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A novel administration route for edaravone: I. Effects of metabolic inhibitors on skin permeability of edaravone

Toshiaki Sato [a], Keizo Mizuno [a], Fumiyoshi Ishii [b,*]

[a] Reserch & Development Division, Mikasa Seiyaku Co., Ltd., 2-3-1 Toyotama-Kita, Nerima-ku, Tokyo 176-8585, Japan
[b] Department of Pharmaceutical Sciences, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo 204-8588, Japan

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ABSTRACT

We examined the effects of metabolic inhibitors on skin permeation of edaravone. SKF-525A, diclofenac sodium (DIC) and indomethacin (IND) were added to supernatant fluid (SF) of hairless rat (HR) skin homogenate. L-Cysteine (L-Cys) and benzotriazole (BTA), as pharmaceutical additives, were added to HR skin homogenate SF, and incubated at 37 °C for 30 min. K_m and V_{max} values were calculated. For determination of edaravone skin permeation from edaravone/hydroxypropyl-β-cyclodextrin (HPβCD) complex solution, HR skin was placed in a Franz diffusion cell, and kept at 37 °C. Edaravone/HPβCD solution that contained L-Cys was put into the donor side. The relative activity in skin homogenate SF after co-treatment with IND and SKF-525A decreased to 40.8% of the control. However, DIC and IND had a weak inhibitory effect. For inhibition of edaravone metabolism, L-Cys and BTA had no effect on K_m value, but V_{max} was significantly decreased compared with controls ($^*P < 0.05$, Tukey–Kramer test). The edaravone skin permeation rate and permeability coefficient from edaravone/HPβCD complex solution with inhibitor were significantly increased compared with those without inhibitor. We suggest that the metabolism inhibitor was useful for the transdermal delivery of edaravone.

1. Introduction

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one, MW 174.20; Radicut® injection 30 mg) is the world's first neuroprotective agent, developed in Japan, and it has a neuroprotective effect (Houkin et al., 1998), an effect on regional cerebral blood flow (Kawai et al., 1997), suppresses delayed neuronal death, and shows antioxidative radical scavenging (Watanabe et al., 1988, 1994; Yamamoto et al., 1997). It has also been reported that its scavenging action is different from that of vitamins C and E (Watanabe et al., 2004). Edaravone has been reported to have protective effects against cisplatin-induced nephrotoxicity in rats (Sueishi et al., 2002). In addition, edaravone has been reported to counteract the development of multiple low-dose streptozotocin-induced diabetes in mice (Fukudome et al., 2008).

Moreover, when a patient with rheumatoid arthritis (RA) developed acute stroke and was treated with edaravone, polyarthralgia improved, with a reduction in serum C-reactive protein concentration soon after the start of treatment, and the disease activity score of 28 also decreased (Arii et al., 2006a). RA is a chronic inflammatory disease associated with immune abnormalities, and it is reported that oxidative stress appears to be involved in the pathogenesis of RA (Arii et al., 2006b; Cimen et al., 2000; Ozturk et al., 1999; Mazzetti et al., 2001; Miesel et al., 1996). Therefore, edaravone, an antioxidant agent, might be a novel therapeutic agent from which to develop a new strategy for RA treatment.

A phase III clinical trial of edaravone in amyotrophic lateral sclerosis (ALS) is in progress, and the effects on an ALS patient have already been reported (Yoshino and Kimura, 2006).

There is evidence to suggest that allergic and inflammatory skin diseases such as atopic dermatitis, urticaria and psoriasis are mediated by oxidative stress (Okayama, 2005). Human skin has a protective function, and is therefore, a potential site for the production of free radicals. The role played by topically applied antioxidants as inhibitors of oxidative stress damage was felt to be worth investigation. In addition, hydroxyl radical scavenging and antipsoriatic activity of benzoic acid derivatives have already been reported (Haseloff et al., 1990). Therefore, edaravone, as a radical scavenger, might be a novel therapeutic agent from which to develop a new strategy for treatment of skin diseases.

Edaravone is presently only available commercially in an injectable form. However, it is thought that a route of administration other than by injection is necessary when edaravone is used to treat skin diseases. Furthermore, topical administration is thought to be the preferred route. Therefore, we studied the possibility of transdermal absorption of edaravone.

* Corresponding author. Tel.: +81 424 95 8468; fax: +81 424 95 8468.
E-mail address: fishii@my-pharm.ac.jp (F. Ishii).

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Cyclodextrin improves solubility and skin permeability when it is administered in combination with edaravone (Sato et al., 2005, 2006) have been reported in pharmaceutical studies. Moreover, we have already reported participation of the non-enzymatic reaction in interaction of edaravone and skin (Sato et al., 2008). In this study, the inhibition of edaravone metabolism in the skin by pharmaceutical additive agents was examined, along with the effect of these additives on skin permeation of edaravone.

2. Materials and methods

2.1. Materials

Edaravone was synthesized from ethyl acetoacetate and phenylhydrazine (Komatsu et al., 1996). Superoxide dismutase (SOD, Zn type) from *Bacillus* sp., SKF-525A, diclofenac sodium (DIC) and indomethacin (IND) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Catalase (CAT) from bovine liver was purchased from Merck Ltd. (Tokyo, Japan). Sodium hydrogen sulfite (SHS), L-Cysteine (L-Cys), L-arginine (L-Arg), 2-mercaptobenzimidazole (MBI) and benzotriazole (BTA) were purchased from Kanto Chemical Co. (Tokyo, Japan). Hydroxypropyl-β-cyclodextrin (HPβCD) was obtained from Nihon Shokuhin Kako Co. Ltd. (Tokyo, Japan). All other reagents and solvents were of special grade and used without further purification.

2.2. Animals

Ten-week-old 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 #5002; PMI Nutrition International, USA) and water were given *ad libitum*. Ten-week-old hairless male rats were used for studies of skin metabolism and permeation of edaravone. All experiments were performed under the regulations of the Animal Ethical Committee of Mikasa Seiyaku Co. Ltd.

2.3. Preparation of skin homogenate supernatant fluid (SF)

One gram of abdominal skin removed from hairless rats (HR) was homogenized in 10 ml saline, and centrifuged at 10,000 × *g* for 20 min, and the SF was examined. In addition, HR skin homogenate SF was heated for 15 min at 80 °C to inactivate uridine diphosphate glucuronosyltransferase (UGT). Then, it was centrifuged at 10,000 × *g* for 20 min, and the SF was examined. Skin removed from rats was stored at −80 °C until the experiment, and SF of centrifuged skin homogenate was stored at −20 °C until the experiment.

2.4. Effects of SOD, CAT, SKF-525A, DIC and IND on metabolism of edaravone in HR skin

SOD (4500 U), CAT (4500 U) or SKF-525A (0.1 mmol) in a volume of 0.05 ml were added to 0.25 ml HR skin homogenate SF heated at 80 °C, and 0.175 ml isotonic phosphate buffer solution (pH 7.5) was added. The SFs were incubated at 37 °C for 30 min. Then, 0.025 ml edaravone (60 nmol:2.4 μmol/ml) was added (0.5 ml total volume), and the SFs were incubated at 37 °C for 30 min. Moreover, DIC (0.1 mmol) and IND (0.1 mmol)were examined in the same way, and they had radical scavenging activity and affinity with UGT.

The amount of edaravone was quantified by high-performance liquid chromatography (HPLC).

HPLC chromatograms of edaravone in HR skin SF that was not heated to 80 °C were also investigated. DIC (0.2 mmol), IND (0.2 mmol) or SKF-525A (0.1 mmol) in a volume of 0.05 ml was added to HR skin homogenate SF, and 0.175 ml isotonic phosphate buffer solution (pH 7.5) was added. Then, SF was incubated at 37 °C for 30 min. Edaravone solution (60 nmol:2.4 μmol/ml), 0.025 ml, was added (0.5 ml total volume), and SF was incubated at 37 °C. The amount of edaravone at 30 min was quantified by HPLC.

2.5. Effects of L-Cys, L-Arg, MBI and BTA as pharmaceutical additives on inhibition of edaravone metabolism in HR skin

L-Cys, L-Arg, MBI or BTA (0.01–1.0 μmol/0.05 μl) was added to 0.25 ml HR skin homogenate SF, along with 0.175 ml isotonic phosphate buffer solution (pH 7.5), and incubated at 37 °C for 30 min. Then, edaravone solution (2.4 μmol/ml) 0.025 ml (60 nmol) was added (0.5 ml total volume), and the SFs were incubated at 37 °C for 30 min. Edaravone was quantified by HPLC after addition of 0.3 ml methanol containing methylparaben as an internal standard.

On the other hand, the effect of L-Cys and MBI on edaravone metabolic rate in skin homogenate SF was investigated. The edaravone solution (7.5–60 nmol/0.025 μl) with L-Cys and MBI(0.1–1.0 μmol/0.05 μl) were added to 0.25 ml hairless rat skin homogenate SF. Then 0.175 ml pH 7.5 isotonic phosphate buffer solution was added in these SF (0.5 ml total volume). Then, they were incubated at 37 °C for 30 min, and after 0.3 ml methanol containing methylparaben (internal standard) was added to 0.1 ml of each samples quantified by HPLC.

2.6. Edaravone HR skin permeation from edaravone/HPβCD complex

Abdominal skin removed from HR was placed in a Franz diffusion cell with an effective area of 3.14 cm^2. Then, 1.0 ml edaravone/HPβCD solution containing L-Cys was put into the donor side, and distilled water into the receiver side, and these were kept at 37 °C. 0.2 ml of receiver liquid was extracted after 2, 4, 6, 8 and 24 h, and 0.6 ml methanol containing methylparaben (internal stan-

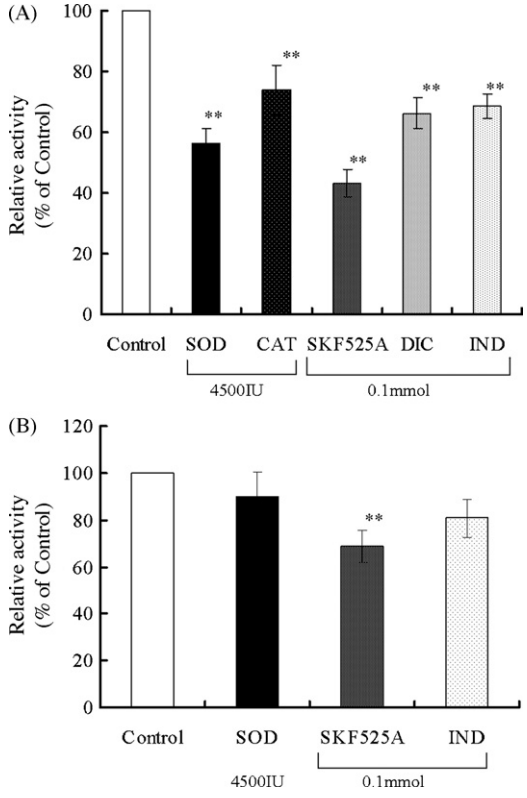

Fig. 1. Effects of various drugs on metabolism of edaravone in HR skin. (A) Skin homogenate SF heated at 80 °C; (B) skin homogenate SF (mean ± S.D.; $n = 3$; vs. control; $^{**}P < 0.01$, Tukey–Kramer test).

dard) was added to each sample, and these samples was measured by HPLC.

2.7. Stability of edaravone in edaravone/HPβCD complex solution

Edaravone/HPβCD solution was stored in brown glass bottles at 50 °C. Samples were taken on days 14 and 28 for analysis of edaravone content at 50 °C by HPLC. Methanol containing methylparaben (internal standard) was added to each sample. We then showed the stability of edaravone at the residual rate, which was calculated as measured/initial concentration × 100.

2.8. Determination of edaravone in samples

The HPLC LC10 system (Shimadzu) was used to determine edaravone in samples. Samples were analyzed on a 5-μm Nucleosil 100 C18 column (4.6 mm × 250 mm; GL Sciences, Tokyo, Japan), at 40 °C. Edaravone was detected by UV at 243 nm. For measuring the metabolism and skin permeation of edaravone, the mobile phase used methanol/distilled water/acetic acid (35:65:0.1, v/v/v), and the flow rate of the mobile phase was 1 ml/min. Then, after methylparaben (internal standard) was added to each sample, 20 μl was injected onto the column. In addition, the mobile phase used methanol and 10 mmol sodium acetate (35:65, v/v), pH 5.5 (Komatsu et al., 1996), to compare the chromatogram t_R value, with a mobile-phase flow rate of 0.5 ml/min.

Other HPLC conditions were the same as those used for metabolism and skin permeation of edaravone.

2.9. Statistical analysis

Inhibition of edaravone metabolism in skin was evaluated by Tukey–Kramer test. Then, HR skin permeation was evaluated by *t* test. In addition, $P < 0.05$ was considered statistically significant in all statistical analyses. The results are presented as means ± S.D.

3. Results

3.1. Determination of edaravone

During the skin metabolism, skin permeation and stability studies of edaravone, the retention time of edaravone was almost 10 min. On the other hand, in comparison of HPLC chromatograms, the retention time was almost 19.5 min. The concentration of edaravone was 1.5–100 nmol for the skin metabolism studies, and 0.125–20 μg/ml for the skin permeation and stability studies. HPLC demonstrated good linearity, with a correlation coefficient of >0.9999.

3.2. Effects of SOD, CAT, SKF-525A, DIC and IND on metabolism of edaravone in HR skin

The effects of inhibition by SOD, CAT, SKF-525A, DIC and IND on metabolism of edaravone in skin homogenate SF heated at 80 °C and skin homogenate SF are shown in Fig. 1A and B. In skin homogenate SF heated at 80 °C, although the skin homogenate SF inactivated an UGT by heating, it showed activity for metabolism of edaravone. SOD and CAT treatments significantly decreased edaravone metabolism in skin compared with the control level ($P < 0.01$, Tukey–Kramer test). SKF-525A, which is a non-specific P450 inhibitor, significantly decreased edaravone metabolism compared with the control level ($P < 0.01$, Tukey–Kramer test). DIC and IND significantly decreased edaravone metabolism compared with the control level ($P < 0.01$, Tukey–Kramer test). However, they had a weak effect compared with that of SKF-525A. In addition, the effect of SKF525A on skin homogenate SF was lower than that with skin

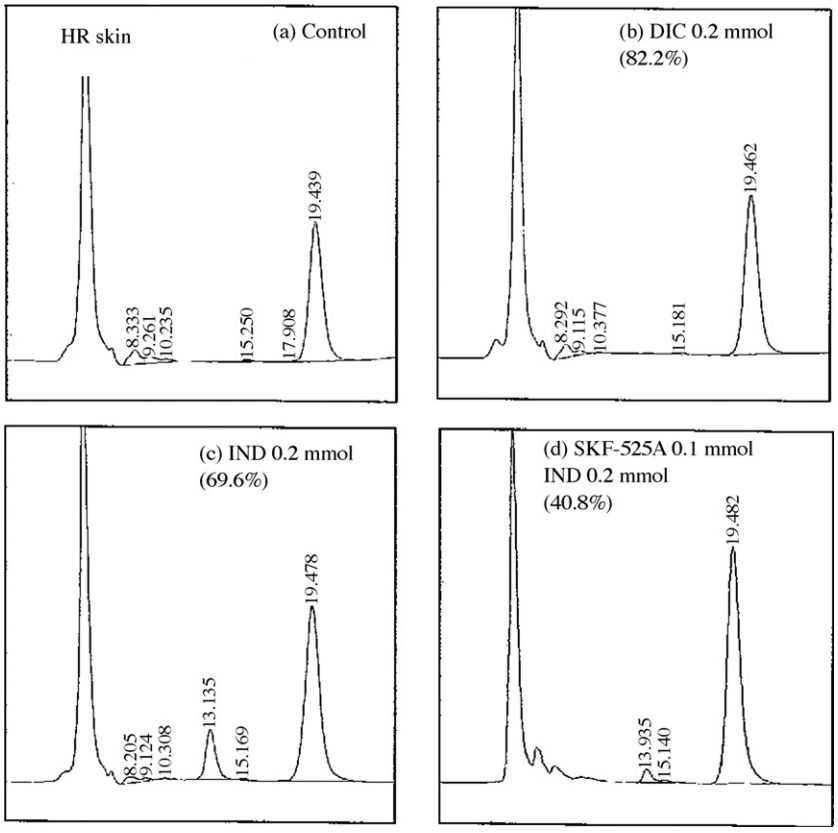

Fig. 2. HPLC chromatograms of edaravone in HR skin homogenate SF treated by DIC, IND or SKF-525A. ( ) Relative activity (% of control).

Table 1
K_m and V_{max} of inhibition of edaravone metabolism in the HR skin for various inhibitors vs. 0 μM, Tukey–Kramer test.

	K_m (nM)		V_{max} (nM/min/mg tissue)	
	L-Cys	BTA	L-Cys	BTA
0 μM	17.54 ± 6.57		11.04 ± 4.70	
0.1 μM	14.43 ± 2.78	13.85 ± 4.67	1.55 ± 0.33**	3.27 ± 0.43**
1.0 μM	12.73 ± 1.87	18.09 ± 4.44	0.85 ± 0.37**	1.28 ± 0.30**

Mean ± S.D. $n = 3$.
** $P > 0.01$.

homogenate SF heated at 80 °C, and SOD and IND had no effect on skin homogenate SF.

HPLC chromatograms of edaravone in HR skin homogenate SF are shown in Fig. 2. The retention time of edaravone was ~19.5 min (Fig. 2A). Peak t_R value of 8–9 min in skin homogenate SF after treatment with DIC did not differ from that of the controls, and inhibitory activity did not appear (Fig. 2B). Following treatment with 0.2 mmol IND, the relative activity of skin homogenate SF was 69.6% of the control, and peak t_R value of 8–9 min was decreased compared with that of the control and DIC-treated sample (Fig. 2C). The relative activity in skin homogenate SF after co-treatment with 0.2 mmol IND and 0.1 mmol SKF-525A decreased to 40.8% of the control level, and peak t_R value of 8–9 min disappeared (Fig. 2D).

3.3. Effects of L-Cys, L-Arg, MBI and BTA as pharmaceutical additives on inhibition of edaravone metabolism in HR skin

The inhibitory effects of amino acids and the azole compounds as pharmaceutical additives on edaravone metabolism in HR skin are shown in Fig. 3. Metabolism of edaravone in skin homogenate SF was decreased in a dose-dependent manner by amino acids. It was significantly decreased to 94.84 ± 8.93, 70.35 ± 3.28, 27.69 ± 10.63% (mean ± S.D.) of the control level by 0.01, 0.1 and 1.0 μM L-Cys, respectively (*$P < 0.05$, Tukey–Kramer test). Edaravone metabolism was significantly decreased to 62.5–66.6% of the control level by 0.05–1.0 μM L-Arg (*$P < 0.05$, Tukey–Kramer test), but this did not appear to be dose-dependent. L-Cys decreased edaravone metabolism more than IND or SKF525A. Edaravone metabolism was significantly decreased in a dose-dependent manner by MBI and BTA (*$P < 0.05$, Tukey–Kramer test). Moreover, MBI and BTA decreased edaravone metabolism more than did IND and SKF525A.

The effects of L-Cys and BTA on rate of edaravone metabolism in HR skin homogenate SF are shown in Table 1. L-Cys and BTA had no effect on K_m value, but V_{max} was significantly decreased compared with the controls (*$P < 0.05$, Tukey–Kramer test).

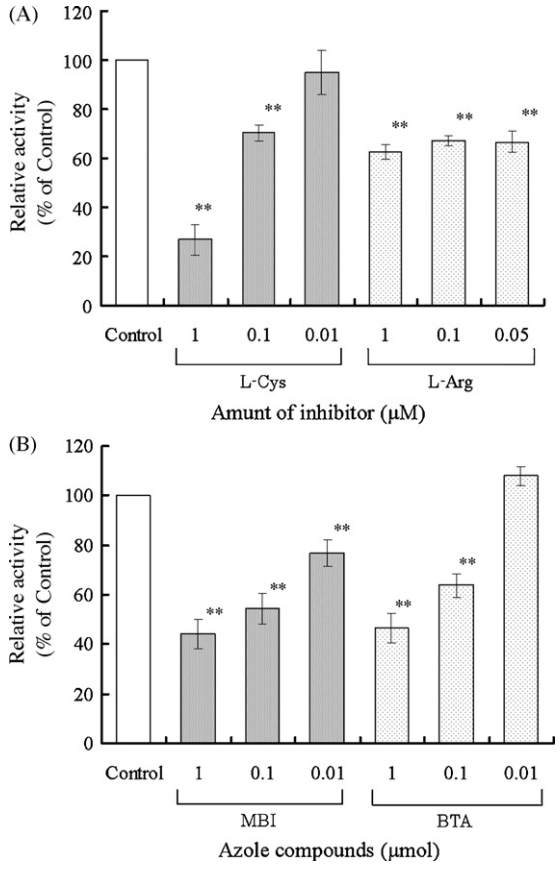

Fig. 3. Effects of pharmaceutical additives on metabolism of edaravone in skin homogenate SF. (A) Amino acids; (B) azole compounds (vs. control, Tukey–Kramer test *$P > 0.05$. Mean ± S.D., $n = 3$).

3.4. Edaravone HR skin permeation from edaravone/HPβCD complex

Effect of L-Cys on edaravone HR skin permeation from edaravone/HPβCD complex solution is shown in Fig. 4 Edaravone skin permeation rate and permeability coefficient from edaravone/HPβCD complex solution with L-Cys were significantly increased compared with those without L-Cys (t test **$P < 0.01$), but diffusion coefficient did not differ (Fig. 5).

3.5. Stability of edaravone in edaravone/HPβCD complex solution

Stability of edaravone in samples stored in brown glass bottles at 50 °C is shown in Table 2. The mean residual level of edaravone in the sample without inhibitors stored at 50 °C was 93% of the initial level. On the other hand, through the initial 14 days, the mean residual level of edaravone in the samples that contained several inhibitors stored at 50 °C was 100% of the initial level. By day 28, the mean residual level was >99%. Therefore, edaravone was stable under both conditions.

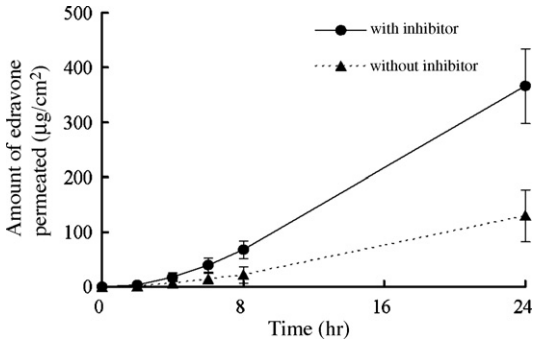

Fig. 4. HR skin permeation profiles of edaravone from edaravone/HPβCD complex with inhibitor (edaravone/HPβCD solution (0.7% edaravone) containing L-Cys shown in Table 2) and without inhibitor (edaravone/HPβCD solution (0.7% edaravone) not containing L-Cys).

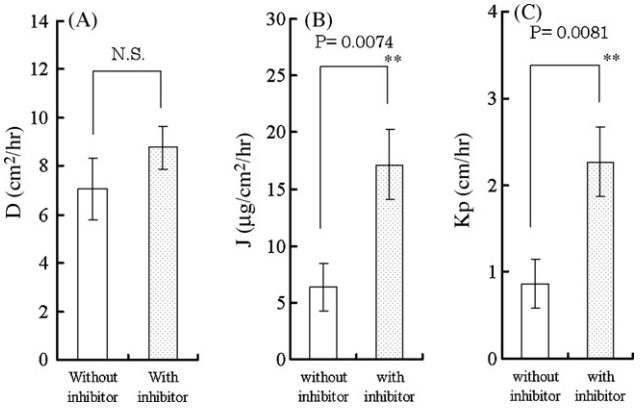

Fig. 5. Effects of inhibitor on edaravone skin permeability from edaravone/HPβCD complex. (A) Diffusion coefficient; (B) permeation rate; (C) permeability coefficient (*t* test $^{**}P < 0.01$, mean ± S.D., $n = 3$).

Table 2
Stability of edaravone in cyclodextrin solution.

	w/v (%)				
	1	2	3	4	5
Edaravone	0.7	0.7	1.0	1.0	2.0
HPβCD	10.0	10.0	20.0	20.0	40.0
SHS	0.1	0.1		0.1	1.2
L-Cys	0.6				0.6
BTA		0.6			
Total volume (ml)	100.0	100.0	100.0	100.0	100.0
50 °C storage	Residual ratio%				
14 days	100.3 ± 0.8	100.7 ± 1.7	93.7 ± 0.6	100.1 ± 0.5	102.5 ± 2.4
28 days	103.2 ± 2.0	103.5 ± 0.2	93.6 ± 1.4	99.8 ± 0.8	99.6 ± 1.8

These samples were added to pH 4.5 citric acid buffer, which was diluted to 1/10, to give a total volume of 100 ml.

4. Discussion

SKF-525A as a non-specific P450 inhibitor inhibits oxidization of P450, but SOD and CAT do not have this effect (Hayashi et al., 2005). The metabolism of edaravone by intravenous administration in the rat does not involve oxidation by P450, and it is mediated by glucuronic acid and sulfuric acid conjugation (Komatsu et al., 1996). It was thought that peak t_R value of around 8.3 min shown in Fig. 2 was glucuronic acid, because glucuronic acid has already been reported to have a peak t_R value of 8–9 min (Komatsu et al., 1996; Sato et al., 2008). On the other hand, IND and DIC have affinity to UGT, but to different UGT family members (Kuehl et al., 2005). In IND, the decrease in peak t_R value of 8–9 min, suggested that the production of the glucuronide may have been inhibited. The metabolism of edaravone in HR skin is considered to be mediated via UGT and reactive oxygen species (ROS) (Sato et al., 2008).

IND, DIC and SKF-525A have radical scavenging actions, which have been reported previously (Prasad and Laxdal, 1994; Towner et al., 2002). However, we examined skin metabolism of edaravone in the presence of compounds already used as pharmaceutical additives because we cannot generally use IND and SKF-525A as pharmaceutical additives. In these experiments, we found that amino acids and azole compounds acted as pharmaceutical additives, and they inhibited skin metabolism of edaravone.

In the skin, L-Cys, MBI and BTA had a higher inhibition effect in a dose-dependent manner than did IND and SKF-525A. For inhibition of edaravone metabolism by L-Cys and BTA, K_m values did not differ significantly compared with the K_m values in the absence of inhibitors, but V_{max} decreased. Therefore, it was thought that these inhibitors acted by non-competitive inhibition.

After intravenous administration of edaravone, keto–enol, glucuronide and sulfate metabolites of edaravone have been reported as detected; therefore, it is thought that metabolism of edaravone is related to keto–enol transformation (Komatsu et al., 1996). Furthermore, 50% of edaravone is present as anions under physiological conditions. Therefore edaravone scavenges various radicals, and this produces the reaction product (2-oxo-3-(phenylhydrazono)-butanoic acid, OPB) which does not have a radical scavenging action (Watanabe et al., 2004). Therefore, in the skin, it is suggested that these drugs inhibited metabolism of edaravone by stabilizing the ketone that is a reactive site of edaravone. However, stability was not thought to play a role in skin permeation of edaravone. On the other hand, sulfhydryl compounds enhance skin permeation of Spantide II, by interacting with keratin in the corneous cell, and that it is thought that the permeation route is transcellular (Babu et al., 2004). Therefore, in skin permeation of edaravone as a radical scavenger, it is necessary to consider inhibition of edaravone metabolism by the skin and interaction between the inhibitor and keratin.

Metabolism of edaravone by ROS is rapid compared with that by the conjugation enzyme UGT. Therefore, for transdermal administration of edaravone, it is thought that metabolism must be minimized following topical treatment in order to increase drug absorption. In the edaravone/HPβCD complex solution, which contained an inhibitor, the edaravone concentration did not decrease for 28 days at 50 °C. Therefore, in this study, it was thought that the metabolic inhibitor was useful for the transdermal administration of edaravone.

5. Conclusion

We suggest that the metabolic inhibitor was useful for the transdermal administration of edaravone. Therefore, transdermal administration of edaravone as a radical scavenger might be a novel therapeutic approach from which to develop a new strategy for the treatment of skin diseases.

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