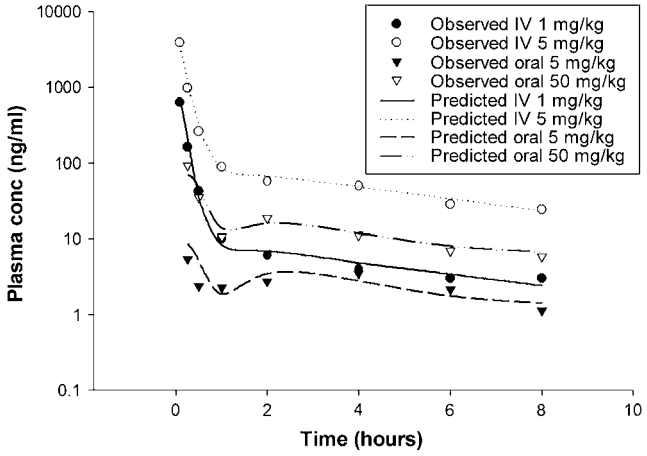

Figure 7. Model fitting of the unchanged plasma biochanin A concentration-time profile following IV and oral administration.

Table 6. Mean Pharmacokinetic Parameter Estimates Obtained From the Enterohepatic Recirculation Model of BCA Disposition (Model 3)*

Parameter	Estimate	CV
CL_1 (L/h/kg)	1.85	17.0
CL_d (L/h/kg)	3.40	18.2
k_a (h^{-1})	2.04	16.4
K_m (ng/mL)	24.3	42.5
V_{max} (ng/h)	2.55×10^5	29.3
F1 (5 mg/kg)	0.0162	12.7
F2 (50 mg/kg)	0.00838	11.4
V_p (L/kg)	0.832	18.6
V_t (L/kg)	16.4	25.9
K_0 (h)	0.100	38.1

*BCA indicates biochanin A; CV, coefficient of variation.

The dose-normalized plasma concentration-time profiles of BCA, determined at 2 IV dose levels, did not appear to be superimposable (Figure 3); the AUC_{0-t}/dose increased with dose, perhaps because of capacity-limited metabolism/excretion or saturable gastrointestinal-lumen efflux pumps. In keeping with this proposal, the nonlinear clearance model with 10 fitted parameters (incorporating a CL_2 term) had lower AIC and SC values (30.2 and 44.2, respectively) than did the linear clearance model with 9 fitted parameters (AIC 44.9, SC 57.5).

It is known that isoflavones undergo enterohepatic recirculation following formation of glucuronide conjugates.[28,42,46] Since a prominent reentry peak in the plasma BCA concentration after oral administration was also shown in our study, a 2-compartment model including enterohepatic recirculation was proposed. Even though no reentry peak (or only a small reentry peak following a 5 mg/kg BCA dose) was observed after IV administration, the existence of a reentry peak after IP administration of 5 mg/kg BCA (Figure 6A) indicates that the absorption mechanism alone cannot explain the existence of a reentry peak. Comparisons of fittings of the 2-compartment model including and excluding enterohepatic recirculation revealed that the plasma data were better described by an enterohepatic recirculation model. Similar models have been developed for roquinimex[47] and morphine 3-glucuronide.[48] To confirm enterohepatic recirculation of BCA, further study to compare biliary excretion and urinary excretion in bile duct–cannulated rats and control animals is needed.

In conclusion, our study is the first evaluation of the pharmacokinetics and metabolism of BCA in rats. Oral BCA pharmacokinetics is complex because 2 opposite effects (capacity-limited elimination and bioavailability) influence the plasma concentration-time profiles. BCA also undergoes enterohepatic recirculation. The major metabolic pathways are 4'-*O* demethylation followed by conjugation, as well as direct conjugation of BCA. Although BCA has poor bioavailability, enterohepatic recycling may provide a longer period of exposure. Additionally, comparison of our study results with those of Mallis et al[42] suggests the possibility that bioavailability may be improved if combinations of flavonoids are administered. BCA and its metabolite GEN are widely ingested as isoflavone herbal preparations and are being investigated for their cardiovascular and chemopreventive effects, as well as in the treatment of menopausal symptoms. Evaluation of the pharmacokinetics of BCA will be useful in assessing concentration-effect relationships for the potential therapeutic applications of BCA.

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