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Systemic Availability and Pharmacokinetics of Surfactin, a Lipopeptide Produced by *Bacillus subtilis* BC1212 in Rats

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ABSTRACT. The aim of the present study was to evaluate systemic bioavailability of surfactin and to determine its pharmacokinetic profiles. The stability of surfactin to pH, temperature and protease was evaluated. Surfactin was resistant to high temperature, a wide range of pH and the action of hydrolytic enzymes. The pharmacokinetic natures of surfactin which were shown the short half-life, rapid clearance and poor bioavailability. The results of study should provide preliminary data of surfactin for further dose-finding studies and for the design of application forms. It is also be important to a context of the safety of surfactin.

Keywords: Surfactin, *Bacillus subtilis*, Pharmacokinetics, Bioavailability, Stability.

INTRODUCTION

Surfactin is a macrolide lipopeptide produced by *Bacillus subtilis* (Arima *et al.*, 1968). It has a strong surface tension-lowering activity, together with antitumor, fibrinolytic and antiviral activities (Arima *et al.*, 1968; Desai and Banat, 1997; Vollenbroich *et al.*, 1997a; Kikuchi and Hasumi, 2002). In solution, the surfactin exhibits a characteristic 'horse saddle' conformation that accounts for its surfactant activity and its numerous biological properties (Bonmatin *et al.*, 1994; Peypoux *et al.*, 1999). The recent finding of its antimycoplasmic and antiviral activities as well as its forming property attains increasing biotechnological and pharmaceutical interests (Vollenbroich *et al.*, 1997a, b).

Natural surfactin is a mixture of isoforms which slightly differ in their physiological properties due to a variation in the chain length and branching of its hydroxy fatty acid component as well as substitutions of the amino acid components of the peptide ring (Baumgart *et al.*, 1991; Kanatomo *et al.*, 1995; Kowall *et al.*, 1998). These variations, rather than being genetically determined, depend on the specific *B. subtilis* strain (Sullivan, 1998). Originally isolated from soil strain *Bacillus subtilis*, standard surfactin contains a heptapeptide linked via a lactone bond to a β-hydroxy-fatty acid with 14 or 15 carbon atoms (Baumgart *et al.*, 1991; Kanatomo *et al.*, 1995; Kowall *et al.*, 1998). Kanatomo *et al.* (1995) revealed that *Bacillus natto* KMD 2311 contained at least 8 homologus depsipeptides with a *n-, iso, anteiso*-β-hydroxy fatty acids of carbon numbers 13 to 16 as part of the ring system. In the present study, *Bacillus subtilis* BC1212 was isolated from soybean paste and surfactin C was the main component of surfactin which was produced by *Bacillus subtilis* BC1212 (Fig. 1).

Various pharmacodynamic activities for surfactins have been demonstrated *in vitro*, but bioavailability of surfactins has not been proven. In addition, the clinical relevance of theses activities depends on the systemic availability of this compound in the respective target organs. Thus, investigation of absorption, distribution and elimination would provide a significant link between *in vitro* effects and *in vivo* efficacy. It may also be important in context of the safety of surfactins. The objective of study is to evaluate systemic bioavailability of surfactin and to determine its pharmacokinetic profiles after intravenous (i.v.), intramuscular (i.m.), subcutaneous (s.c.)

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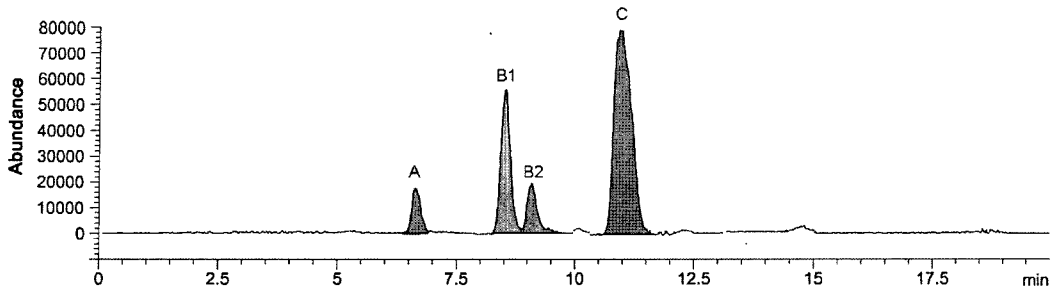

Fig. 1. Separation of surfactin isoforms isolated from *Bacillus subtilis* BC1212 with a mobile phase consisting of 0.1% trifluoracetic acid-acetonitrile. There are three different types of surfactins, A, B and C, which are classified according to the differences in their amino acid sequences. *Bacillus subtilis* BC1212 isolated from soybean paste mainly excreted surfactin C.

and oral administration (p.o.).

MATERIALS AND METHODS

Chemicals

Surfactin as a standard was purchased from Sigma (Missouri, USA). For purification of surfactin isoforms, the surfactin was purified on the analytical reverse column. The purified surfactin isoforms were greater than 90% pure. HPLC grade methanol and acetonitrile were purchased from Mallinckrodt Baker (New Jersey, USA). Other analytical grade chemicals were purchased from Sigma (Missouri, USA).

Bacterial culture and purification of surfactin

Bacillus subtilis BC1212 was isolated from soybean paste and was grown on a shaking incubator (150 rpm) in Erlenmeyer flasks containing 100 ml of the trypticase soy broth during 30 h at 28°C (Korean Patent 10-2004-0092258). Cell-free culture was concentrated by an Vivaspin20 ultra-filtration cell (Viva Science, Hanover, Germany) with polyethersulfone membrane of 3000 molecular weight cut-offs. The concentrated extract was extracted with a mixture of ethyl acetate-methanol (4 : 1), and the organic phase was evaporated to dryness. The crude lipopeptide mixture was purified by gel filtration on silica gel 60 (70~230 mesh, Merck, Germany) with a mixture of n-hexane-acetone (6 : 4) as the eluents. The extract was purified using a preparative reversed phase HPLC column Watcheres 120 ODS-BP (5 μm, 250 × 16 mm, Daiso, Japan) and a HP1100 series HPLC system with mobile phase of 0.1% trifluoroacetic aicd-acetonitrile (10 : 90, v/v). Surfactin was obtained as a yellow powder by lyophilization.

Stability of surfactin to heat, pH and proteolytic enzymes

The surfactin produced by *B. subtilis* BC1212 was analyzed after heat-treatment, treatment with proteolytic enzymes and at different pH values. The concentration of surfactin was determined by LC/MS after treatment. The sample was heated, at 70 and 100°C for 15, 30, 45 and 60 min and at 121°C for 30 min before the determination of surfactin concentration. To test the sensitivity to proteases, 10 mg of surfactin were dissolved in 1 ml of phosphate buffer (pH 8) and then to the solution 0.5 mg of pepsin or trypsin were added. The reaction mixture was kept at 37°C and the concentration of the tested compound was monitored at 2, 4, 8, 12 and 24 h. An untreated surfactin served as controls. To test the influence of pH, the surfactin was adjusted to various pH values ranging from 3 to 10 using either HCl or NaOH, and incubated for 20 min at 37°C. For the assessment of storage stability, the surfactin was kept at room temperature (18°C) for more than 1 months and in frozen state (-20°C) over 3 months.

Animals and sample collections

Male Spraue-Dawlety (SD) rats, weighing between 160 and 220 g at the age of 6~7 weeks, were used for this study. They were obtained from Samtaco Biokorea Co. (Anyang, Korea) and acclimated for one week before experiments in the Animal Environmental Control Unit (temperature, 23 ± 3°C; relative humidity, 50 ± 10%; light-dark cycle, 12 h). Healthy SD rats were randomly allocated into 4 groups of each 4 male rats. To evaluate the bioavailability and pharmacokinetic profiles of surfactin C, a single dose of surfactin extract (25 mg as surfactin C/kg of body weight) was administered to rats. Blood samples were collected in caudal vein at the predetermined time (pre-dosing, 0.25, 0.5, 0.75, 1, 2, 3, 4, 6 and 8 h). The collected blood samples were centrifuged at 6,000 rpm for 10 min for separation of plasma. Immediately separated plasma was stored at -40°C until assay.

Analytical method

Two hundred μl of plasma samples were pipetted into

a polypropylene tube (1.5 ml) and then added 800 μl of methanol. The mixture was shaken for 30 min at room temperature. The sample was centrifuged at approximately 12,000 *g* for 15 min. After centrifugation, the supernatant was injected onto the HPLC column after filtration. Plasma samples were analyzed on a Hewlett-Packard 1100 series LC/MSD system. Separation was achieved on Watchers120 ODS-BP C_{18} reverse phase column (5 μm, 4.6 mm × 150 mm, Daiso, Japan) and equilibrated with 10% solution A (0.1% triflouroacetic acid) and 90% solution B (acetonitrile). The instrument was tuned and optimized for the transmission of the positive ion of surfactin C at *m/z* 1036.7.

Pharmacokinetic analysis

The plasma surfactin C concentration vs. time was analyzed using model-independent standard methods. The elimination rate constant (λ_z) was derived by the unweighted least square regression analysis of at least last four time points of the semi-logarithmic plasma concentration-time curves. The body tissue elimination half-life ($t_{1/2\lambda z}$) was calculated according to $t_{1/2\lambda z} = \ln 2/\lambda_z$. Non-compartmental analysis based on statistical moments was also performed. The area under plasma concentration-time curve (AUC) and the area under the first moment curve (AUMC) were calculated by the method of trapezoids and extrapolation to infinity. The mean residence time (MRT) was determined as: MRT = AUMC/AUC.

RESULTS

Method validation for the determination of surfactin C

As a result of analysis of blank plasma samples, matrix interference was not shown. The peak of surfactin C was shown at about 8.7 min and increased in proportion to its concentrations. The limit of quantitation of surfactin C was 10 ng/ml, the inter-day and intra-day precision (CV, %) was 15% and calibration curve was linear ($r > 0.99$) from 0.01 to 10 μg/ml. The method was successfully utilized for determining the plasma concentration of surfactin in rat plasma.

Stability of surfactin produced by *Bacillus subtilis* BC1212

The sensitivity of surfactin produced by *Bacillus subtilis* BC1212 to heat treatment showed that the concentration of surfactin was not altered by heat treatment after 60 min at 70°C or 100°C. These results suggest that the surfactin is strongly heat resistant. The pH stability of the surfactin in the culture supernatant was studied in the pH range 3~10. The activity remained unchanged at pH values from 3 to 10. To determine the proteinaceous nature of the surfactin, the effects of some proteolytic enzymes (trypsin and pepsin) were tested. Incubation of the samples for 2, 4, 8, 12 and 24 h at 37°C with each of these enzymes was not able to destroy the surfactin. Neither the pH-adjustment, nor the proteolytic enzymes treatment affected the stability of surfactin. Storage of the surfactin at 4°C for more than 2 months and in frozen state over 6 months did also not influence the concentration of surfactin.

Pharmacokinetic analysis

The time course of the surfactin C concentration in rat plasma is shown in Fig. 2. The plasma concentrations of surfactin C following intravenous (i.v.), intramuscular (i.m.) and subcutaneous (s.c.) injection were declined with mean elimination half-life of 0.65 h, 1.45 h and 1.63 h, respectively. The AUC was 57.4 μg·h/ml (i.v.), 1.27 μg·h/ml (i.m.) and 1.53 μg·h/ml (s.c.). After i.v. administration, volume of distribution was 0.13 l/kg and plasma

Table 1. Pharmacokinetic parameters of surfactin C in SD rats after intravenous, intramuscular and subcutaneous administration with 25 mg/kg of body weight

Pharmacokinetic parameters	Units	The routes of administration		
		Intravenous	Intramuscular	Subcutaneous
t_{max}	h		3	3
C_{max}	μg/ml	250.6 ± 23.7	0.29 ± 0.10	0.31 ± 0.08
λ_z	1/h	1.06 ± 0.58	0.48 ± 0.07	0.43 ± 0.12
$t_{1/2\lambda z}$	h	0.65 ± 0.34	1.45 ± 0.34	1.63 ± 0.21
AUC	μg·h/ml	57.4 ± 10.2	1.27 ± 0.57	1.53 ± 0.67
V_z	l /kg	0.13 ± 0.04	-	-
Cl	l /h/kg	0.43 ± 0.07	-	-
MRT	h	0.17 ± 0.05	3.84 ± 1.23	3.80 ± 1.43
V_{ss}	l/kg	0.08 ± 0.02	-	-

† t_{max}, the time to reach peak or maximum plasma concentration; C_{max}, peak plasma concentrations; λ_z, terminal elimination coefficient; $t_{1/2\lambda z}$, terminal elimination half-life; AUC, area under curve; V_z, volume of distribution; Cl, total body clearance; MRT, mean residence time; V_{ss}, volume of distribution at the steady state.

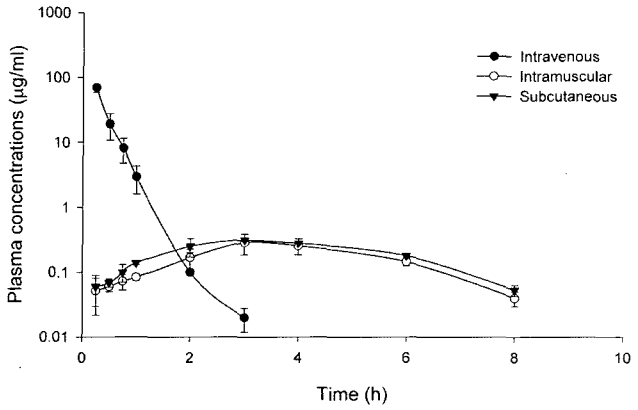

Fig. 2. Plasma concentrations of surfactin C in SD rats after intravenous, intramuscular and subcutaneous administration with 25 mg/kg of body weight. Values represent mean ± SD in 4 rats.

clearance was 0.43 l/h·kg. No surfactin C could be detected in plasma after oral administration.

DISCUSSION

Surfactin, a cyclic lipopeptide produced by *Bacillus subtilis* strains, is one of the other biosurfactants with well-known antimicrobial properties (Vollenbroich *et al.*, 1997a; Singh and Cameotra, 2004). There are three different types of surfactins, A, B and C, which are classified according to the differences in their amino acid sequences. Surfactin-A has L-leucine, surfactin-B has L-valine and surfactin-C has L-isoleucine at the amino acid position involved in lactone ring formation with the C14~C15 β-hydroxy fatty acid (Baumgart *et al.*, 1991; Kanatomo *et al.*, 1995; Kowall *et al.*, 1998; Singh and Cameotra, 2004). *Bacillus subtilis* BC1212 isolated from soybean paste mainly excreted surfactin C. Cyclic peptides are known to be hardly hydrolyzed by common protease because of their unique structures containing D amino acid residues (Grangemard *et al.*, 1999). Bacteria of the genus *Bacillus* are known as producers of a number of peptides with antibiotic properties effective against bacteria, fungi and yeasts and also with a high stability attributable to their structure. Surfactin was also resistant to high temperature, a wide range of pH and the action of hydrolytic enzymes.

Surfactin inhibits fibrin clot formation, induces formation of ion channels in lipid bilayer membranes, inhibits cyclic adenosine monophosphate, inhibits platelet and spleen cytosolic phospholipase A_2 and exhibits antiviral and antitumor activities apart from antifungal and moderate antibacterial properties (Singh and Cameotra, 2004). Surfactin also has antimycoplasmic properties and is active against several viruses, including semliki forest virus, herpes simplex virus (HSV-1 and HSV-2), suid herpes virus, vesicular stomatitis virus, simian immunodeficiency virus, feline calicivirus and murine encephalomyocarditis virus (Vollenbroich *et al.*, 1997a, b). Although these advantages of surfactin, the pharmacokinetic natures of surfactin in the present study which were shown the short half-life, rapid clearance and poor bioavailability limit its potential use as an orally administered therapeutics. In the previous reports, bacitracin, a polypeptide antibiotic produced by *Bacillus lichenformis* was also absorbed very little or not at all from the intestines as demonstrated for rats and swine (Donoso *et al.*, 1970; Froyshov *et al.*, 1986). The main reasons for the low oral bioavailability of peptide drugs are pre-systemic enzymatic degradation and poor penetration of the intestinal mucosa (Hamman *et al.*, 2005). Peroral dosing, the most common route of application for the systemic delivery of drugs, is limited for most peptides due to extensive metabolism and poor mucosal permeability (Lee, 1990). Recently, alternative routes, such as the nasal, buccal, rectal, vaginal, conjunctival and pulmonary routes, are being investigated for systemic delivery of peptides and proteins, since the oral absorption of these compounds is typically poor (LeCluyse and Sutton, 1997). The study for enhancing oral absorption or other delivery systems should be performed to develop the drug containing the surfactin.

In conclusion, the systemic bioavailability study of surfactin C, the main compound of the surfactins produced from *Bacillus subtilis* BC1212, after intravenous, intramuscular, subcutaneous and oral administration of single dose was carried out. Surfactin was stable compound to heat, pH and proteolytic enzyme and was not absorbed from the intestines. The pharmacokinetic natures of surfactin were shown the short half-life, rapid clearance and poor bioavailability restricting its biomedical use, but it is favorable for the viewpoint of toxicity. This study should also provide preliminary data for further dose-finding studies and for the design of application forms.

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