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Comparative assessment of distribution of blackcurrant anthocyanins in rabbit and rat ocular tissues

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Abstract

Anthocyanins (ACs) are phenolic compounds that are distributed widely in fruits and vegetables. Although consumption of these compounds has been shown to improve visual function, the distribution of ACs in ocular tissue has not been examined in detail. The aim of this study was therefore to evaluate the ocular distribution of blackcurrant anthocyanins (BCAs) in rats and rabbits after oral, intravenous (i.v.) and intraperitoneal (i.p.) administration. Identification and quantification of ACs were carried out using high-performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-ESI-MS) and high-performance liquid chromatography (HPLC) with UV-visible detection, respectively. BCAs were identified in the plasma and whole eye after oral and i.p. administration in rats. No other peaks were detected in either plasma or ocular tissues after administration when the absorbance of the eluate was monitored at 520 nm. This finding indicates that intact forms of ACs were present in rats after administration of BCA. In rats given i.p. administration, the concentration of total ACs in the whole eye and some ocular tissues was higher than that measured in plasma. These results suggested that ACs detected in the ocular tissues were not due to residual blood. Following i.v. administration in rabbits, four ACs were identified in the plasma and several ocular tissues including the aqueous humor, cornea, sclera, choroid, ciliary body, iris and retina. A small amount of ACs was also detected in the vitreous and lens. In conclusion, this study demonstrated that BCAs were absorbed and distributed in ocular tissues as intact forms. Our data show clearly that intact forms of BCAs pass thorough the blood–aqueous barrier and blood–retinal barrier in both rats and rabbits.

Keywords: blackcurrant; anthocyanin; ocular distribution; ciliary body; sclera; retina

1. Introduction

Anthocyanins (ACs) are a group of naturally occurring phenolic compounds responsible for the color of many flowers, fruits (particularly berries) and vegetables. Due to their widespread distribution and occurrence in fruits and vegetables, the daily intake of ACs in humans in the United States has been estimated to be 200 mg/day (Kühnau, 1976). Dietary ACs have attracted considerable interest due to their health-promoting benefits, such as reducing the risk of coronary heart disease and preventing several chronic diseases (Renaud and de Lorgeril, 1992).

Blackcurrant (*Ribes nigrum* L.) berries and juice are rich in ACs and are consumed in many countries of the world. The composition of blackcurrant anthocyanins (BCAs) is summarized in Fig. 1 and consists of four AC components. The typical AC profile in blackcurrant fruits is 47% of delphinidin-3-rutinoside (D3R), 13% of delphinidin-3-glucoside (D3G), 35% of cyanidin-3-rutinoside (C3R) and 5% of cyanidin-3-glucoside (C3G). These four ACs have been isolated and purified (Matsumoto et al., 2001a) and we have demonstrated in both humans and rats that they are absorbed through the gastrointestinal tract and are detectable in blood as unmetabolized forms (Matsumoto et al., 2001b). The bioavailability and metabolism

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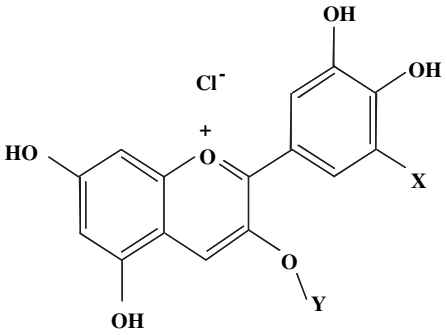

Fig. 1. Structure of the four anthocyanins in blackcurrants. Delphinidin-3-rutinoside (D3R; X = OH, Y = glucose-rhamnose), delphinidin-3-glucoside (D3G; X = OH, Y = glucose), cyanidin-3-rutinoside (C3R; X = H, Y = glucose-rhamnose) and cyanidin-3-glucoside (C3G; X = H, Y = glucose).

of ACs has been reported in several studies and although these mainly investigated cyanidin-glycosides, ACs were shown to be absorbed only as intact forms in human plasma. Neither cyanidin aglycone, conjugated form nor methylated anthocyanins were detected (Miyazawa et al., 1999; Felgines et al., 2003; Morazzoni et al., 1991; Mulleder et al., 2002). Recently, several metabolites were reported to form after oral ingestion of anthocyanins. These metabolites include a glucuronyl conjugate from C3G and both glucuronyl conjugate (Wu et al., 2002) and sulfate conjugate from pelargonidin (Felgines et al., 2003). However, the intake of D3G, D3R or C3R did not produce any glucuronides in rats (Ichiyanagi et al., 2004).

Recently, we reported a series of studies that showed oral intake of BCAs prevented myopic refractory shift caused by work on visual display terminals (VDT) (Nakaishi et al., 2000). In addition, we demonstrated that D3R, the main component of BCAs, has a relaxing effect on bovine ciliary smooth muscles (Matsumoto et al., 2005). Ciliary muscle relaxation has been studied extensively in the search for drug therapies to treat both myopia and glaucoma (Beauregard et al., 2001). We have also reported that C3G and C3R stimulate the regeneration of rhodopsin in frog rod outer segment (ROS) membranes (Matsumoto et al., 2003). Although ACs are widely available in the United States and Japan as nutritional supplements for improving visual function, their bioavailability in ocular tissue has not been studied. In order to clarify the physiological ability of BCAs to improve visual function, we investigated the ocular absorption, distribution and elimination of BCAs in rats after oral and intraperitoneal (i.p.) administration and in rabbits after i.v. administration.

2. Materials and methods

2.1. Chemicals and solutions

The BCA powder was prepared from commercial blackcurrant juice by the methods described in our previous report (Matsumoto et al., 2001a). The total AC content in the BCA was 21.6%, consisting of D3R (10.2%), C3R (7.5%), D3G (2.9%) and C3G (1.0%) (Fig. 1).

2.2. Oral administration in rats

Thirty-five male Wistar rats aged 8 weeks with a mean body weight of 218.38 ± 2.82 g were obtained from Clea Japan Co., Ltd. (Tokyo). The rats were housed individually in stainless-steel wire-mesh cages at 23 ± 2 °C with a 12-h light–dark cycle. The animals were given free access to tap water and a commercial diet (MF, Oriental Yeast. Co. Ltd, Tokyo, Japan). All rats were handled in accordance with the NIH Guide for the Care and Use of Laboratory Animals. After a 1 week feeding period, food was withheld for 12 h. The rats were then assigned randomly to seven groups. BCA powder dissolved in physiological saline (23.15 mg/ml) was administered orally to the rats in the designated group by direct stomach intubation at a dosage of 463 mg BCA powder per kg body weight (100 mg ACs per kg body weight). Five rats were killed at each of the following time points after administration of BCA (0 min, 30 min, 1 h, 2 h, 4 h, 8 h and 24 h) by withdrawing blood from the inferior vena cava under diethyl ether anesthesia using a heparinized needle and syringe. The whole eyes were then enucleated, with both eyes from each animal being pooled. In order to minimize AC contamination from blood, the blood samples were withdrawn from a vessel on the sclera. The plasma and eyes were used to determine the AC levels at various times after administration of BCAs.

2.3. Intraperitoneal administration in rats

Thirty-five male Wistar rats aged 9 weeks, with a mean body weight of 255.24 ± 4.35 g (Clea Japan) were used for these experiments. The rats were housed using the same domestication procedures as in the oral administration study. After the feeding period, food was withheld for 12 h. BCA powder dissolved in physiological saline (500 mg/10 ml) was then administered intraperitoneally at a dosage of 500 mg BCA powder per kg body weight (108 mg AC per kg body weight). Five rats were killed at each of the following time points after administration of BCAs (0 min, 30 min, 1 h, 2 h, 6 h and 24 h). Blood and whole eye samples were collected for measurement of ACs using the same procedures as in the oral administration study.

To examine the distribution of ACs in ocular tissue, the remaining five rats were killed at 60 min post-administration by withdrawing blood under diethyl ether anesthesia from the inferior vena cava using a heparinized needle and syringe. The aqueous humor was aspirated into a sterile syringe after enucleation of the eye globe and then acidified with a 1/40 volume of 6 N HCl. The aqueous humor samples from both eyes of the animals were pooled from each time point and kept at 5 °C for measurement of ACs. The eyes were then dissected carefully into six parts (cornea, sclera with choroid, ciliary body with iris, retina, vitreous and lens). The plasma and corresponding tissues of both eyes from each animal were pooled, with the

tissues being used to measure ACs in the different eye tissues at various times after administration of BCAs.

2.4. Intravenous administration in rabbits

A total of 16 Japan White male rabbits, weighing 1.42 ± 0.02 kg were assigned randomly to six groups (five groups consisted of three rabbits and the control group of one rabbit). The rabbits were fed a commercial diet (Laboratory Rabbit HF EQ 5L95, Japan SLC Co. Ltd, Hamamatsu, Japan) under the same domestic conditions used in the rat study. All the rabbits except for the control received 92.6 mg/kg body weight of BCA (AC 20 mg/ kg body weight) solution in 1 ml physiological saline via an ear vein. Blood samples were collected at 30 min, 1 h, 2 h, 4 h and 24 h post-administration from the inferior vena cava under pentobarbital anesthesia using a heparinized needle and syringe. The animals were then sacrificed by withdrawing blood from the carotid artery, followed by enucleation of the eye globe and aspiration of the aqueous humor into a sterile syringe. The aqueous humor samples were acidified with a 1/40 volume of 6 N HCl, with the samples from both eyes of each animal being pooled from each time point and stored at 5 °C for measurement of ACs. The eyes were then dissected carefully into eight parts (cornea, sclera, choroid, ciliary body, iris, retina, vitreous and lens). The plasma and corresponding tissues of both eyes from each animal were pooled and used to determine AC levels in the different eye tissues at various times after administration of BCAs.

2.5. Sample preparation

Plasma samples were prepared using minor modifications of the method of Tsuda et al. (1999). Both rat and rabbit plasma was obtained immediately from the blood samples by centrifugation at 1600 × *g* for 15 min at 4 °C. Separation of the plasma was completed within 30 min and was then acidified with a 1/40 volume of 6 N HCl. A 400 µl aliquot of rat plasma was mixed with 400 µl of 10 mmol/l oxalic acid. In addition, each sample of the acidified plasma was applied to Sep-Pak C_{18} cartridges (Waters, Milford, MA), which had been washed with 10 ml of methanol containing 5% trifluoroacetic acid (TFA) and equilibrated with 10 ml of 10 mmol/l oxalic acid prior to use. After washing with 10 mmol/l oxalic acid, the ACs were eluted with methanol containing 5% TFA, and the eluate evaporated carefully to dryness in vacuo below 35 °C. The dried residue was redissolved in 200 µl of 3% phosphoric acid and a 20 µl aliquot of this solution was then injected into an HPLC system for AC analysis. The method of AC analysis in plasma was validated by performing a recovery test (Matsumoto et al., 2001b). To determine the AC content of either the whole eye or in each of the ocular tissues, the tissues were weighed, minced gently with scissors and then homogenized in 10 volumes of an 80% methanol–HCl solution using a Polytron homogenizer (Kinematica AG, Switzerland). The homogenates were centrifuged and the supernatant was applied to ACs analysis using the same procedure as the plasma samples. The aqueous humor samples were acidified with 10 volumes of the 80% methanol–HCl solution and the ACs analyzed using the same procedure as the plasma samples.

In an earlier study we validated the method of AC measurement in plasma by performing a recovery test (Matsumoto et al., 2001b). In the present study, the extraction recovery in ocular tissues was also determined. AC solutions were prepared by dissolving BCA powder (15 µg/ml, 1.5 µg/ml and 0.15 µg/ml) in the homogenate of each tissue (500 µl) obtained from an untreated rat or rabbit. The resulting solutions were processed in an identical manner as tissues from the AC-treated rats or rabbits. HPLC analysis of the extracts showed that the recovery of ACs from aqueous humor, cornea, sclera and vitreous was greater than 90%, while recovery from the iris, ciliary body and retina was greater than 80%. The choroid and lens showed good linearity but the worst recovery at 33% and 65%, respectively. In this study, correction factors were therefore not applied to the data.

2.6. Determination of anthocyanins

Total ACs were calculated as the sum of the individual ACs. Identification and quantification of the four ACs were performed using an HP 1100 series high-performance liquid chromatography (HPLC) system (Hewlett Packard, Palo Alto, CA) equipped with a Zorbax SB C-18 column (250 × 4.6 mm ID, particle size 5 µm) and a photodiode array detector at 520 nm (Matsumoto et al., 2001a). Injection was carried out using an autosampler with a 100 µl fixed loop. Elution was performed using a solvent system consisting of a mixture of solvent A (0.5% phosphoric acid) and solvent B (methanol), applied at a flow rate of 1.0 ml/min as a linear gradient from 80% A/20% B (v/v) to 77% A/23% B (v/v) for 15 min, then held at 77% A/23% B (v/v) for a further 8 min. The eluted constituents were identified by measuring the photodiode array UV-vis spectra from 200 to 600 nm. The areas of the peaks of D3R, C3R, D3G, and C3G were proportional to the amounts injected within the range 0.2–400 ng, with a detection limit of 1 ng in each instance.

Mass spectrometry (MS)-analysis was carried out using a minor modification of the method of Felgines et al. (2003). The samples were analyzed using an HP-1100 series HPLC connected to a mass selective detector with an atmospheric pressure ionization-electrospray (API-ES) ionization chamber. A 5 µl aliquot of each solution was applied to a -Capcellpack UG120 column (Shiseido, Tokyo, Japan; 150 × 2.0 mm ID, particle size 5 µm). The solvents used were (A) 2% formic acid in water and (B) 2% formic acid in acetonitrile. Elution was carried out at a flow rate of 0.2 ml/min using a linear gradient from 5 to 20% (v/v) B for 20 min, followed by isocratic elution with 20% (v/v) B for 5 min. Mass spectrometric data of the column eluent were acquired in the positive mode for ACs. In the API-ES method, the eluted compounds were mixed with nitrogen in the heated nebulizer interface and polarity was tuned to positive. Adequate calibration of the ES parameters (needle potential, gas

temperature, nebulizer pressure, and fragmentator voltage) was required in order to optimize the response and to obtain a high sensitivity for the molecular ion. The selected values were as follows: capillary voltage, 4000 V; drying gas temperature, 350 °C; drying gas flow, 11 ml/min; nebulizer pressure, 40 psig; fragmentor voltage, 115 V.

3. Results

3.1. Oral administration in rats

The plasma and whole eye concentrations of ACs were measured by HPLC after oral administration of BCA powder (100 mg/kg body weight). Four blackcurrant ACs were detected as intact forms in both plasma and the whole eyes. Only intact ACs peaks were detected in the analyses of rat plasma and whole eyes after oral administration when absorbance of the eluate was monitored at 520 nm (Fig. 2). Fig. 3 shows the time-course of changes in total AC concentration in rat plasma (Fig. 3(A)) and whole eyes (Fig. 3(B)) after oral administration of BCA powder. None of these ACs were detected in the plasma prior to administration of the powder. Following oral administration there was a rapid increase in the plasma concentration of total ACs that reached a maximum of 1.94 ± 0.44 μg/ml (C_{max}) after 30 min (T_{max}), followed by a gradual decrease. The concentration of total ACs in the whole eye also reached a maximum of 115 ± 32 ng/g (C_{max}) after 30 min (T_{max}) administration and then decreased. The $t_{1/2}$ of ACs in the plasma and whole eye were 1.4 and 1.1 h, respectively. The $AUC_{0\text{-inf}}$ of ACs in the plasma and whole eye were 2.56 μg h/ml and 0.23 μg h/g, respectively.

3.2. Intraperitoneal administration in rats

The plasma levels of ACs after i.p. administration in rats are shown in Fig. 4(A). The mean maximum plasma concentration of total ACs reached 2.30 ± 0.76 μg/ml (C_{max}), after 1 h (T_{max}). Thereafter the decrease in plasma AC levels was found to be slower than with oral administration.

The concentration of total ACs in the whole eyes was twice as high as the plasma AC concentration throughout the study, and reached a maximum mean concentration of 4.99 ± 0.48 μg/g (C_{max}) 2 h post-administration (T_{max}) (Fig.4(B)). The $t_{1/2}$ of ACs in the plasma and whole eye were 2.8 and 2.6 h, respectively. The $AUC_{0\text{-inf}}$ of ACs in the plasma and whole eye were 12.3 μg h/ml and 25.0 μg h/g, respectively.

The ocular distribution of ACs following i.p. administration is summarized in Table 1 as mass concentration and as percent of total ACs in ocular tissues. Sixty minutes after administration, the majority of ACs (93%) were found in connective tissue, such as the sclera with choroid (245.04 μg/g fresh tissue) and cornea (20.62 μg/g fresh tissue). These concentrations were markedly higher than the levels of ACs in plasma

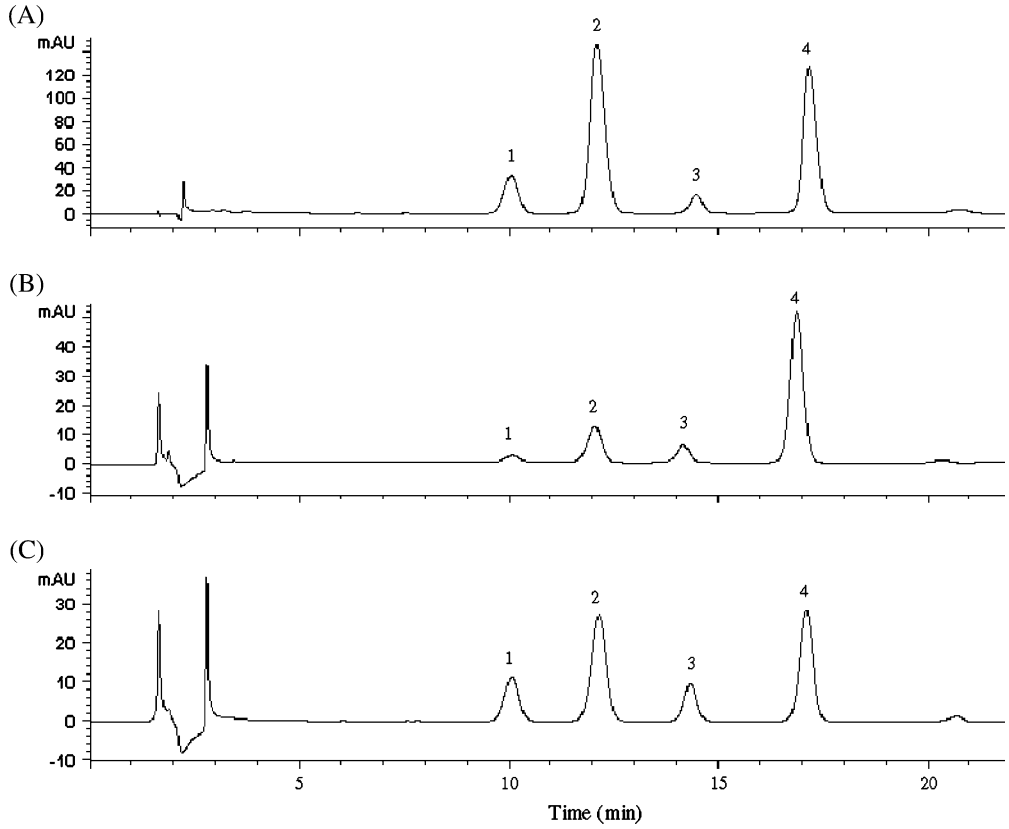

Fig. 2. Representative HPLC chromatograms of BCA (A), rat plasma (B) and rat whole eyes (C) after oral administration of BCA. Detection was performed at 520 nm. Peaks are as follows: 1, D3G; 2, D3R; 3, C3G; 4, C3R.

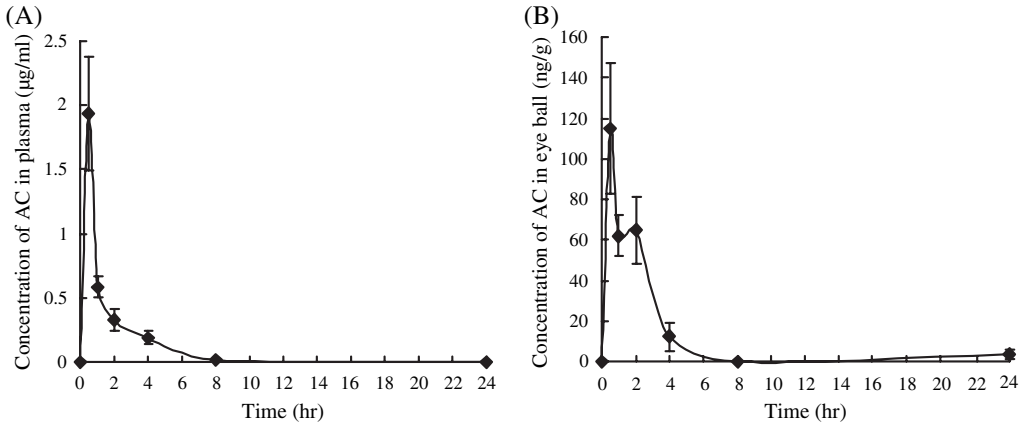

Fig. 3. Time course of changes in AC concentration after oral administration in rat. Values are means ± SE of five rats. (A) plasma AC concentration (B) whole eye AC concentration.

(2.30 μg/ml). The level of ACs in the ciliary body with iris (12.93 μg/g fresh tissue) was also higher than plasma levels, whereas the AC content of the aqueous humor (6.72 μg/g fresh tissue) and retina (6.89 μg/g fresh tissue) was similar to plasma levels. Only small amounts of ACs were found distributed in the vitreous (0.60 μg/g fresh tissue) and lens (0.36 μg/g fresh tissue).

3.3. Intravenous administration in rabbits

As in the rat, rabbit plasma and ocular tissue contained no detectable ACs prior to intake of BCA. Fig. 5A shows the time-course of changes in plasma total AC concentration after i.v. administration of BCA, while Fig. 5(B) shows the associated changes in each ocular tissue of the rabbits. The distribution rate in ocular tissue is summarized in Table 1. As in the rat i.p. study, we found ACs were distributed mainly in the sclera (3.02 μg/g fresh tissue) and choroid (3.00 μg/g fresh tissue) 30 min after administration. However, these concentrations were lower than the plasma level of 12.42 μg/ml. The distribution rates in the sclera and choroid (total 53.3%) were also lower than that measured in the rat i.p. study (89.09%). The AC levels in the ciliary body (2.04 μg/g fresh tissue), aqueous humor (1.19 μg/g fresh tissue) and iris (1.11 μg/g fresh tissue) were higher than in the rat i.p. study. On the other hand, the AC levels in the retina (0.27 μg/g fresh tissue) and cornea (0.55 μg/g fresh tissue) were lower than in the rat i.p. study. Only trace amounts were detected in the vitreous (0.11 μg/g fresh tissue), while no ACs were found in the lens. Twenty-four hours after i.v. administration no ACs were detected in any tissue in rabbit.

Table 2 summarizes the time of observed maximum concentration (T_{max}), maximum concentration (C_{max}), elimination half-life ($t_{1/2}$), total area under the concentration-time curve (AUC) and AUC ratio (tissue/plasma and tissue/aqueous humor) of ACs in the aqueous humor and ocular tissues. The $t_{1/2}$ of ACs in the aqueous humor and eye tissues ranged between 0.5 and 5.6 h. Of all the tissues examined, AUC was

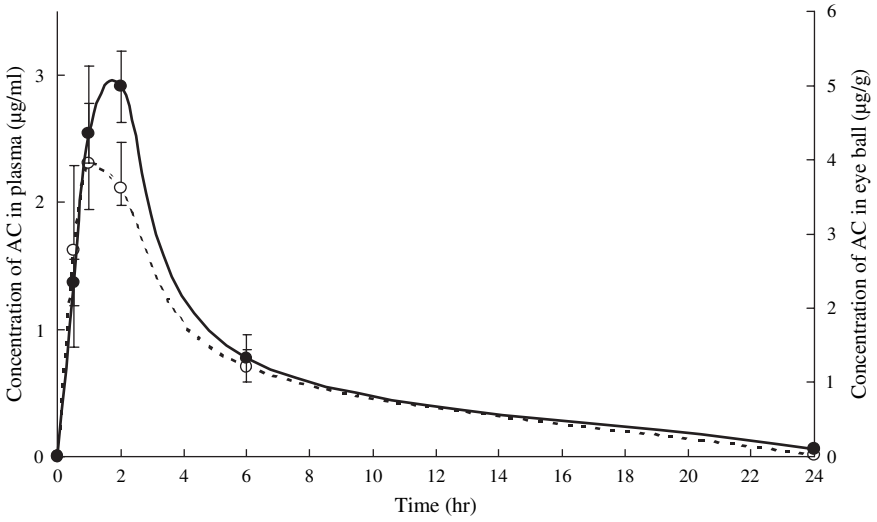

Fig. 4. Time course of changes in AC concentration after intraperitoneal administration in rat. Values are means ± SE of five rats. Open circle, plasma AC concentration; closed circle, whole eye AC concentration.

Table 1
The ocular particular distribution of AC at 1 h post-intraperitoneal administration of 500 mg/kg of BCA (equivalent to 108 mg/kg of AC) in rats and at 30 min post-intravenous administration of 20 mg/kg of BCA (equivalent to 4.32 mg/kg of AC) in rabbits. Mean ± SE of five rats per group, and of three rabbits per group

Ocular tissues or body fluid	Intraperitoneal administration in rat		Intravenously administration in rabbit	
	AC of tissue (μg/g tissue)	Distribution ratio (%)	AC of tissue (μg/g tissue)	Distribution ratio (%)
Aqueous humor	6.72	0.88	1.19 ± 0.21	10.54
Cornea	20.62	3.67	0.55 ± 0.05	4.89
Sclera with Choroid	245.04	89.09	3.02 ± 0.09 (Sclera)	26.73
			3.00 ± 0.06 (Choroid)	26.57
Ciliary body with Iris	12.93	1.39	2.04 ± 0.28 (Ciliary body)	18.07
			1.11 ± 0.08 (Iris)	9.81
Retina	6.89	4.76	0.27 ± 0.02	2.41
Vitreous	0.60	0.14	0.11 ± 0.02	0.98
Lens	0.36	0.06	0.00 ± 0.00	0.00
Plasma (μg/ml)	2.30 ± 0.76		12.42 ± 1.25	

highest in the choroid (9.08 μg h/g), with values of 7.22, 4.69 and 4.35 μg h/g tissue being found in the sclera, ciliary body and cornea respectively. These values were considerably higher than that measured in the aqueous humor (2.61 μg h/g). The iris and retina had AUC values of 1.98 and 0.84 μg h/g tissue, respectively. A low AUC value was observed in the vitreous (0.38 μg h/g), while no ACs were detected in the lens. After 30 min C_{max} was seen in the sclera (3.02 μg/g) and the choroid (3.00 μg/g), followed by the ciliary body (2.04 μg/g) and aqueous humor (1.19 μg/g). After 1 h C_{max} in the iris was 1.18 μg/g. Mean C_{max} values 30 min after application of the BCAs were 0.55 μg/g in the cornea, 0.27 μg/g in the retina, and 0.11 μg/g in the vitreous. These values were lower than in the aqueous humor. T_{max} in the majority of the eye tissues was observed at 30 min with the exception of the iris, in which it occurred at 1 h.

3.4. Identification of ACs

HPLC-ESI-MS was used to identify the ACs in plasma and ocular tissues. Fig. 6 shows the LC-DAD profile at 520 nm (A) and ion chromatograms (B) of the ciliary body. All other ocular tissues, plasma and standard anthocyanins had the same LC-DAD profiles (data not shown). The presence of small amounts of D3G, D3R, C3G and C3R was confirmed by identification of the respective parent and product ion pairs with those of standard anthocyanins (Matsumoto et al., 2001a) (*m/z* values: 465/303, 611/303, 449/287 and 595/287, respectively.

4. Discussion

ACs are widely available as nutritional supplements for visual function. However, there are no published reports on the distribution of ACs in ocular tissue following ingestion by any route. This study in rat and rabbit investigated the ocular absorption, distribution and elimination of ACs following i.p., i.v., and oral administration. These compounds were identified in both plasma and whole eyes as intact forms in studies on oral and i.p. administration of BCAs in rats. In an i.v. administration study in rabbits, we also identified ACs in the plasma and several ocular tissues including the cornea, aqueous humor, iris, ciliary body, choroid, sclera and retina. These results suggested that ACs accumulate in these ocular tissues after

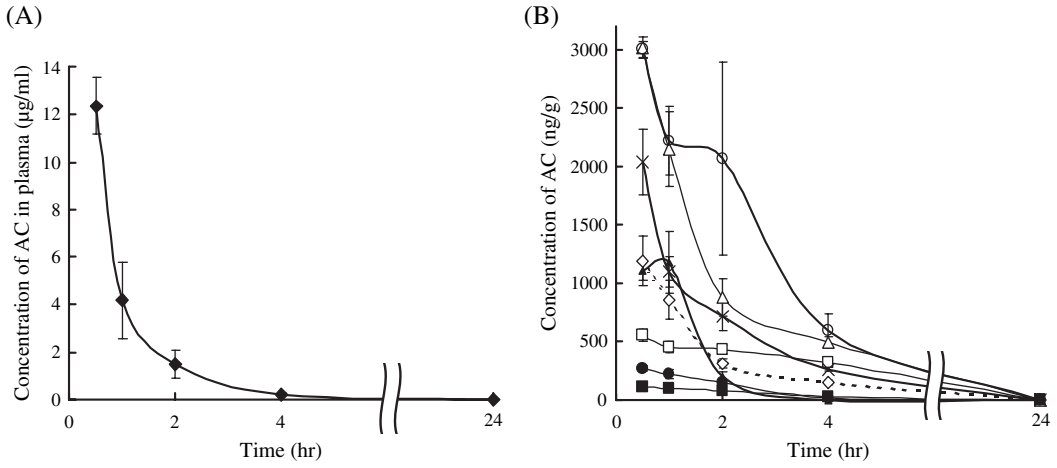

Fig. 5. Time course of changes in AC concentration after intravenous administration in rabbits. Values are means ± SE of three rabbits. (A) Plasma AC concentration, (B) AC concentration in the respective eye tissues; aqueous humor (◇), cornea (□), sclera (△), iris (▲), ciliary body (×), retina (●), choroid (○), vitreous (■).

Table 2
Pharmacokinetic parameters of anthocyanins after intravenous administration to rabbits

Ocular tissues or body fluid	T_{max} (h)	C_{max} (μg/g)	$t_{1/2}$ (h)	$AUC_{0\text{-inf}}$ (μg h/g)	AUC ratio (tissue/plasma)	AUC ratio (tissue/aqueous humor)
Aqueous humor	0.5	1.19	1.5	2.61	0.12	1.00
Cornea	0.5	0.55	5.6	4.35	0.21	1.67
Sclera	0.5	3.02	1.7	7.22	0.34	2.77
Choroid	0.5	3.00	1.4	9.08	0.43	3.48
Ciliary body	0.5	2.04	1.4	4.69	0.22	1.80
Iris	1.0	1.18	0.5	1.98	0.09	0.76
Retina	0.5	0.27	1.8	0.84	0.04	0.32
Vitreous	0.5	0.11	1.6	0.38	0.02	0.14
Plasma	0.5	12.36[a]	0.7	21.15[b]	1.00	8.11

[a] μg/ml.
[b] μg h/ml.

intake of BCAs and that ACs pass thorough the blood–aqueous barrier and blood–retinal barrier. Tests showed a good recovery rate in the aqueous humor, cornea, sclera, vitreous, retina, iris and ciliary body. In contrast, recovery rate was minimal in the choroid and lens, with the levels being at the sensitivity limit of our assay. Hagerman and Butler (1981) reported that proanthocyanidin interacts and binds to some unidentified protein, with this anthocyanin–protein interaction being a possible mechanism to account for the poor recovery we observed in our study. In the present study, a correction factor was not applied to the data as there were only trace amounts of ACs in the lens. However, the AC content of the choroid may be three-fold higher than that shown in Tables 1, 2 and Fig. 5.

Recently, several studies have been carried out on the bioavailability and biotransformation of ACs following oral administration. We showed in humans that the BCAs, D3G, D3R, C3G and C3R are absorbed orally and then distributed in the blood as intact forms (Matsumoto et al., 2001b). This indicates that the organs of the digestive tract possess a metabolic pathway for ACs that incorporates enzymatic conversions such as methylation and/or glucurono-conjugation. It has also been shown in rats that a small amount of C3G is absorbed in the plasma as glucuronyl conjugate and methylated forms (Wu et al., 2002; Ichiyanagi et al., 2005). In addition, Ichiyanagi et al. (2004) reported that D3G was absorbed in rats as an intact glucoside form and a small amount as 4′-*O*-methyl-D3G. The intake of D3G, D3R or C3R did not produce any glucuronides in rat (Ichiyanagi et al., 2004). Since the AC composition of BCA was D3R (47.2%), C3R (34.7%), D3G (13.5%) and C3G (4.6%), the amount of C3G was minimal. Taken together, it appears that intact forms of the four ACs were the only compounds detected in our study.

The retina is a highly differentiated tissue that plays a key role in vision, and has a blood–retinal barrier (BRB) in order to maintain a constant milieu and also shield the neural retina from the circulating blood (Hosoya and Tomi, 2005). In addition to the BRB, the blood–aqueous barrier (BAB), formed by epithelial barriers of the ciliary body and the iridial endothelial cells, is present in the anterior segment of the eye and maintains conditions in the aqueous humor (Hosoya and Tomi, 2005). In order to clarify whether oral intake of ACs has the potential to improve visual function it is important to verify that ACs can pass through these two barriers.

In the study on oral administration in rats, four ACs of BCA were detected in the plasma and whole eye as intact forms. This result suggested that ACs may accumulate in the eye after oral intake of BCA. However, since the concentration of ACs

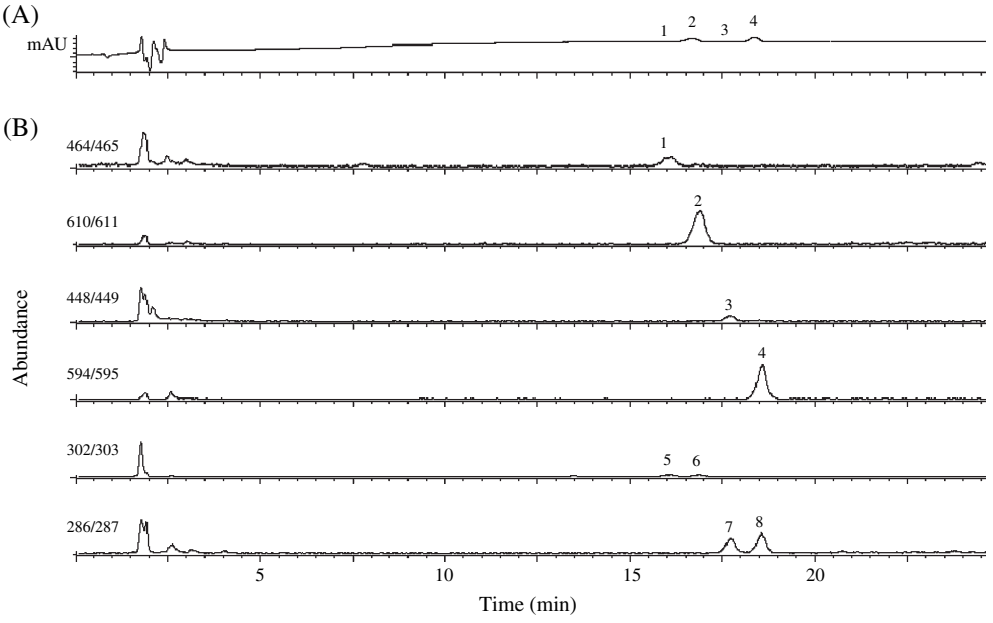

Fig. 6. LC-DAD-MS analysis of rabbit ciliary body at 30 min after intravenous administration of BCA. LC-DAD profile at 520 nm (A) and ion chromatograms (B). Detection of the respective *m/z* values of parent and product ions. Peaks are as follows: 1, D3G; 2, D3R; 3, C3G; 4, C3R; 5, product ion of D3G; 6, product ion of D3R; 7, product ion of C3G; 8, product ion of C3R.

was low, it proved difficult to determine the quantity and type of ACs in the ocular tissues.

Four ACs of BCA were also detected in the plasma, whole eye and each ocular tissue in rats following i.p. administration (Table 1). One hour after administration of BCA, total ACs in the various ocular tissues was graded in the following order: sclera with choroid > cornea > ciliary body with iris > retina > aqueous humor > vitreous > lens. Twenty-four hours after i.p. administration, no ACs were detected in either the whole eye or plasma, indicating that ACs remain in the tissues for only short periods. In the rat study, as a consequence of the small size of the eyes, it proved difficult to isolate the choroid from the sclera and the iris from the ciliary body. However, it is noteworthy that the concentration of total ACs in the whole eyes, sclera and cornea was markedly higher than in the plasma. In the i.p. study, as ACs were absorbed continuously into the blood, the reduction rate of ACs may be slower in ocular tissues than in plasma. These results suggested that ACs detected in the ocular tissues were not due to contamination from blood. In our study, the ACs were detected mainly in connective tissue, such as the sclera and cornea. As ACs also have a strong affinity for kidney and the skin (Lietti and Forni, 1976), this suggests that ACs may have increased affinity for collagen fibers. Tanaka et al. (2004) reported that fluoroquinolones have a high affinity for melanin-containing ocular tissues, such as the iris, ciliary body stratum pigment, chorioides and sclera. ACs may also have an affinity for melanin-containing ocular tissues. As treatment with a single dose of C3G has been shown to decrease cell proliferation without affecting cell viability or inducing apoptosis or necrosis (Serafino et al., 2004), further experiments are therefore necessary in order to demonstrate the potential of collagen to bind high concentrations of ACs in the sclera and cornea.

The distribution of ACs in the ciliary body and iris was also higher than plasma, while levels in the aqueous humor and retina were similar to that in plasma. These results suggest that ACs pass thorough the BAB and BRB as intact forms in rats. As a consequence, ACs accumulate in the eye after being absorbed from the plasma, with this deposition being a possible mechanism to account for the beneficial effects that AC intake may have on visual function. We detected only trace amounts of ACs in the vitreous and lens and it is probable these were contaminants. It is likely small compounds such as ACs are released from tissues during dissection resulting in trace amounts of AC contamination of the vitreous and lens. In order to reduce tissue to tissue contamination seen in the small eyes of rats we investigated the AC ocular profile after i.v. administration of BCA in rabbits. In a study of oral administration to rabbits we found ACs were poorly absorbed and were near the limit of detection in plasma (1 ng, data not shown). Inspection of the stomach contents of the rabbits administered BCA showed the AC solution was present as solid material in the stomach. This accounts for the low absorption rate in these animals. Accordingly we decided not to study oral administration of BCAs in rabbits and chose to use i.v. administration as an alternative.

As shown in Table 1, the distribution of ACs in ocular tissues in rabbits was found to be similar to that observed in rats. Fig. 5 shows the time-course of changes in plasma total AC concentration (Fig. 5(A)) and each ocular tissue (Fig. 5(B)) after i.v. administration of BCA. Thirty minutes after administration of BCAs to rabbits, the total AC accumulation in ocular tissues was ranked: sclera > choroid > ciliary body > aqueous humor > iris > cornea > retina > vitreous > lens. This distribution pattern also appeared to be the result of an affinity between ACs and collagen fibers. The distribution ratios in the sclera and cornea were lower than in the rat i.p. study, since ACs were absorbed continuously in the i.p. study. The small amount of ACs distributed in the vitreous was also a contaminant of small particles of retina.

The pharmacokinetics parameters of AC after i.v. administration of BCA are summarized in Table 2. The AUC in the various ocular tissues was ranked in the following order: choroid > sclera > ciliary body > cornea > aqueous humor > iris > retina > vitreous > lens. The $t_{1/2}$ values of ACs of the various ocular tissues were similar and ranged between 1.4 and 1.8 h with the exception of the cornea and iris. The $t_{1/2}$ values for clearance of AC were three-fold higher in the cornea and three-fold lower in the iris compared to the other tissues. This suggests that ACs have weak binding affinity for iris protein and strong binding affinity for cornea. We reported that ACs stimulate regeneration of rhodopsin from 11-*cis*-retinal and opsin at the ROS membrane (Matsumoto et al., 2003). Regeneration of 11-*cis*-retinal occurs in adjacent retinal pigment epithelial (RPE) cells (McBee et al., 2000). The ROS membrane is connected to RPE cells on the choroid. In the present study we found the choroid including RPE cells also had a high distribution of ACs. It appears that ACs pass from blood vessels in the choroid through BRB which is at the choroid/RPE juncture and then attain to the ROS. These results support the concept that oral intake of ACs may affect rhodopsin regeneration at the ROS membrane.

In an earlier publication we speculated that AC glycoside may be absorbed through the hexose transport pathway in the small intestine (Matsumoto et al., 2001b). A facilitative D-glucose transporter had been identified immunohistochemically near the inner BRB (Hosoya and Tomi, 2005) and it is possible that ACs may be absorbed by this transport pathway.

The distribution ratio of ACs in the ciliary body, aqueous humor and iris were also higher than plasma in the rat i.p. study. This implies that ACs are taken up by the ciliary body via the BAB and then excreted into the aqueous humor. The ciliary body is the tissue that produces aqueous humor thereby regulating intraocular pressure. It is well known that ascorbic acid is highly concentrated into the aqueous humor where it serves as an antioxidant by scavenging free radicals. ACs in aqueous humor may play a similar antioxidant role as ascorbic acid (Matsumoto et al., 2001b). We reported that D3R exerted a sustained and progressive relaxation during contraction induced by endothelin-1 in bovine ciliary bodies (Matsumoto et al., 2005). Taken together, these results indicate that administration of BCA may be useful as drug therapy for treating both myopia and glaucoma.

In conclusion, this is the first report demonstrating that ACs are absorbed and distributed as intact forms into ocular tissues

including the cornea, aqueous humor, iris, ciliary body, choroid, sclera and retina. This study demonstrated that BCAs pass through the BAB and BRB as intact forms in both rats and rabbits. Oral intake of BCAs may have potential as a drug therapy for treating ophthalmological diseases such as myopia and glaucoma. However, further experiments are necessary in order to obtain more detailed information.

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References

Beauregard, C., Liu, Q., Chiou, G.C., 2001. Effects of nitric oxide donors and nitric oxide synthase substrates on ciliary muscle contracted by carbachol and endothelin for possible use in myopia prevention. J. Ocul. Pharmacol. Ther. 17, 1–9.

Felgines, C., Talavera, S., Gonthier, M.P., Texier, O., Scalbert, A., Lamaison, J.L., Remesy, C., 2003. Strawberry anthocyanins are recovered in urine as glucuro- and sulfoconjugates in humans. J. Nutr. 133, 1296–1301.

Hagerman, A.E., Butler, L.G., 1981. The specificity of proanthocyanidin-protein interactions. J. Biol. Chem. 256, 4494–4497.

Hosoya, K., Tomi, M., 2005. Advances in the cell biology of transport via the inner blood-retinal barrier: establishment of cell lines and transport functions. Biol. Pharm. Bull. 28, 1–8.

Ichiyanagi, T., Rahman, M.M., Kashiwada, K., Ikeshiro, Y., Shida, Y., Hatano, Y., Matsumoto, H., Hirayama, M., Tsuda, T., Konishi, T., 2004. Absorption and metabolism of delphinidin 3-*O*-β-D-glucopyranoside in rats. Free Radic. Biol. Med. 36, 930–937.

Ichiyanagi, T., Shida, Y., Rahman, M.M., Hatano, Y., Matsumoto, H., Hirayama, M., Konishi, T., 2005. Metabolic Pathway of Cyanidin 3-*O*-β-D-glucopyranoside in rats. J. Agric. Food Chem. 53, 145–150.

Kühnau, J., 1976. The flavonoids. A class of semi-essential food components: their role in human nutrition. World Rev. Nutr. Diet 24, 117–191.

Lietti, A., Forni, G., 1976. Studies on *Vaccinium myrtillus* anthocyanosides. Arzneimittelforschung 26, 832–835.

Matsumoto, H., Hanamura, S., Kishi, M., Kawakami, T., Sato, Y., Hirayama, M., 2001a. Preparative-scale isolation of four anthocyanin components in blackcurrant (*Ribes nigrum* L.) fruits. J. Agric. Food Chem. 49, 1541–1545.

Matsumoto, H., Inaba, H., Kishi, M., Tominaga, S., Hirayama, M., Tsuda, T., 2001b. Orally administered delphinidin 3-rutinoside and cyanidin 3-rutinoside are directly absorbed in rats and humans and appear in the blood as the intact forms. J. Agric. Food. Chem. 49, 1546–1551.

Matsumoto, H., Nakamura, Y., Tachibanaki, S., Kawamura, S., Hirayama, M., 2003. Stimulatory effect of cyanidin 3-glycosides on the regeneration of rhodopsin. J. Agric. Food Chem. 51, 3560–3563.

Matsumoto, H., Kamm, K.E., Stull, J.T., Azuma, H., 2005. Delphinidin-3-rutinoside relaxes the bovine ciliary smooth muscle through activation of ET(B) receptor and NO/cGMP pathway. Exp. Eye Res. 80, 313–322.

McBee, J.K., Kuksa, V., Alvarez, R., de Lera, A.R., Prezhdo, O., Haeseleer, F., Sokal, I., Palczewski, K., 2000. Isomerization of all-trans-retinol to cis-retinols in bovine retinal pigment epithelial cells: dependence on the specificity of retinoid-binding proteins. Biochemistry 39, 11370–11380.

Miyazawa, T., Nakagawa, K., Kudo, M., Muraishi, K., Someya, K., 1999. Direct intestinal absorption of red fruit anthocyanins, cyanidin-3-glucoside and cyanidin-3,5-diglucoside, into rats and humans. J. Agric. Food Chem. 47, 1083–1091.

Morazzoni, P., Livio, S., Scilingo, A., Malandrino, S., 1991. *Vaccinium myrtillus* anthocyanosides pharmacokinetics in rats. Arzneimittelforschung 41, 128–131.

Mulleder, U., Murkovic, M., Pfannhauser, W., 2002. Urinary excretion of cyanidin glycosides. J. Biochem. Biophys. Methods 53, 61–66.

Nakaishi, H., Matsumoto, H., Tominaga, S., Hirayama, M., 2000. Effects of blackcurrant anthocyanosides intake on dark adaptation and VDT work-induced transient refractive alternation in healthy humans. Altern. Med. Rev. 5, 553–562.

Renaud, S., de Lorgeril, M., 1992. Wine, alcohol, platelets, and the French paradox for coronary heart disease. Lancet 339, 1523–1526.

Serafino, A., Sinibaldi-Vallebona, P., Lazzarino, G., Tavazzi, B., Rasi, G., Pierimarchi, P., Andreola, F., Moroni, G., Galvano, G., Galvano, F., Garaci, E., 2004. Differentiation of human melanoma cells induced by cyanidin-3-O-beta-glucopyranoside. FASEB J. 18, 1940–1942.

Tanaka, M., Takashina, H., Tsutsumi, S., 2004. Comparative assessment of ocular tissue distribution of drug-related radioactivity after chronic oral administration of ^{14}C-levofloxacin and ^{14}C-chloroquine in pigmented rats. J. Pharm. Pharmacol. 56, 977–983.

Tsuda, T., Horio, F., Osawa, T., 1999. Absorption and metabolism of cyanidin-3-*O*-β-D-glucoside in rats. FEBS Lett. 449, 179–182.

Wu, X., Cao, G., Prior, R.L., 2002. Absorption and metabolism of anthocyanins in elderly women after consumption of elderberry or blueberry. J. Nutr. 132, 1865–1871.