Pharmacokinetics and Biotransformation of Beraprost Sodium I: Plasma Level Profile of Beraprost Sodium in Rat.

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Summary
Beraprost sodium (TRK-100: Sodium (±)-(1R*, 2R*, 3aS-, 8bS)-2, 3, 3a, 8b-tetrahydro-2-hydroxy-1-[(E)-(3S*)-3-hydroxy-4-methyl-l-octen-6-ynyl]-1H-cyclopenta[b]benzofuran-5-butyrate) labeled with tritium was orally and intravenously administered to male and female rats to examine plasma level profile and metabolism.

After oral administration at doses of 0.04, 0.2 and 1.0mg/kg to male rats, concentration of the unchanged drug achieved maxima at 10-30min (18.4±11.4, 42.7±15.9 and 220.5±68.5ng/ml, respectively), and then declined biphasically. AUC at a dose of 0.2mg/kg was 98.2±23.7ng•hr/ml, which accounted for 81% of AUC after intravenous injection at the same dose. In female rats, higher concentrations were observed than in male.

Metabolites found in plasma were 2,3-dinor-beraprost, 13, 14-dihydro-15-oxo-beraprost and 13,14-dihydro-2,3-dinor-15-oxo-beraprost. Among the three metabolites, the 2,3-dinor-beraprost showed the highest AUC and Cmax, and thus seemed to be a major metabolite of beraprost sodium.
Introduction

Prostacyclin (PGI₂) is a potent vasodilator and an inhibitor of platelet aggregation. It is, however, chemically unstable and rapidly hydrolyzed to inactive 6-oxo-PGF₁α even in buffer solutions or physiological saline. The instability has limited clinical use of PGI₂. Therefore extensive efforts have been made to synthesize stable analogs of PGI₂ which exhibit same features as the natural product.

Beraprost sodium (BPS) has been reported to be a chemically stable and potent analogue of PGI₂. Therefore, we were interested in its pharmacokinetics. In this article, we report on the plasma levels of beraprost sodium and some metabolites in rats.

Materials and Methods

1. Materials.

³H-Beraprost sodium (³H-BPS) was synthesized by the NEN Research Products (USA) according to a synthetic route shown in Scheme 1. Specific activity of the ³H-beraprost sodium thus synthesized was 46.8 mCi/mg and radiochemical purity was more than 98% by TLC (Merck art No. 5715; 99.07% [chloroform/methanol/acetic acid = 85/15/0.5], 98.11% [chloroform/ethyl acetate/ethanol/acetic acid = 20/20/4/1] and 98.06% [upper layer of ethyl acetate/water/iソooctane/acetic acid = 110/100/40/20]) and HPLC methods. Unlabeled beraprost sodium was prepared by the Basic Research Laboratories of Toray. D₃-Beraprost sodium (D₃-BPS) was used in identification of metabolites. APS-308 ((±)-(1R*, 2R*, 3aS*, 8bS*)-2, 3, 3a, 8b-tetrahydro-2-hydroxy-1-[(E)-(3S*)-3-hydroxy-3-cyclopentyl-1-propenyl]-1H-cyclopenta[b]benzofuran-5-butyrate)-labeled with tritium was used as internal standard for plasma analysis. These compounds were also synthesized by the laboratories. Other reagents and solvents were obtained from commercial sources. The solvents were of HPLC grade, and were used without further purification.

2. Animals and Dosing.

Male and female rats of Wistar strain were purchased from Japan SLC, Inc., and were housed in temperature- and humidity-controlled rooms (24 ± 2°C, 55 ± 10%) with 12 hr light-dark cycles for more than a week. The animals were given laboratory chow (MF or NMF, Oriental Yeast, Japan) and water ad libitum, and were fasted for 18
hours before and 4 hours after dosing.

A group of five or four rats (8 weeks old) was used in experiments for measurement of plasma levels. $^3$H-Beraprost sodium was diluted to specific activity of 22.0 mCi/mg with unlabeled beraprost sodium for dosing of 1.0 mg/kg, and was used without dilution for dosing of 0.2 and 0.04 mg/kg. In experiments on oral dosing, an aqueous solution of the drug was given to male rats at doses of 0.04, 0.2 and 1.0 mg/10 ml/kg, and to female rats at a dose of 0.2 mg/10 ml/kg. Blood samples were withdrawn from jugular vein at 10, 30 min, 1, 2, 3, 4, 6, 10 and 24 hr using heparinized syringe, and were immediately centrifuged to obtain plasma. In intravenous dosing, the drug in 0.9% saline was injected into tail vein of male and female rats at a dose of 0.2 mg/5 ml/kg. Blood samples were collected at 5, 15, 30 min, 1, 2, 4, 6, 10 and 24 hr, and treated as mentioned above. All samples were stored at -20°C until analysis.

In another experiment, in order to accumulate an enough amount of metabolites, 51 male rats were given a 1:1 mixed solution of D$_3$-beraprost sodium and unlabeled beraprost sodium in saline (0.9%) at a dose of 1.0 mg/kg by injection into tail vein. At 30 min after dosing, blood was withdrawn from jugular vein, and then centrifuged to obtain plasma. The pooled plasma was stored at -20°C until isolation of metabolites.


Radioactivity of all samples was determined with liquid scintillation counters (Packard 1500). Quenching was corrected by an external standard method. An aliquot of plasma samples was solubilized using Soluene 350 (Packard) in isopropanol (50% v/v, 0.2 ml), decolorized (30% H$_2$O$_2$, 0.1 ml) and acidified with 1N HCl. Radioactivity of the samples was counted in AQUASOL-2 (NEN). Eluent from HPLC was directly counted in AQUASOL-2.

4. HPLC Analysis of Plasma.

Procedure of the plasma analysis was illustrated in Scheme 2. An aliquot of the radioactive plasma was mixed with $^3$H-APS-308 as an internal standard for a calculation of concentration and unlabeled beraprost sodium and APS-308 as carriers of radioactive materials. The plasma was adjusted to pH 3 with 1N HCl, and was diluted with water. And then, the sample was applied to SEP-PAK C18 cartridge (Waters) which was pre-washed with methanol and then with water containing 0.1% of acetic acid. Following sample application, the cartridge was washed with 5 ml of water, and radioactive materials were recovered with 3 ml of methanol. The methanol eluent was concentrated under nitrogen stream. The resulting residue was applied to re-
Scheme 2 Flow chart of the plasma analysis.

Table I HPLC conditions for the plasma analysis and the isolation of metabolites.

<table>
<thead>
<tr>
<th>Column</th>
<th>Mobile phase</th>
<th>Flow rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC-1 YMC PACK A-212 (C8) (150mm × 6mm id)</td>
<td>MeOH/Water/AcOH 0~65min (46:54:1)</td>
<td>1 ml/min</td>
</tr>
<tr>
<td></td>
<td>65~110min (53:47:1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>110~125min (62:38:1)</td>
<td></td>
</tr>
<tr>
<td>HPLC-2 YMC PACK A-K03 (Chiral polymer) (250mm × 4.6mm id)</td>
<td>n-Hexane/EtOH/Water (100:2:0.01)</td>
<td>1 ml/min</td>
</tr>
<tr>
<td>HPLC-3 YMC PACK A-222 (C8) (150mm × 10mm id)</td>
<td>MeOH/Water/AcOH (58:42:0.1)</td>
<td>2 ml/min</td>
</tr>
<tr>
<td>HPLC-4 YMC PACK A-212 (C8) (150mm × 6mm id)</td>
<td>MeOH/Water/AcOH (55:45:0.1)</td>
<td>1 ml/min</td>
</tr>
<tr>
<td>HPLC-5 YMC PACK A-012 (Silica) (150mm × 6mm id)</td>
<td>n-Hexane/EtOH (95:5)</td>
<td>1 ml/min</td>
</tr>
</tbody>
</table>
verse phase HPLC. HPLC eluent was collected with the fraction collector. A part of the fractions of $^3$H-beraprost was sampled for further analysis of metabolite eluted along with the unchanged drug, and radioactivity remained in all fractions was measured. The part of $^3$H-beraprost fractions thus sampled were combined, methylated with diazomethane and extracted with ethyl acetate. After concentration of the extract, the resulting residue was further analyzed by HPLC to obtain ratios of methylated $^3$H-beraprost and metabolite. Instrument of HPLC consisted of LC-6A or LC-3A system (Shimadzu), 7125 sample injector (Rheodyne) and FRAC-100 fraction collector (Pharmacia). HPLC columns and mobile phase were summarized in Table I.

Concentrations of the unchanged drug were calculated from radioactivities in HPLC fractions of $^3$H-beraprost using calibration lines which were prepared as follows. Plasma samples containing known amounts of $^3$H-beraprost sodium and the internal standard were analyzed as mentioned above, and the weight ratios of beraprost sodium to the standard were plotted against ratios of radioactivity measured. A regression analysis of the plot by least-square method gave parameters of the line which allowed to determine unknown concentrations. The concentrations of the unchanged drug were calculated as that of beraprost sodium. Concentrations of metabolites were also evaluated from ratios of radioactivity to the internal standard using the calibration lines for beraprost sodium.

5. Pharmacokinetic Analysis.

Values of Cmax and Tmax were directly read from concentration data. Values of area under the concentration-time curve (AUC) were calculated by trapezoidal method. Half-lives were determined using a computer program for a nonlinear regression analysis (MULTI) reported by K. Yamaoka. The concentrations were best fitted by 2-compartment model or 3-compartment model. These parameters were tested for statistical significance by Student's t-test.

6. Isolation of Metabolites.

The plasma collected after dosing of the mixture of D$_3$-beraprost sodium and beraprost sodium was applied to SEP-PAK C18 cartridges in a similar manner to the analysis of plasma. One cartridge was used for treatment of 10ml of plasma. After sample application, the each cartridge was washed with 20ml of water and then with 7.5ml of methanol. The methanol eluent.

![Scheme 3 Isolation of the metabolites in plasma.](image)
was washed with 5ml of n-hexane. The methanol solutions were combined and evaporated to dryness under reduced pressure. Metabolites in the resulting residue were isolated by HPLC as shown in Scheme 3. Shimadzu LC-4A HPLC system was used in this experiment and HPLC conditions were shown in Table I.

7. Derivatization Procedure.

The methyl derivatives were prepared with an ethereal diazomethane at ambient temperature. Trimethylsilyl (TMS) derivatives for GC–MS were prepared by treatment with N,O-bis(trimethylsilyl)trifluoroacetamide in pyridine at 50°C for 30min.

8. Mass spectrometry.

GC–MS was performed on D300 mass spectrometer (JEOL, Japan) equipped with a glass column (1m x 3mm id) packed with 2% OV–17 on Gaschrom Q (80/100mesh). Mass spectrometry of the methylated metabolites was performed by direct injection on DX303 mass spectrometer (JEOL, Japan). In the both measurements, the ion source temperature was 200°C, the ionizing voltage was 70 volts, the ionizing current was 300 μA and the ion acceleration potential was 3kV.

Results

1. Plasma Levels.

Following oral administrations of 3H–beraprost sodium to male rats at the doses of 0.04, 0.2 and 1.0mg/kg, peak levels of total radioactivity occurred at 30–60 min (26.5 ± 11.5, 67.9 ± 17.8 and 359.6 ± 93.5ng eq. of BPS/ml, respectively). Concentrations of the unchanged drug achieved maxima at 10–30 min, and then declined biphasically. Pharmacokinetic parameters of the unchanged drug were summarized in Table II. In experiment at a dose of 1.0mg/kg, two of five rats showed extremely longer half-lives of β–phase (160hr and 36hr), while the
half-lives of the others were ranging from 2.6hr to 4.8hr. The values of AUC after 24hr were estimated by exponential extrapolation were less than 5% of AUCo−24hr except for AUC of the two animals exhibiting longer half-lives at a dose of 1.0mg/kg. After intravenous injection at a dose of 0.2mg/kg to male rats, plasma level of total radioactivity was 325.0 ± 34.9ng eq. of BPS/ml at 5min. Concentration of the unchanged drug showed 299.5 ± 42.0ng/ml at 5min, which accounted for 92% of the total radioactivity, and then fallen to 0.060 ± 0.032ng/ml by 24hr with tri-phasic decline (Fig. 3).

In female rats, after oral dosing at a dose

Fig. 4 Concentration of beraprost sodium in the plasma after oral administration or intravenous injection of 3H-beraprost sodium to female rats (mean±s.d., n=4 or 5).

| Table II Pharmacokinetic parameters of beraprost sodium after oral administration or intravenous injection of 3H-beraprost sodium to male and female rats (mean±s.d., n=4 or 5) |
|---|---|---|---|---|---|---|---|---|
| Sex | Dose | Cmax (ng/ml) | Tmax (min) | AUC0−24hr (ng•hr/ml) | T1/2Ka*(hr) | T1/2α* (hr) | T1/2β*(hr) | T1/2γ*(hr) |
| Male | 0.04mg/kg p.o. | 18.4 ± 11.4 | 18 ± 11 | 18.0 | 0.052 | 0.41 | 5.9 |
| | 0.2mg/kg p.o. | 42.7 ± 15.9 | 26 ± 9 | 98.2 ± 23.7 | 0.18 ± 0.05 | 0.54 ± 0.22 | 6.6 ± 3.1 |
| | 1.0mg/kg p.o. | 220.5 ± 68.5 | 30 ± 0 | 496 ± 50 | 0.12 ± 0.06 | 0.47 ± 0.29 | 42 ± 68 |
| | 0.2mg/kg i.v. | 121.5 ± 5.5 | 0.10 ± 0.02 | 0.43 ± 0.19 | 5.3 ± 1.1 |
| Female | 0.2mg/kg p.o. | 113.6 ± 36.4 | 26 ± 9 | 453 ± 167 | 0.16 ± 0.09 | 0.64 ± 0.55 | 4.0 ± 0.2 |
| | 0.2mg/kg i.v. | 350.3 ± 53.1 | 0.30 ± 0.03 | 3.3 ± 0.3 |

*) These parameters were obtained from a nonlinear regression analysis to fit following equations.
Cp= Ae−αt+B e−βt− (A+B)e−κt (for oral administration)
Cp= Ae−αt+B e−βt+C e−γt (for intravenous injection to male rats)
Cp= Ae−αt+B e−βt (for intravenous injection to female rats)
Fig. 5 Radiochromatogram of the plasma at 30 min after intravenous injection of $^3$H-beraprost sodium to male rat (dose: 0.2mg/kg).

Table III Chemical structures of the metabolites present in the plasma

<table>
<thead>
<tr>
<th>Chemical structures</th>
<th>M$^+$ in mass spectrum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Me derivatives</td>
</tr>
<tr>
<td>2, 3-Dinor-beraprost (D1, D2)</td>
<td>m/z 384</td>
</tr>
<tr>
<td></td>
<td>$C_{22}H_{26}O_5=370.4$</td>
</tr>
<tr>
<td>13, 14-Dihydro-2, 3-dinor-15-oxo-beraprost (P2)</td>
<td>m/z 384</td>
</tr>
<tr>
<td></td>
<td>$C_{22}H_{26}O_5=370.4$</td>
</tr>
<tr>
<td>13, 14-Dihydro-beraprost (P3)</td>
<td>m/z 412</td>
</tr>
<tr>
<td></td>
<td>$C_{24}H_{30}O_5=398.5$</td>
</tr>
</tbody>
</table>
of 0.2 mg/kg, peak level of radioactivity was 153.7 ± 35.4 ng eq. of BPS/ml at 10–30 min. The unchanged drug reached maxima at 10–30 min (113.6 ± 36.4 ng/ml), and then decreased biphasically. After intravenous injection at the same dose, radioactivity level in plasma was 354.9 ± 14.8 ng eq. of BPS/ml at 5 min. The unchanged drug accounted for 91% of the radioactivity (323.4 ± 35.0 ng/ml) at 5 min after dosing, and then eliminated biphasically (Fig. 4). Pharmacokinetic parameters of the unchanged drug in female rats were also shown in Table II.


Typical radiochromatogram of plasma was illustrated in Fig. 5. Beraprost sodium is a mixture of two racemates which can be separated by HPLC as shown in the chromatogram. Two major peaks found around 58 min and 64 min were identified as 2,3-
dinor-beraprost, $\beta$-oxidation products of the two racemates of beraprost sodium, by HPLC and mass spectrometry. The metabolites were also present in urine and feces, and the structural assignment of the metabolites will be described in a subsequent article on urinary metabolites$^8$. Furthermore, two of the other minor metabolites

Fig. 8 Mass spectrum of the methylated P2.

Fig. 9 Mass spectrum of the methylated and trimethylsilylated P2.
were isolated and the structures were determined. Table III showed the structures of metabolites.

**Metabolite P3.** In mass spectrum of methylated P3 (Fig. 6), ion pair derived from D₀⁻ and D₃⁻ substances was observed at m/z 412 and m/z 415. In GC-MS of methyl and trimethylsilyl derivative, the ion pair shifted to m/z 484 and m/z 487 (Fig. 7). Difference by the trimethylsilylation was 72 amu, which indicates the presence of only one hydroxyl group. Further-
more, P3 exhibited reactivity to methoxylamine, which suggests the presence of a carbonyl group. Thus, P3 was elucidated to be 13,14-dihydro-15-oxo-beraprost. The spectra of P3 were identical with those of the synthetic standard except for several differences due to D3-material. Thus the structure was confirmed.

**Metabolite P2.** In mass spectrum of methylated P2, the ion pair was found at m/z 384 and m/z 387 (Fig. 8). Mass spectrum of methyl and trimethylsilyl derivative showed the ion pair at m/z 456 and m/z 459 (Fig. 9). The values corresponded to M+ of the derivatives of 13,14-dihydro-2,3-dinor-15-oxo-beraprost, a product of β-oxidation of P3. Mass spectra of synthetic standards of this compound and the spectra of P2 were identical except for several differences due to D3-material, and thus the structure was confirmed.

Fig.10 and Fig.11 illustrated time-courses of concentrations of the metabolites in plasma, and their pharmacokinetic parameters were summarized in Table IV. Among the three metabolite thus identified, 2,3-dinor-beraprost showed the highest Cmax and AUC.

**Discussion**

In the present article, we sought to clarify plasma levels of beraprost sodium and its metabolite. After oral dosing to male rats, maximum concentrations of the unchanged drug were observed at 10-30min. The results suggest that 3H-beraprost sodium given orally is absorbed rapidly. The values of Cmax after oral administrations to male rats at doses of 0.04, 0.2 and 1.0 mg/kg were not correlated with the doses administered. This may be mainly due to an inhibitory effect of beraprost sodium on gastric emptying which is observed at higher doses than 0.2mg/kg9). On the other hand, the mean values of AUC after oral dosing at doses of 0.04, 0.2 and 1.0mg/kg to male rats were well correlated with the doses administered, although two animals given at a dose of 1.0mg/kg exhibited extremely larger values. Furthermore, there was no significant difference between the AUC after oral dosing and the AUC after intravenous injection at a dose of 0.2mg/kg. The result suggests that 3H-beraprost sodium given orally is absorbed nearly completely.

The structures of the metabolites in plasma indicate that beraprost sodium is metabolized by β-oxidation of α-side chain, oxidation of hydroxyl group at C15-position and hydrogenation of double bond at ω-side chain. These metabolic pathways are
the same as those of natural PGI2 reported by Sun and Taylor\textsuperscript{10,11}. Among the three metabolites, 2,3-dinor-beraprost exhibited the highest AUC and Cmax, and thus seemed to be a major metabolite of beraprost sodium. We have been further investigating the metabolism of beraprost sodium, and will report the results in the subsequent article.

In female rats, the higher concentrations of the unchanged drug were observed. There was no significant difference in the value of T\textsubscript{max} and half-lives after oral administration between both sexes. On the other hand, in experiments on intravenous injection, the elimination rate of the unchanged drug at early phase was slower in female than in male. We suspect that metabolic capacity or biliary excretion for beraprost sodium would differ between both sexes in rats, and that the higher concentrations observed in female may be caused by the difference. With respect to this, we are going to investigate metabolic pattern of liver and bile in both sexes.

Acknowledgments

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References