In Vitro Metabolism Study of Edaravone in Wistar and Hairless Rat Skin

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We investigated the skin metabolism of edaravone as a radical scavenger in Wistar and hairless rat skin. Approximately 1 g of abdominal skin was excised from 10-week-old Wistar and hairless rats, homogenized in 10 ml saline, and centrifuged at 10000 g for 20 min. The supernatant fluid was used for the examination of edaravone metabolism in the skin, and we also used supernatant fluid that was heated at 80 °C. Edaravone solution (0.05 ml, 2.4 μmol/ml) was added to 0.95 ml Wistar rat and hairless rat skin homogenate supernatant fluids. In Wistar rats, the residual amount of edaravone in skin homogenate supernatant fluid at 37 °C after 0, 5, 10, 20 and 30 min was 61.58±1.65, 41.84±8.52, 35.54±8.62, 19.73±5.99 and 13.89±4.40%, respectively. In hairless rats, the residual amount of edaravone in skin homogenate supernatant fluid at 37 °C after 0, 5 and 10 min was 50.19±14.17, 6.71±5.82 and 0.89±0.80%, respectively, and edaravone was not detected after 20 min. Although it was thought that metabolic enzyme activity in skin homogenate supernatant fluid was lost following heat treatment at 80 °C, the residual amount of edaravone in our skin homogenate supernatant fluid decreased with time. It is suggested that edaravone metabolism in the skin is necessary for non-enzymatic reactions.

Key words edaravone; Wistar rat skin; hairless rat skin; HPLC; drug metabolism

3-Methyl-1-phenyl-2-pyrazolin-5-one (edaravone, Radicut injection) acts as an antioxidative radical scavenger, and has been developed as a treatment for cerebrovascular disease. In addition, edaravone has been reported to have protective effects on functional damage caused by hyperglycemia to human dermal microvascular endothelial cells, inflammation-induced pain and cisplatin-induced nephrotoxicity in rats. There are also many reports that edaravone has a protective effect on ischemic diseases caused by oxidative stress. Edaravone administration in patients with rheumatoid arthritis leads to a reduction in serum C-reactive protein and disease activity score. Therefore, edaravone is expected to have a role in the prevention and treatment of acute and chronic diseases.

Transdermal absorption can be considered as an administration route for prolonged edaravone action. We have already reported the formation of an edaravone/cyclodextrin complex and its skin permeability. It has previously been clearly shown that the edaravone/cyclodextrin complex increases the amount of edaravone on the skin surface, as well as its skin permeability. These results suggest the possibility of transdermal and transmucosal treatment with edaravone. On the other hand, it has been reported that the pharmacokinetics of edaravone after intravenous administration in rats and humans involve excretion of glucuronide and sulfate. Moreover, the activity of UDP-glucuronosyltransferase (UGT) and sulfotransferase (SULT) in human skin has also been reported. Therefore, it is considered that skin metabolism of edaravone in humans may be similar to that in rat skin. However, there have been no reports on skin metabolism of edaravone.

In this study, we used Wistar and hairless rat skin to investigate the metabolism of edaravone in vitro.

MATERIALS AND METHODS

Materials Edaravone was synthesized from ethyl acetooacetate and phenylhydrazine according to the method of Komatu et al. All other reagents and solvents were of special grade and used without further purification.

Animals Wistar/ST and hairless (HWY) male rats were obtained from Japan SLC (Shizuoka, Japan). These animals were housed in rooms controlled at 23±2 °C and 55±15% relative humidity. Pelleted chow (Certified Rodent Diet #1002, PMI Nutriton International, U.S.A.) and water were given ad libitum. 10- and 24-Week-old Wistar rats and 10-week-old hairless rats were used for experiments. All experiments were performed under the regulations of the Animal Ethical Committee of Mikasa Seiyaku (based on the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Science and Technology).

Residual Edaravone in Skin Homogenate and Intact Skin One gram of abdominal skin was excised from three or four 10- and 24-week-old Wistar or 10-week-old hairless rats, homogenized in 10 ml saline (Physcotron NS-50; Microtec, Chiba, Japan), and centrifuged at 10000 g for 20 min. The excised skin was stored at −80 °C, and skin homogenate supernatant fluid was stored at −20 °C until use. To establish whether the concentration of edaravone affected the level of edaravone in skin homogenate supernatant fluid, 0.05 ml edaravone solution (1.2 or 2.4 μmol/ml) was added to 0.95 ml 24-week-old Wistar rat skin homogenate supernatant fluid (60 or 120 nmol/ml final concentration). To assess whether rat strain had any effect on the decrease in edaravone in skin homogenate supernatant fluid, edaravone solution (0.05 ml, 2.4 μmol/ml) was added to 0.95 ml 10-week-old Wistar and hairless rat skin homogenate supernatant fluids (120 nmol/ml final concentration). These samples were incubated at 37 °C. The intact skin was used and examined to consider the influence by homogenized the skin. In abdominal intact skin excised from four hairless rats, edaravone solution 0.05 ml (2.4 μmol/ml) was injected using a 1-ml syringe with a 25G needle. The intact skins were incubated at 37 °C, then they were homogenized in methanol to inactivate the metabolic
enzymes, and centrifuged at 3500 \( g \) for 20 min. Then, the residual amount of edaravone in these supernatant fluids was quantified by high performance liquid chromatography (HPLC).

The effect of pre-heating on the residual amount of edaravone in skin homogenate supernatant fluids was determined. In a study using four 10-week-old hairless rat skin homogenate supernatant fluids heated to 80 °C, 0.05 ml edaravone solution (2.4 \( \mu \)mol/ml) was added to 0.95 ml supernatant fluid, and these samples were incubated at 37 °C.

Furthermore, the samples in these examinations were mixed with methanol containing methylparaben (internal standard, IS) at 1 : 3, and these samples were quantified by HPLC.

**Residual Edaravone in Plasma** Three 10-week-old Wistar and hairless rats were used to determine the edaravone concentration in plasma. Plasma obtained from rat blood by centrifugation at 3500 \( g \) for 20 min was stored at \(-20^\circ C\) until needed. After 0.05 ml edaravone solution (2.4 \( \mu \)mol/ml) was added to 0.9 ml plasma, the samples were incubated at 37 °C. The amount of edaravone in the plasma was measured after mixing plasma and methanol (content IS) at a ratio of 1 : 3, and these samples were quantified by HPLC.

**HPLC of Edaravone in Wistar and Hairless Rat Skin Homogenate Supernatant Fluids and Plasma** Residual edaravone in skin homogenate supernatant fluids and plasma was measured using the HPLC LC10 system (Shimadzu, Kyoto, Japan). Samples were analyzed on a 5-\( \mu \)m Inertsil ODS-2 column (4.6×250 mm; GL Sciences, Tokyo, Japan), at a temperature of 40 °C. Edaravone was detected by UV at 240 nm. The mobile phase consisted of a methanol: distilled water: acetic acid mixture (35:65:0.1, v/v). The flow rate of the mobile phase was 1 ml/min, and after methanol (content IS) was added to each sample, these samples 20 \( \mu l \) were injected onto the column. Residual edaravone ratio in these samples at each time was calculated by the following equation:

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\text{percentage residual edaravone} = \frac{\text{residual amount of edaravone in samples}}{\text{amount of edaravone added}} \times 100
\]

**Comparison of HPLC of Edaravone in Wistar and Hairless Rat Skin Homogenate Supernatant Fluids** Samples were analyzed on a 5-\( \mu \)m Nucleosil 100 C18 column (4.6×250 mm; GL Sciences), at a temperature of 40 °C. To compare HPLC of edaravone in Wistar and hairless rat skin homogenate supernatant fluids, the mobile phase consisted of a mixture of methanol: 10 mmol sodium acetate (35:65, v/v, pH 5.5, adjusted with 10% acetic acid), and the flow rate of the mobile phase was 0.5 ml/min.

**Statistical Analysis** Results were evaluated by the Tukey–Kramer test. \( p<0.05 \) was considered statistically significant.

**RESULTS**

**HPLC of Edaravone in Wistar and Hairless Rat Skin Homogenate Supernatant Fluids and Plasma** The retention time of edaravone was 10 min. Over the concentration range 4.5—144 nmol/ml, HPLC demonstrated good linearity, with a correlation coefficient of \( >0.9999 \).

**Residual Edaravone in Skin Homogenates and Intact Skin** The effect of edaravone concentration on the decrease in edaravone in edaravone (60, 120 nmol/ml) in 24-week-old Wistar rat skin homogenate supernatant fluids was shown in Fig. 1. The residual amount of edaravone in 24-week-old Wistar rat skin homogenate supernatant fluids decreased rapidly after addition of 0.05 ml edaravone solutions (1.2 or 2.4 \( \mu \)mol/ml). These results show that edaravone was metabolized in the skin homogenate supernatant fluids. The residual amount of edaravone in skin homogenate supernatant fluids decreased rapidly to \( ca. \) 40% after addition of edaravone. This decreased to \( ca. \) 10% \( 37^\circ C \) for 3 h.

Therefore, we investigated the residual amount of edaravone after a shorter period of time. The residual amount of edaravone in skin homogenate supernatant fluids from 10- and 24-week-old Wistar rats is shown in Fig. 2. The residual amount of edaravone in skin homogenate supernatant fluids at 37 °C at 0, 5, 10, 20 and 30 min in 10-week-old rats was 61.58±1.65, 41.84±8.52, 35.54±8.62, 19.73±5.99 and 13.89±4.40% (mean±S.D.), respectively, and in 24-week-old rats it was 35.75±12.63, 28.16±13.77, 16.36±12.23, 8.19±6.11 and 5.46±4.68% (mean±S.D.), respectively. There were no significant differences in these results. The decrease in residual rate edaravone in 24-week-old rat skin homogenate supernatant fluids was more rapid at each time.
point compared with that in 10-week-old rat skin homogenates, but the difference was not significant.

Hairless rats have been used in several experiments for transdermal drug delivery. Accordingly, we investigated hairless rat skin homogenate supernatant fluids. The residual amount of edaravone in skin homogenate supernatant fluids from 10-week-old hairless rats is shown in Fig. 3. The residual amounts of edaravone in 10-week-old hairless rat skin homogenate supernatant fluids at 37 °C are 50.19 ± 14.17, 6.71 ± 5.82 and 0.89 ± 0.80, respectively, and edaravone was not detected after 20 min. The residual amount of edaravone in 10-week-old hairless rat skin homogenates decreased significantly compared with the residual amount of edaravone in 10-week-old Wistar rat skin homogenate supernatant fluids (*p < 0.05).

The residual amount of edaravone in intact skin is shown in Fig. 4. The residual amount of edaravone did not differ significantly from that in skin homogenate supernatant fluids (unheated). The residual amounts of edaravone in hairless rat skin homogenate supernatant fluids heated to 80 °C are shown in Fig. 5. The decrease in the residual amount of edaravone was significantly less than that in unheated skin homogenate supernatant fluids (*p < 0.05).

Residual Edaravone in Plasma The residual amount of edaravone in plasma in 10-week-old Wistar and hairless rats is shown in Figs. 6A and B. The decrease in edaravone was slower than that in skin homogenate supernatant fluids at 37 °C for 30 min. The residual amount of edaravone in Wistar and hairless rat plasma samples at each time point was significantly higher than that in the skin homogenate supernatant fluids of both rat types (*p < 0.05). These results showed that metabolism of edaravone in plasma was low.

Comparison of Edaravone in Wistar and Hairless Rat Skin Homogenate Supernatant Fluids HPLC of edaravone standards and edaravone in Wistar and hairless rat skin homogenate supernatant fluids is shown in Fig. 7. Peaks for edaravone appeared at around 20 min. Both rat skin homogenates incubated with edaravone at 37 °C for 30 min had a tR value of 8.9 min; however, this tR value did not apply to skin homogenate supernatant fluids without edaravone. Hairless rat skin homogenate supernatant fluids incubated with edaravone had three different tR values of 8.9, 10 and 18 min, and these did not apply to the hairless rat skin homogenate supernatant fluids without edaravone. However, these tR values were not seen in the 80 °C pre-heated skin homogenate.

Fig. 3. Percentage Residual Edaravone in 10-Week-Old Wistar (n = 3) and Hairless Rat (n = 4) Skin Homogenate Supernatant Fluids

* *p < 0.05, Tukey–Kramer test; mean ± S.D.

Fig. 4. Percentage Residual Edaravone in Hairless Rat Intact Skin

Intact skins were incubated at 37 °C for 30 min, which were then homogenized with methanol. N.S., not significant, Tukey–Kramer test; mean ± S.D.; n = 4; N.D., not detected.

Fig. 5. Effect of Pre-heating Hairless Rat Skin Homogenate Supernatant Fluid to 80 °C on Skin Metabolism of Edaravone

* *p < 0.05, Tukey–Kramer test; mean ± S.D.; n = 4; N.D., not detected.

Fig. 6. Percentage Residual Edaravone in Plasma and Skin Homogenate Supernatant Fluids

(A) Wistar rat plasma and skin homogenate supernatant fluids; (B) hairless rat plasma and skin homogenate supernatant fluids. *p < 0.05, Tukey–Kramer test; mean ± S.D.; plasma, n = 3; skin, n = 4.
Sulfoconjugation activity increases after birth and reaches a maximum in 3-week-old male and female rats. The activity in 2-year-old male and female rats is intermediate between the 7-week-old male and female rats, and no sex difference was observed. These results indicate that the substrates for sulfoconjugation can be divided into at least two groups, an alcohol and amine type, and a phenol type. Moreover, it has already been reported for edaravone metabolism that the above is not first phase oxidation by P450, but rather second-phase metabolism, such as conjugation. In this study, the difference in the residual amount of edaravone in the skin homogenate supernatant fluids according to rat strain may be due to a difference in expression of the UGT family. In human skin, UGT has been shown to have activity against steroids and androstanediol. SULT has been shown to have activity against minoxidil in human scalp skin. These findings suggest that human skin also has UGT and SULT activity. Although metabolic activity was reduced by methanol in this study, in order to investigate the influence of other substances it may be necessary to use alternative inhibitors of UGT or SULT. The effects of UGT inhibitors such as ketoconazole, and SULT inhibitors such as curcumin and mfenamic acid have been reported. On the other hand, time 0 min in the residual ratio of each time is immediately after edaravone addition. It was thought that the residual ratio of edaravone was decreased at time 0 min by en-
zymic and non-enzyme reactions. In addition, as shown in Fig. 5, although it was thought that metabolic enzyme activity in skin homogenate supernatant fluids was inactivated by heat treatment at 80 °C, the residual amount of edaravone in skin homogenate supernatant fluids decreased with time. Moreover, edaravone residual ratio in unheated skin homogenates decreased immediately after edaravone addition. Therefore, it was thought that involvement in enzymatic and non-enzymatic reaction, and the free radical may be related to the skin metabolism of edaravone. However, we thought that the results in different rats strains shown in Fig. 3 needed to be examined further. In this study, it was suggested that skin metabolism of edaravone had the necessity of consideration to conjugation by enzyme, and also a non-enzymatic reaction.

**CONCLUSION**

In this study, *in vitro* rat skin metabolism of edaravone appeared to lead to production of glucuronide and sulfate, although a detailed investigation is needed. However, it is suggested that edaravone metabolism in the skin is necessary for its effects on the expression of the UGT family and non-enzymatic reactions.

**REFERENCES**