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Absorption and Bioavailability of Artepillin C in Rats after Oral Administration

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Artepillin C (AC), an active ingredient of Brazilian propolis, permeates intact across Caco-2 cells by transcellular passive diffusion. The permeation of AC across Caco-2 cells is as efficient as that of phenolic acids and the microbial metabolites of poorly absorbed polyphenols, which are actively absorbed by the monocarboxylic acid transporter (MCT) (*Biochim. Biophys. Acta* **2005**, *1713*, 138−144). Here, the absorption of orally administered AC in rats has been studied to evaluate its pharmacokinetics and bioavailability in vivo in comparison with those of *p*-coumaric acid (CA), a substrate of MCT. Rats were given 100 μmol/kg of body weight of AC or CA, and blood was subsequently collected from the portal vein and abdominal artery. AC, CA, and their metabolites were quantified by coulometric detection using HPLC−ECD. The serum concentration of intact AC and CA in the portal vein peaked at 5−10 min after administration, with a C_{max} of 19.7 μmol/L for AC and 74.8 μmol/L for CA. The area under the curve (AUC) for intact AC and CA in the portal vein was calculated from the serum concentration as 182.6 and 3057.3 μmol·min·L^{-1}, respectively. The absorption efficiency of CA was about 17-fold higher than that of AC. Furthermore, the bioavailability of CA was about 278-fold higher than that of AC, and the ratio of AUC in the abdominal artery to AUC in the portal vein was 0.04 and 0.70, for AC and CA, respectively. Thus, AC is likely to be more susceptible to hepatic elimination than is CA. The bioactive compound of AC in vivo should be investigated further.

KEYWORDS: Artepillin C; p-coumaric acid; monocarboxylic acid transporter; intestinal absorption; rat

INTRODUCTION

Artepillin C (AC; 3,5-diprenyl-4-hydroxycinnamic acid) is one of the principal phenolic acids present in propolis extract and has been ascribed to various biological activities of the extract, such as its antibacterial, antiviral, and anticarcinogenic properties (*1*−*5*). However, there are very few reports in the literature concerning the absorption, distribution, and excretion of AC. Recently, it was reported that intact AC was readily absorbed by the intestine and gave protection against oxidative stress in vitro, suggesting that AC is the principal bioactive compound in propolis (*6*). As it is considered timely to reevaluate the biological activity and health effects of polyphenols in terms of their bioavailability (*7*), it would be desirable to determine the absorption characteristics and bioavailability of AC in detail.

We have recently elucidated the absorption characteristics of many phenolic acids (i.e. ferulic, *p*-coumaric, gallic, caffeic acids) and related compounds (i.e. chlorogenic, rosmarinic acids) in terms of their affinity for the monocarboxylic acid transporter (MCT) and have demonstrated the diverse nature of the absorption of phenolic compounds in Caco-2 cells (i.e. MCT-mediated absorption, partial MCT-mediated absorption, paracellular diffusion) (*8*−*11*). It has been also shown that the absorption characteristics of these compounds in Caco-2 cells in vitro correlates well with their absorption efficiencies and bioavailabilities in vivo (*12*, *13*). Furthermore, the physiological significance of the microbial metabolites of poorly absorbed parent polyphenols has now been firmly established (*7*); these metabolites are also thought to be absorbed and distributed by MCT, similar to phenolic acids (*14*, *15*). These observations highlight the physiological impact of MCT-mediated absorption and distribution in humans, which involves specific transport systems that act not only for phenolic acids but also for "metabonutrients", that is, microbial metabolites of poorly absorbed polyphenols or dietary fibers having biological activities (*14*, *15*).

The key features of a substrate for MCT are thought to be a monoanionic carboxylic acid group and a nonpolar side chain or aromatic hydrophobic moiety (*16*). Because AC seems to

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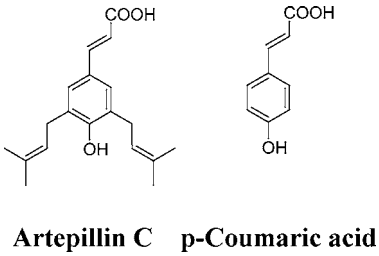

Figure 1. Chemical structures of AC and CA.

fulfill the structural criteria for a MCT substrate (**Figure 1**), we have previously examined whether AC is transported by MCT and have shown that it mainly permeates intact across Caco-2 cells by transcellular passive diffusion with a permeation efficiency as high as those of MCT substrates (*17*). It is generally considered, however, that the first hepatic elimination of hydrophobic compounds that are absorbed by transcellular passive diffusion is high and that their bioavailability is necessarily low. The current study was designed to examine the intestinal absorption efficiency and bioavailability of AC in vivo by means of pharmacokinetics and to compare them with those of the MCT substrate CA.

MATERIALS AND METHODS

Materials. AC, CA, and sulfatase type H-5 were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). The other chemicals used in this study were of analytical grade.

Animals and Diets. Male Wistar rats (7 weeks old, Charles River Japan, Yokohama, Japan) were housed in an air-conditioned room (22 ± 1 °C) under 12-h dark/12-light cycles, with free access to tap water and a commercial nonpurified CE-2 diet (CLEA Japan, Inc., Tokyo, Japan). Three rats to be administered AC or CA were assigned to each time point of each experimental group. This study was approved by the Ethics Committee of Kirin Brewery Co., Ltd.

Sample Preparation. Rats were fasted for 20 h and their body weight was measured (182−213 g). They were given AC or CA (100 μmol/kg in 50% propyleneglycol) by gastric intubation. Blood was withdrawn from the portal vein and abdominal artery at each time point (5, 10, 20, 30, 60, and 90 min) after the administration of AC or CA. Serum was obtained by centrifugation and was stored at −80 °C until analysis.

HPLC−ECD Analysis. An HPLC−ECD fitted with a coulometric detection system was used to measure the amount of AC, CA, and their conjugates in serum samples according to a previously described method (*14*, *15*). In brief, to 25 μL of serum was added 25 μL of 0.1 mol/L sodium acetate buffer (pH 5.0), and 100 μL of 0.83 mol/L acetic acid in methanol. The mixture was vortexed, sonicated, and centrifuged (at 8500*g* for 5 min at 4 °C), and the supernatant was injected onto an HPLC C18 column (ODS150, MC Medical, Inc., Tokyo, Japan). For AC, mobile phase A (solvent A) was 50 mM sodium acetate containing 40% acetonitrile and 20% methanol (pH 3.0), and mobile phase B (solvent B) was 50 mM sodium acetate containing 80% methanol (pH 3.5). The elution profile (0.6 mL/min) was as follows: 0−28.5 min, linear gradient from 85% solvent A/15% solvent B to 0% solvent A/100% solvent B; 28.5−32 min, isocratic elution 0% solvent A/100% solvent B; 32−35 min, isocratic elution 85% solvent A/15% solvent B. Eight electrode detector potentials (from 200 to 760 mV in increments of 80 mV) were used. For CA, mobile phase A (solvent A) was 50 mM sodium acetate containing 5% methanol (pH 3.0), and mobile phase B (solvent B) was 50 mM sodium acetate containing 40% acetonitrile and 20% methanol (pH 3.5). The elution profile (0.6 mL/min) was as follows: 0−28.5 min, linear gradient from 85% solvent A/15% solvent B to 20% solvent A/80% solvent B; 28.5−31 min, isocratic elution 0% solvent A/100% solvent B; 31−35 min, isocratic elution 85% solvent A/15% solvent B. Eight electrode detector potentials (from 0 to 700 mV in increments of 100 mV) were used. The quantitative determination of AC and CA was performed by using an external standard method, which verified that the detector response was linear for concentrations of up to 400 μmol/L for AC and 600 μmol/L for CA.

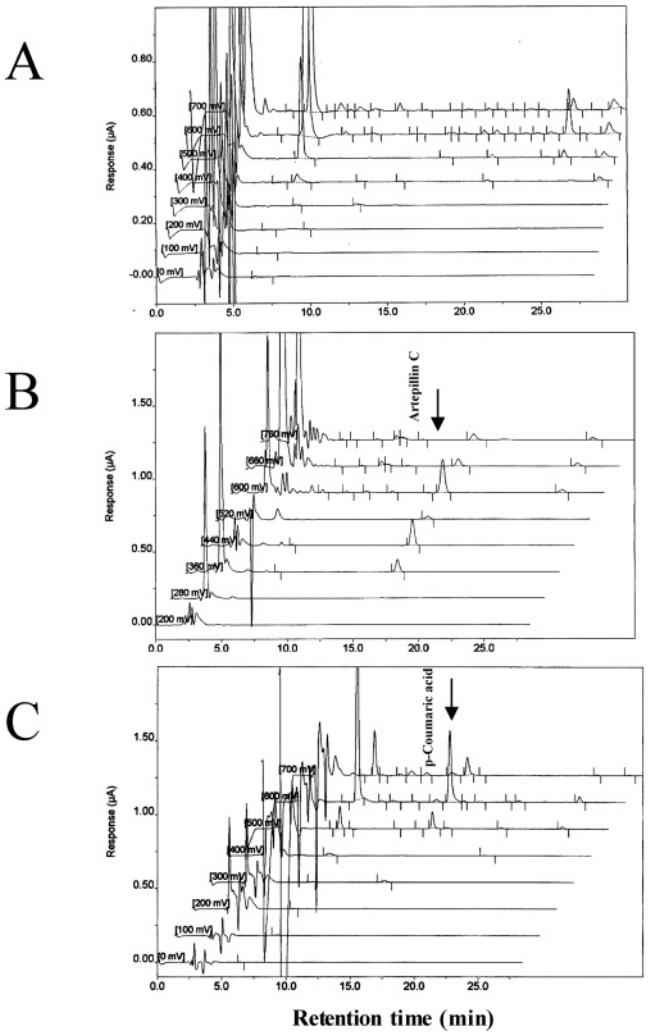

Figure 2. Chromatograms obtained by HPLC–ECD analysis of rat serum before (**A**) and after the administration of AC (**B**) or CA (**C**).

Enzymatic Hydrolysis and Determination of AC or CA Conjugates. Serum (25 μL) was mixed with 25 μL of sulfatase type H-5 solution in 0.1 mol/L acetate buffer (pH 5.0) containing both 12.5 units of sulfatase and about 270 units of β-glucuronidase activity. The mixture was incubated at 37 °C for 45 min. The difference in AC or CA content before and after sulfatase treatment was assumed to be due the amount of the respective sulfate and glucuronide conjugates in the sample.

Data Analysis. Noncompartmental pharmacokinetic parameters were calculated from the serum concentration−time data by using WinNonlin. The measured values were used to determine the maximum serum concentration, C_{max}, and the time, t_{max}, taken to reach C_{max}. The results of C_{max} are expressed as the mean ± SEM of three determinations. The area under the curve (AUC) for the serum concentration−time data from zero to the final sampling time at 1.5 h ($AUC_{0-1.5h}$) was calculated by using the linear/log trapezoidal rule. The elimination half-life ($t_{1/2}$) was calculated from a log−linear regression of the terminal phase of the serum concentration−time profile. AUC and $t_{1/2}$ were calculated by using the mean concentration value at each time point.

RESULTS

Determination of AC and CA in Serum Samples. Figure 2 shows representative HPLC profiles of serum from a control rat (A), and serum from rats given AC (B) and CA (C). On the basis of a comparison in two dimensions (i.e. chromatographic and voltammetric), the identity of the AC or CA peak was

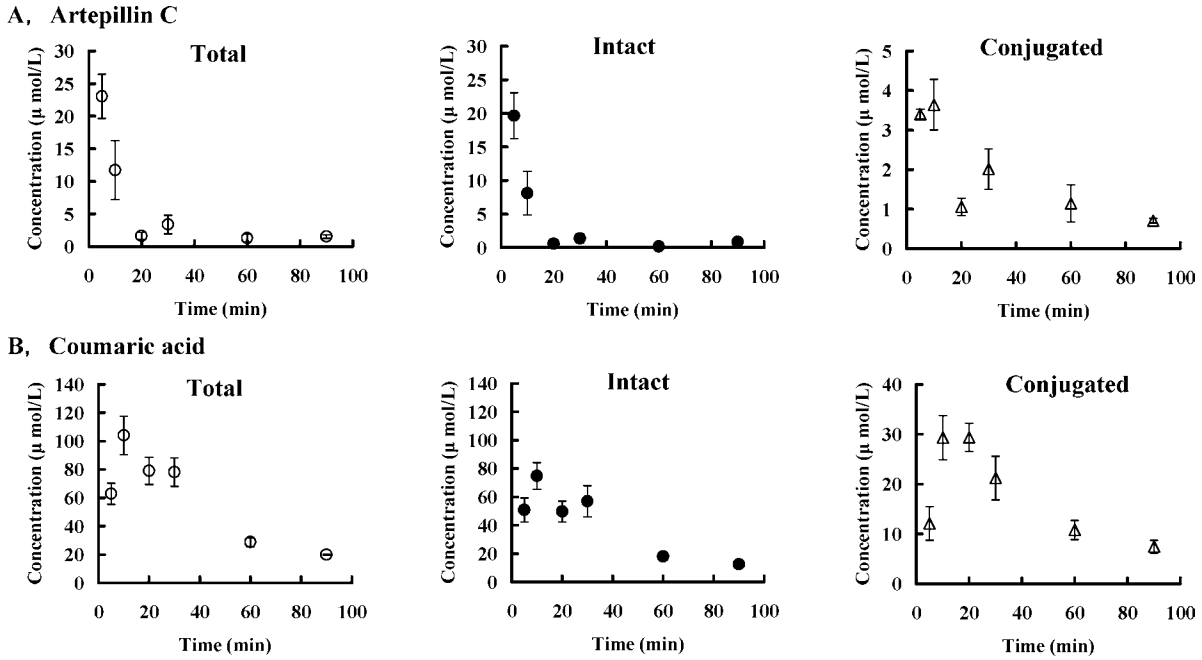

Figure 3. Serum concentration of phenolic acid in the portal vein as a function of time after the administration of AC (**A**) or CA (**B**). Each point is expressed as the mean ± SEM, $n = 3$.

determined by evaluating the peak area ratio for the oxidation channels (lower or upper) adjacent to the dominant oxidation channel. An accuracy in the ratio of more than 70% was considered to support peak purity (*18*). The retention time (t_R) and dominant oxidation potential were, respectively, 16.3 min and 440 mV for AC and 14.9 min and 600 mV for CA. Experiments with AC- or CA-spiked serum showed that this procedure gave more than 97% recovery for both compounds throughout the detection range.

Quantitative Changes in AC, CA, and Their Metabolites in Rat Serum. The mean serum concentrations of AC, CA, and their metabolites of sulfate and glucuronide in the portal vein as a function of time after administration are shown in **Figure 3**. The concentrations of total and intact AC and CA were measured after and before deconjugation with sulfatase treatment, and the results of the noncompartmental pharmacokinetics analysis are given in **Table 1**. The intestinal absorption of intact AC and CA was fast: the first and second peak levels were 5 min (19.67 μmol/L) and 30 min (1.39 μmol/L) for AC and 10 min (74.75 μmol/L) and 30 min (56.94 μmol/L) for CA. There was a difference in the AUC in the portal vein (AUC_{portal}) calculated for intact AC and CA. The absorption efficiency of CA was estimated to be 17-fold greater than that of AC. This finding shows that the intestinal absorption efficiency of CA is much higher than that of AC (**Table 1**).

Furthermore, the concentration of AC, CA, and their metabolites in the abdominal artery was investigated as a function of time after administration to clarify hepatic elimination (**Figure 4**), and the results of the noncompartmental pharmacokinetics analysis are also given in **Table 1**. At all time points after administration, there was much less intact AC in the abdominal artery than in the portal vein. In contrast, a large amount of intact CA was detected in the abdominal artery at all time points after administration, and the concentration of CA in the abdominal artery showed the same trend over time as that in the portal vein. Intriguingly, the AUC in the abdominal artery ($AUC_{abdominal}$) for CA was about 278-fold greater than that for AC (**Table 1**).

Table 1. Pharmacokinetic Parameters of Intact AC and CA in the Portal Vein and Abdominal Artery after the Administration of a Single Oral Dose of 100 μmol/kg AC or CA[a]

	AC	CA
Portal Vein		
C_{max},[b] μmol/L	19.67 ± 3.43	74.75 ± 9.27
t_{max}, min	5	10
$AUC_{0-1.5h}$, μmol·min·L^{-1}	182.6	3057.3
$t_{1/2}$, min	86.6	27.5
relative absorption efficiency	16.7	
Abdominal Artery		
C_{max}, μmol/L	0.55 ± 0.12	47.36 ± 9.54
t_{max}, min	5	10
$AUC_{0-1.5h}$, μmol·min·L^{-1}	7.72	2143.0
$t_{1/2}$, min	23.2	27.5
relative bioavailability	277.6	

[a] Abbreviations: AC, artepillin C; CA, *p*-coumaric acid; C_{max}, maximum serum concentration; t_{max}, time to reach the C_{max}; AUC, area under the serum concentration–time curve; $t_{1/2}$, elimination half-life. Relative absorption efficiency was calculated as follows: AUC_{portal} of CA/AUC_{portal} of AC. Relative bioavailability was calculated as follows: $AUC_{abdominal}$ of CA/$AUC_{abdominal}$ of AC. [b] Values of C_{max} are the mean ± SEM, $n = 3$.

The concentration of conjugated AC in the abdominal artery was also much lower than that in the portal vein, although the concentration of conjugated AC in the abdominal artery exhibited the same trend over time as that in the portal vein (**Figures 3** and **4**). In contrast, the concentration profile of conjugated CA in the abdominal artery was nearly the same as that in the portal vein. The noncompartmental pharmacokinetics analysis of conjugated AC and CA is shown in **Table 2**. The AUC and C_{max} of conjugated CA in the portal vein were nearly the same as the corresponding values in the abdominal artery, whereas the values of AUC and C_{max} of conjugated AC were much lower in the abdominal artery than those in the portal vein (**Table 2**).

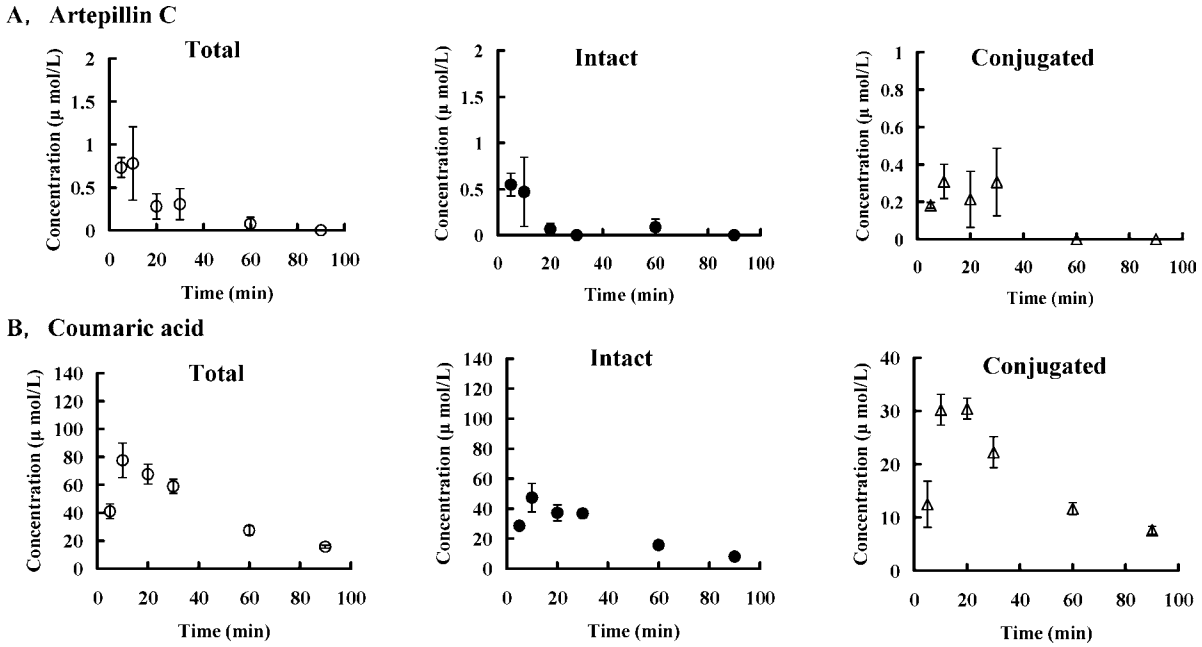

Figure 4. Serum concentration of phenolic acid in the abdominal artery as a function of time after the administration of AC (**A**) or CA (**B**). Each point is expressed as the mean ± SEM, $n = 3$.

Table 2. Pharmacokinetic Parameters of AC and CA Conjugates in the Portal Vein and Abdominal Artery after the Administration of a Single Oral Dose of 100 μmol/kg AC or CA[a]

	AC	CA
Portal Vein		
C_{max},[b] μmol/L	3.64 ± 0.64	29.36 ± 2.83
t_{max},min	10	20
$AUC_{0-1.5h}$, μmol·min·L^{-1}	135.4	1410.7
$t_{1/2}$, min	40.0	39.8
Abdominal Artery		
C_{max}, μmol/L	0.31 ± 0.09	30.43 ± 1.94
t_{max},min	10	20
$AUC_{0-1.5h}$, μmol·min·L^{-1}	6.8	1477.8
$t_{1/2}$, min	nc	38.6

[a] Abbreviation: AC, artepillin C; CA, *p*-coumaric acid; C_{max}, maximum serum concentration; t_{max}, time to reach the C_{max}; AUC, area under the serum concentration–time curve; nc, not calculated. [b] Values of C_{max} are the mean ± SEM, $n = 3$; $t_{1/2}$, elimination half-life.

DISCUSSION

In this study, we have demonstrated that the in vivo absorption efficiency of intact AC is lower than that of intact CA, indicating that there is a specific difference in the absorption characteristics of these two phenolic acids in vivo. Indeed, the absorption characteristics of AC are different from those of CA in Caco-2 cells (i.e. transcellular passive diffusion for AC versus MCT-mediated active transport for CA) (*9*, *17*). However, the absorption efficiency of intact AC and CA in vivo is apparently different from that observed in the in vitro Caco-2 cell system, because the absorption efficiency of AC was found to be as high as that of an MCT substrate such as CA in Caco-2 cells (*17*), in contrast to the lower absorption efficiency of rosmarinic and gallic acids, which are absorbed by paracellular diffusion (*9*, *11*). It is possible that this discrepancy in absorption efficiency between in vivo and in vitro studies might originate from such differences as an unstirred water layer, a mucin layer, or some other feature of the evaluation method employed. Further, the difference of absorption efficiency in stomach between CA and AC might cause this discrepancy. Indeed, it was reported that MCT expression was detected in mouse stomach (*19*) and that both transport systems for ferulic acid via transcellular passive diffusion and MCT-mediated absorption might operate (*20*), but at present the exact reason is unknown.

For gut absorption of dietary polyphenols, it is generally considered that the partition coefficient seems to govern their permeation across epithelial cells, because only passive diffusion appears to be involved (*21*). Indeed, it was reported that the lipophilicity of flavonoids and their affinity for liposomal membranes were well-correlated with their absorptivity into Caco-2 cells (*22*). Quercetin, one of the most typical and prevalent flavonoids in the human diet, and its glucosides were taken up into Caco-2 cells according to their own liphophilicity and were further conjugated across the epithelium (*23*). It was also reported that quercetin glucosides might be taken up intracellularly by SGLT1 (*24*) and transported by efflux into the intestinal lumen by MRP2 (*25*), indicating that the absorption and metabolism of quercetin and its glucosides are complex. Nevertheless, it is reasonable to assume that quercetin permeates across epithelial cells by transcellular passive diffusion (*22*), although a precise analysis of its absorption characteristics has not been performed, as it has in the case of AC (*17*). The same mechanism appears to govern the transport of isoflavonoids such as genistein and diazein, although isoflavones are transported as intact aglycons more efficiently than as flavonoids owing to their steric structure (*26*). In contrast to the deduced absorption characteristics in vitro, the absorption efficiency of quercetin or daizein in vivo is low in human or rat (*27*−*29*), which is in keeping with our findings for AC in this study. It is possible that an unknown but specific elimination mechanism might be involved in the low absorption efficiency in vivo to preserve homeostasis, because it would be harmful to living things if a xenobiotic compound with affinity for biomembranes could permeate across the epithelium easily and enter into enterocytes.

Furthermore, we also have demonstrated that the in vivo bioavailability of intact AC is much lower than that of intact CA. We found that the ratio of $AUC_{abdominal}$ to AUC_{portal} for intact AC and CA was 0.04 and 0.70, respectively, which suggests that intact AC is much more susceptible than intact

Table 3. Absorption Characteristics, Absorption Efficiency, and Bioavailability of Phenolics[a]

	CA[b]	CFA[c]	GA or RA[d]	AC[e]
phenolic characteristics	MCT-mediated active transport	paracellular diffusion[f]	paracellular diffusion	transcellular diffusion
efficiency	high	low	low	low
bioavailability	high	low	low	low

[a] Abbreviation: CA, p-coumaric acid; CFA, caffeic acid; GA, gallic acid; RA, rosmarinic acid; AC, artepillin C. [b] From refs *9*, *12*. [c] From refs *10*, *13*. [d] From refs *9*, *11*–*13*. [e] From ref *17*. [f] MCT-mediated active transport, in part.

CA to hepatic elimination. It is likely that a mechanism for eliminating AC in vivo might be involved, as mentioned above.

Considerable amounts of AC and CA conjugates were observed in the portal vein (**Figure 3**), although no AC and CA conjugates were transported in Caco-2 cells (*9*, *17*). This finding indicates that conjugation of AC and CA occurs during their permeation across the rat epithelium, consistent with results obtained in our previous in vivo studies (*12*, *13*). The discrepancy in conjugate formation between the in vivo and the in vitro Caco-2 cell system also might originate from differences in the evaluation method, as discussed above for the absorption efficiency or bioavailability. The $AUC_{abdominal}$ of conjugated AC was much lower than the AUC_{portal} of conjugated AC (ratio 0.05), and similar results were obtained for intact AC. This finding also suggests that AC may be eliminated by a specific mechanism. In contrast, because the AUC_{portal} of conjugated CA was nearly the same as the $AUC_{abdominal}$ of conjugated CA (**Table 2**), it is possible that CA is conjugated mainly during the absorption process and that further conjugation does not occur in the liver.

In this study, apart from the propyleneglycol concentration of the vehicle, which was 50% due to the lipophilicity of AC, the other experimental conditions were exactly the same as those used in previous in vivo studies (propyleneglycol concentration 10%). It has been reported that the vehicle used for oral administration can affect the absorption efficiency of flavonoids (*30*). Indeed, there are differences in the C_{max} values and concentration profiles of CA between this study and previous in vivo studies (*12*), but the AUC and t_{max} values were almost constant, indicating the high absorption efficiency and bioavailability of CA, similar to the results of the previous in vivo study. These results, together with a series of our previous works (*9*−*13*, *17*), have been used to summarize the diversity of absorption characteristics, absorption efficiency, and bioavailability of dietary phenolic compounds in **Table 3**, highlighting the unique physiological significance of the MCT-mediated transport system. We have focused on the physiological impact of MCT-mediated absorption and distribution in humans, which involves specific transport systems that act not only for phenolic acids but also for microbial metabolites of poorly absorbed polyphenols or dietary fibers (*14*, *15*). It is highly desirable to assess in full the health effects of phenolic acids and "metabo-nutrients", in other words, microbial metabolites with the biological activities of polyphenols or dietary fibers (*15*).

In conclusion, we have demonstrated that the absorption efficiency and bioavailability of AC are extremely low in vivo, in comparison to those of CA, which is absorbed and distributed by the MCT-mediated transport system. To evaluate the health effect of AC or propolis, further identification and characterization of its bioactive compounds in vivo are required.

ABBREVIATIONS USED

MCT, monocarboxylic acid transporter; AC, artepillin C; CA, *p*-coumaric acid; ECD, electrochemical detector.

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