Pharmacological Evaluation of Hydroxypropylcellulose–Ethylcellulose Micro-capsules Containing Piretanide*

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Hydroxypropylcellulose (HPC)–ethylcellulose (EC) microcapsules containing piretanide prepared by a solvent evaporation technique, were evaluated on pharmacokinetic, pharmacodynamic and pharmacological parameters in spontaneously hypertensive rats (SHR).

HPC–EC₁₀ (5:3) microcapsules showed sustained plasma piretanide levels and almost the same AUC (area under the curve) as compared with piretanide solution. Effect of treatment with the microcapsules (single oral administration per day in doses of 10 and 30 mg/kg) and the solution (double oral administration per day in doses of 5 and 15 mg/kg) was examined for 4 weeks on urine volume and urinary electrolytes excretion and blood pressure. The microcapsules and solution induced dose-dependent diuresis throughout the experimental period and a reduction in blood pressure from 2 weeks of the treatment.

HPC–EC₁₀ (5:3) microcapsules containing piretanide were satisfactory as a sustained-release preparation in the light of the anti hypertensive effect even at a half frequency of daily dosing of the solution.

Keywords — microcapsule; piretanide; hydroxypropylcellulose; ethylcellulose; pharmacokinetic comparision; pharmacodynamic comparison; pharmacological comparison; sustained release

Introduction

In recent years, development of sustained-release preparations was stimulated by the need to improve patient compliance. Attempts have been made to obtain ideal formulations showing highly constant and reproducible effects in combination with a reduced frequency of administration. The microencapsulation of solid, active ingredients is an effective method for producing such preparations and has been studied extensively.¹⁻³⁰ Many of the studies on microcapsules, however, have been carried out in vitro, and only a few papers deal with their in vivo evaluation.

We have investigated physicochemical and pharmacokinetic properties of orally active drugs suitable for microencapsulation, and devised simple and reproducible pharmaceutical methods required for this purpose.⁴⁻⁸)

Piretanide, a recently marketed diuretic acting on the loop of Henle, is one of the drugs we have investigated. The in vitro evaluation and pharmacokinetic study of gelatin microcapsules of piretanide in beagle dogs gave satisfactory results as regards their sustained-release effect.⁹) In addition, a pharmacodynamic study in beagle dogs indicated long-lasting effects of gelatin microcapsules of piretanide on the urinary piretanide excretion rate, urinary electrolyte (Na⁺, K⁺, Cl⁻) excretion rate, and urine excretion rate after oral administration.¹⁰)

In the pharmacokinetic study including the gastrointestinal mode of action of the drug, the rate-determining step for the ordinary piretanide tablet was the absorption process and that for the gelatin microcapsules of piretanide was the release process in both rabbits and beagle dogs as regards the transfer of piretanide to blood. In beagle dogs, the gelatin microcapsules showed favorable slow-release properties and similar values in the time to reach the maximum drug concentration (Tₘₐₓ) and elimination rate constant to those in human.¹¹) However, formal-
dehyde used as the membrane-hardening agent in the microencapsulation process with gelatin remains in about 2% of the resultant microcapsules.\textsuperscript{12)} Therefore, we have prepared microcapsules of piretanide with hydroxypropylcellulose (HPC) and ethylcellulose (EC) instead of gelatin.\textsuperscript{13)} The HPC-EC microcapsules of piretanide could control both in vitro and in vivo dissolution rate in beagle dogs by changing the proportion of HPC and EC contents. As compared with the ordinary tablet, the HPC-EC\textsubscript{10} (5:3) microcapsules have favorable sustained release properties with a prolonged $T_{\text{max}}$, low maximum plasma drug concentration ($C_{\text{max}}$), low dissolution rate constant (in vitro and in vivo), almost the same $AUC$, low maximum values in urine and urinary electrolytes excretion rate (acute diuresis, a side effect of the tablet is reduced), and no difference in cumulative urine volume and cumulative electrolytes excretion in beagle dogs.\textsuperscript{13)}

This study aims to evaluate the HPC-EC\textsubscript{10} (5:3) microcapsules of piretanide in terms of the pharmacokinetic, pharmacoodynamic and pharmacological, especially blood pressure, aspects using spontaneously hypertensive rats (SHR).

Materials and Methods

Materials — Piretanide was supplied from Hoechst Japan Ltd. HPC (Nisso HPC-M\textsuperscript{®}) and decaglyceryl hepta oleate (Decaglyn 7-0\textsuperscript{®}) were purchased from Nihon Soda Ltd. and Nikko Chemicals Ltd., respectively. EC having a viscosity grade of 10 cps (EC\textsubscript{10}) was obtained from Wako Pure Chemical Ind., Ltd. Bumetanide (Lunetron Injection\textsuperscript{®}) was obtained from Sankyo Co. The other reagents were of analytical grade.

Preparation of HPC-EC\textsubscript{10} Microcapsules — Microcapsules of 20% piretanide made up with HPC and EC\textsubscript{10} (5:3) were prepared in the same manner as reported previously\textsuperscript{13)} and these microcapsules were spherical particles with a smooth surface. This microencapsulation method aims at preparing dissolution-controlled microcapsules consisting of dispersed piretanide in HPC-EC\textsubscript{10} matrix and is schematized in Chart 1. The particle size of the microcapsules used in the present study ranged from 250 to 1000 $\mu$m.

Animals — Male SHR aged 7 weeks were purchased from Charles River Japan and maintained in cages with 2 animals at 23 °C and 55% of relative humidity. All animals except those for urine collection were allowed free access to a solid diet (CE-2, CLEA Japan Inc.) and tap water.

Measurement of Piretanide Levels in Plasma — Piretanide levels in plasma samples were determined by high performance liquid chromatography (HPLC). A 0.1 ml portion of blood was collected from the tail vein of SHR with a heparinized syringe, mixed with 0.25 ml of heparin (1000 U/ml), and centrifuged at 5000 rpm for 3 min to obtain the supernatant. The plasma piretanide level was determined with 0.2 ml of the supernatant by the method described below. Separately, the plasma/blood ratio of the blood collected from SHR before dosing was measured with a hematocrit capillary tube. To 0.2 ml of the supernatant, 0.5 ml of the internal standard solution (bumetanide, 0.5 $\mu$g/ml), 1 ml of 0.5 N hydrochloric acid and 5 ml of dichloromethane were added, and mixed by shaking for 15 min. After 15 min centrifugation at 3000 rpm, the dichloromethane layer was passed through a silica cartridge (Sep Pak 51900, Waters). The piretanide adsorbed cartridge was washed with 10 ml of dichloromethane, and the cartridge was treated with 5 ml of
Evaluation of Microcapsules of Piretanide

a dichloromethane solution containing acetic acid in 4% (v/v) to elute piretanide. The eluate was evaporated to dryness at 50 °C under N₂ flow until the odor of acetic acid disappeared. The residue was dissolved in 0.25 ml of the mobile phase for HPLC, and this solution was used as the sample solution. The HPLC system consisted of a solvent pump (TRI-ROTAR V, Jasco), an autoinjector (710B, Waters), a 5 μm particle size reversed phase octadecyl column (4.6 mm × 150 mm, Nucleosil C₁₈, Machery & Nagel), a precolumn (Bondapack C₁₈, Corasil, Waters), a fluorescence detector (350 nm excitation wavelength, 430 nm emission fluorescence wavelength, 850, Hitachi), and an integrator (peak height mode, 3392A, Hewlett-Packard). The mobile phase was 1/30 M phosphate buffer (pH 6.5) containing acetonitrile in 45% (v/v). The flow rate was 1.0 ml/min and injection volume was 0.20 ml.

Single Oral Administration for Pharmacokinetic Study — Piretanide solution and microcapsules were orally administered to SHR in a dose of 30 mg/kg by gavage with 2 ml of water. The animals were given only water ad libitum for 18 h before the start of the experiment. Blood was collected from the tail vein for 6 h after the administration.

Training of SHR for Pharmacological Study — After 1-week acclimation period, SHR were fixed in a holder for about 2 h twice a day for 1 week. Then the blood pressure was measured for 1 week, and those showing relatively constant blood pressure were used for the experiment.

Multiple Oral Administration — Piretanide solution (5, 15 mg/kg; twice a day) and microcapsules (10, 30 mg/kg, with 2 ml of water, once a day) were orally administered to SHR by gavage for 4 weeks. In the experiments on the plasma renin activity and aldosterone level, both dosage forms were dosed for 5 weeks. Control rats received placebo in a similar manner.

Measurement of Blood Pressure and Heart Rate — SHR were left at 30 °C for 15 min, fixed in a holder, and their mean systolic blood pressure and heart rate measured with a tail-cuff apparatus (DSR 801A, Toiden). The blood pressure and heart rate were measured seven times, and the mean was calculated from medial three values.

Measurement of Urine Volume and Urinary Electrolytes (Na⁺, K⁺ and Cl⁻) Levels — The animals were loaded with 25 ml/kg body weight of water just after dosing and kept individually in metabolic cages to collect urine for 6 h. The urinary Na⁺, K⁺ and Cl⁻ levels were measured by the ion selective electrode method with an automated Na⁺, K⁺ and Cl⁻ analyzer (Sera 300A, Horiba).

Measurement of Plasma Renin Activity — Piretanide solution (5, 15 mg/kg; twice a day) and microcapsules (10, 30 mg/kg; once a day) were daily administered to SHR for 5 weeks. Two hours after the last administration, the animals were anesthetized with pentobarbital, and blood was collected directly from the heart immediately using a syringe moistened with 15% ethylenediaminetetraacetic acid diammonium salt (EDTA·2NH₄). The blood was centrifuged at 3000 rpm for 10 min, and the plasma obtained was kept frozen until the measurement.

The plasma was incubated at 37 and 4 °C, and the plasma renin activity was calculated by subtracting the amount of angiotensin I formed at 37 °C from the formed at 4 °C and recorded as the mean of two measurements. Angiotensin I was determined by radioimmunoassay (RIA, Renin·Riabead, Dainabot) using 125I.

Measurement of Plasma Aldosterone Level — The radioactivity of the 125I-labeled aldosterone combined with aldosterone antibodies previously added to the plasma obtained in the above paragraph was measured to calculate the antigen–antibody binding ratio (%), and the plasma aldosterone level was read from the standard curve (Aldosterone·RIA Kit II, Dainabot) and recorded as the mean of two measurements.

Pharmacokinetic Study — The pharmacokinetic parameters were calculated by the non-linear least-squares method.¹⁴

Statistical Analysis — Data on the cumulative urine volume, cumulative urinary electrolytes amount, blood pressure, plasma renin activity and plasma aldosterone level were analyzed by Student’s t-test to evaluate differences among the groups given the micro-
Fig. 1. Plasma Piretanide Levels after Single Oral Doses of HPC-EC<sub>10</sub> (5:3) Microcapsules (●, 30 mg/kg, n = 5) and Solution (○, 30 mg/kg, n = 5) in SHR.
Each point represents the mean value and each vertical line, the standard error.

capsules, the solution and placebo.

**Results and Discussion**

**Plasma Piretanide Level**

**Single Oral Administration** — Figure 1 shows the time course of plasma piretanide level in SHR after oral administration of the HPC-EC<sub>10</sub> (5:3) microcapsules and solution of piretanide in a dose of 30 mg/kg. The area under the concentration–time curve for 0—∞ h (AUC<sub>0—∞</sub>), maximum plasma piretanide concentration (C<sub>max</sub>), time to reach the maximum plasma piretanide concentration (T<sub>max</sub>) and mean residence time for 0—∞ h (MRT<sub>0—∞</sub>) were 5.36 h·μg/ml, 1.00 μg/ml, 2.0 and 4.68 h, respectively, for the microcapsules and 5.32 h·μg/ml, 1.88 ·μg/ml, 1.0 and 2.20 h, respectively, for the solution. The AUC<sub>0—∞</sub> for the microcapsules was 0.99-fold that for the solution, but desirable plasma piretanide levels lasted for a prolonged time after microcapsule dosing, unlike the pattern obtained with the solution.

**Multiple Oral Administration** — Figure 2

![Graph showing excreted urine volume after oral administration of HPC-EC<sub>10</sub> (5:3) microcapsules, solution and control in SHR.](image)

**Fig. 3.** Excreted Urine Volume after Oral Administration of HPC-EC<sub>10</sub> (5:3) Microcapsules, Solution and Control in SHR.

A. □, control; □, solution, 5 mg/kg × 2/d; □, solution, 15 mg/kg × 2/d.
B. □, control; □, microcapsule, 10 mg/kg/d, □, microcapsule, 30 mg/kg/d. The vertical line indicates the standard error. a) p < 0.05 and b) p < 0.01 compared with control.
shows the time course of plasma piretanide levels after 4 week consecutive administration each of the microcapsules (30 mg/kg, once daily) and the solution (15 mg/kg, twice a day). The pattern of the plasma piretanide level after 4 week dosing of the microcapsules was nearly the same as that after the single dosing of the microcapsules. Though the dosage level of the solution in the multiple oral administration was half the amount of the single dosing, the plasma piretanide level after the 4 consecutive week dosing was nearly half that after the single dosing and the pattern of plasma piretanide levels was nearly equal. At a dose level of 10 mg/kg for the microcapsules and 5 mg/kg for the solution, complete plasma piretanide level–time curves could not be drawn because the plasma piretanide level was under the detection limit (70 ng/ml) in some samples.

**Pharmacodynamic Study**

Figures 3—6 show the cumulative urine excretion volume and urinary electrolytes amounts ($\text{Na}^+$, $\text{K}^+$, $\text{Cl}^-$) for 6 h after the administration of the microcapsules (30, 10 mg/kg; once daily) and the solution (15, 5 mg/kg; twice a day) to SHR at every 2 weeks during the 4-week administration period. The results at 1 and 3 weeks were nearly equal to those at the initial and at 2 weeks, respectively. The total urine excretion volume and urinary electrolytes
Fig. 6. Urinary Chloride Excretion after Oral Administration of HPC-EC₁₀ (5:3) Microcapsules, Solution and Control in SHR

A. □, control; ☐, solution, 5 mg/kg × 2/d; ☐, solution, 15 mg/kg × 2/d.

B. □, control; ☐, microcapsule, 10 mg/kg/d, ☐, microcapsule, 30 mg/kg/d. The vertical line indicates the standard error. a) $p < 0.01$ compared with control.

amounts (Na⁺, K⁺, Cl⁻) increased dose-dependently at each measuring point as compared with those in the control group. However, the increase in the urinary K⁺ amount was less than those in the other electrolytes, which accorded well with the results in beagle dogs reported previously.\(^{13}\)

Pharmacological Study

Fig. 7. Blood Pressure after Multiple Doses of HPC-EC₁₀ (5:3) Microcapsules (●, 30 mg/kg/d, $n = 6$; ▲, 10 mg/kg/d, $n = 6$), Solution (○, 15 mg/kg × 2/d, $n = 6$; △, 5 mg/kg × 2/d, $n = 6$) and Control (□) in SHR

Each point represents the mean and each vertical line, the standard error. a) $p < 0.05$ and b) $p < 0.01$ compared with control.
The blood pressure, heart rate and body weight were measured daily from 2 to 5 h after the administration of the microcapsules (30, 10 mg/kg; once daily) and the solution (15, 5 mg/kg; twice a day) to SHR for the first 4 weeks of the 5-week administration period. The plasma renin activity and plasma aldosterone level were determined just after the last administration at 5 weeks. These parameters were measured also in the control group.

Heart Rate and Body Weight — The heart rate was kept between 400 and 430 beats/min for the first 4 weeks of the administration period, and there was no difference in the heart rate among the groups given placebo, the microcapsules and the solution. The body weight was about 250 g at the start of the experiment and increased, as the experiment went on, up to about 300 g at 4 weeks. No difference was observed among the control, microcapsule and solution groups.

Blood Pressure — Figure 7 shows the time course of the blood pressure in SHR orally given the microcapsules and solution for 4 weeks. The blood pressure raised gradually from the initial value of about 170 mmHg to about 210 mmHg at 4 weeks in the control group. However, the microcapsule and solution groups showed suppressed development of hypertension and, from 3 weeks onward, significantly lower mean blood pressure than the control group in each dosage group in a dose-dependent manner. Though the mean blood pressure in the microcapsule group was generally lower than that in the solution group, no significant difference was seen between the two groups. At any measuring point after the dosing, the mean blood pressure did not fall significantly as compared with the value before the dosing.

The antihypertensive effect of piretanide owes mainly to diuretic effect-based decreases in circulating blood volume and cardiac output, but there are other possible factors \( ^{13} \) such as direct vascular action and declined response to endogenous tensor substance \( ^{16, 17} \). Nevertheless, the mechanism of such antihypertensive effect has not completely been elucidated. Anyway, the present results suggest that the once-a-day dosing of the microcapsules exhibits equal suppressive effect on the development of hypertension to the twice-a-day equivalence dosing of the solution.

Plasma Renin Activity and Plasma Aldosterone Level — Figures 8 and 9 show the plasma renin activity and plasma aldosterone level, respectively, at 2 h after the last administration of the microcapsules and solution to SHR in the 5-week administration period. The plasma renin activity and plasma aldosterone level in the microcapsule group increased dose-dependently as compared with the control group and showed no difference between the groups.

![Fig. 8. Plasma Renin Concentration after Multiple Doses of HPC-EC\(_{10}\) (5:3) Microcapsules (n = 6) and Solution (n = 6) for 5 Weeks in SHR](image1)

- Control: \( \square \)
- Solution, 5 mg/kg × 2/d: \( \square \)
- Solution, 15 mg/kg × 2/d: \( \square \)
- Microcapsule, 10 mg/kg/d: \( \square \)
- Microcapsule, 30 mg/kg/d: \( \square \)

The vertical line indicates the standard error. \( a) \) \( p \leq 0.01 \) compared with control

![Fig. 9. Plasma Aldosterone Concentration after Multiple Doses of HPC-EC\(_{10}\) (5:3) Microcapsules (n = 6) and Solution (n = 6) for 5 Weeks in SHR](image2)

- Control: \( \square \)
- Solution, 5 mg/kg × 2/d: \( \square \)
- Solution, 15 mg/kg × 2/d: \( \square \)
- Microcapsule, 10 mg/kg/d: \( \square \)
- Microcapsule, 30 mg/kg/d: \( \square \)

The vertical line indicates the standard error. \( a) \) \( p \leq 0.01 \) compared with control.
given the microcapsules and the solution. As the piretanide-induced increase in the plasma renin activity accorded well with the report of Unger et al.,\textsuperscript{15} the increase is considered to be attributed to the diuretic effect-based decrease in the circulating blood volume. The increased plasma aldosterone level seems to be due mainly to the renin–angiotensin–aldosterone system.

**Conclusion**

Piretanide, an expected hypotensive diuretic, was successfully microencapsulated with HPC and EC\textsubscript{10}. The HPC–EC\textsubscript{10} microcapsules of piretanide were concluded to have favorable sustained release properties for the following reasons: (1) the time to reach the maximum plasma piretanide concentration (\(T_{max}\)) was prolonged after the administration of the microcapsules over that of the solution used as the standard; (2) the maximum plasma piretanide concentration (\(C_{max}\)) after the microcapsule dosing was lower than that of the solution; (3) the \(AUC^*\)'s for the microcapsules and the solution were almost equal; (4) the cumulative urine volume and cumulative electrolytes excretion from the microcapsules, as well as the solution, increased dose-dependently as compared with those in the control group; and (5) the microcapsules as well as the solution suppressed the development of hypertension in SHR dose-dependently as compared with the control group, exhibited similar antihypertensive action to that of the solution at a half frequency of the dosing of the solution, and showed a significant difference in the mean blood pressure from the control group at 2 weeks onwards after the dosing.

Furthermore, microencapsulation of piretanide using HPC–EC\textsubscript{10} will surely contribute to improvement of patient compliance from the fact that almost equal pharmacological effects (antihypertensive effect, renin activity, aldosterone level) to those by twice-a-day administration of the solution can be obtained by once-a-day administration of the microcapsules.

In our future studies, effects of microencapsulated piretanide in human subjects will be investigated.

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