Sensitive Determination of 4-(4-Bromophenyl)-4-hydroxypiperidine, a Metabolite of Bromperidol, in Rat Plasma by HPLC with Fluorescence Detection after Pre-column Derivatization Using 4-Fluoro-7-nitro-2,1,3-benzoxadiazole

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The purpose of this study was to determine the level of 4-(4-bromophenyl)-4-hydroxypiperidine (BPHP), a bromperidol (BRO) metabolite, in rat plasma by HPLC with fluorescence detection after pre-column derivatization using 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). After basic extraction of the samples with benzene, derivatization with NBD-F was conducted in borate buffer (pH 8.0) at 60 °C for 3 min. Mexiletine was utilized through the procedure as an internal standard (IS). Retention times of the BPHP and IS derivatives were 7.7 and 11.5 min, respectively. The regression equation for BPHP showed good linearity in the range of 0.01—1 mg/ml with the detection limit of 0.003 μg/ml. The coefficient of variation was less than 12.0%. The recovery was satisfactory. This method was applied for a pharmacokinetic study of BPHP in comparison with 4-(4-chlorophenyl)-4-hydroxypiperidine (CPHP), the corresponding haloperidol (HAL) metabolite, in rats. The ratio of the area under the plasma concentration curve (AUC) after *p.o.* administration of BPHP to the AUC after i.p. administration of BPHP (46%) was lower than that of CPHP (56%), indicating that intestinal absorption of BPHP is lower than that of CPHP. The ratio of BRO metabolism to BPHP (48%) was 1.8-fold higher than that of HAL metabolism to CPHP (27%); the ratio was estimated as $(AUC_{p.o.,A\rightarrow B}/AUC_{p.o.,B})\times100$, where $AUC_{p.o.,A\rightarrow B}$ is the AUC value of BPHP or CPHP after *p.o.* administration of BRO or HAL, and $AUC_{p.o.,B}$ is the AUC of BPHP or CPHP after administration of BPHP or CPHP, respectively. Our method provides a sensitive procedure for determination of BPHP in rat plasma and is suitable for pharmacokinetic studies of BPHP after BRO administration.

Key words   4-(4-bromophenyl)-4-hydroxypoperidine; bromperidol; derivatization; 4-fluoro-7-nitro-2,1,3-benzoxadiazole; pharmacokinetic study

Haloperidol (HAL, Fig. 1) has been used extensively as a neuroleptic for more than 40 years, and acts mainly as a blocker of dopamine D1 or D2 receptors.[1,2] The metabolites of HAL include a reduced form, 4-(4-chlorophenyl)-4-hydroxypiperidine (CPHP, Fig. 1) and others.[3,4] Structurally, CPHP resembles 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, which induces severe neurotoxicity, including Parkinson-like disease and dyskinesia.[5,6] Ablordeppey *et al.*[7] reported that CPHP induces a delayed and persistent freezing of movement. This action may involve sigma receptors, but not the dopamine D2 receptor.[8]

Bromperidol (BRO, Fig. 1) is also used in the treatment of patients with psychiatric disease and is a close structural ana-

logue of HAL. The metabolic fate of BRO is similar to that of HAL.[9—11] Acute dystonia is a side effect of BRO treatment.[5,12] 4-(4-Bromophenyl)-4-hydroxypiperidine (BPHP, Fig. 1) produced by *N*-dealkylation of BRO may be partly responsible for the disorders induced by BRO treatment. There is currently no information about the determination or pharmacokinetics of BPHP.

Recently, we determined CPHP levels in biological fluids from rats by HPLC with fluorescence detection after pre-column derivatization using 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), and the method was applied to study the disposition kinetics of CPHP after HAL administration in rats.[13] In addition, BPHP determination in phosphate-
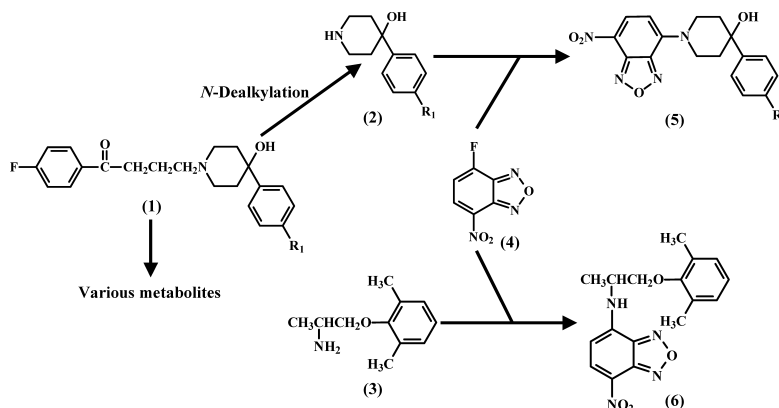

Fig. 1.   Chemical Structures and Fluorescent Derivatization Scheme Using NBD-F

(1) R_1=Br, BRO; R_1=Cl, HAL. (2) R_1=Br, BPHP; R_1=Cl, CPHP. (3) IS. (4) NBD-F. (5) R_1=Br, BPHP-NBD; R_1=Cl, CPHP-NBD. (6) IS-NBD.

buffered saline was established by HPLC—dual UV detection.[14] However, this method was poor in terms of sensitivity (0.01 μg/ml at 200 nm and 0.02 μg/ml at 220 nm). In this study, we determined BPHP levels in rat plasma by means of a similar procedure using NBD-F. The reaction scheme is presented in Fig. 1. The method was applied to evaluate the disposition kinetics of BPHP after administration of BPHP or BRO in rats.

MATERIALS AND METHODS

Materials BPHP, BRO and mexiletine hydrochloride as an internal standard (IS) were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). NBD-F and general reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Equipment The HPLC-fluorescence detection system consisted of an L-6200 pump (Hitachi, Tokyo, Japan), a Mightysil RP-18GP ODS column (150×4.6 mm i.d., 5 μm, Kanto Chemical, Tokyo), a Rheodyne injection valve (Cotati. CA, U.S.A.) with a 20-μl loop and a model RF-10A fluorometer (Shimadzu, Kyoto, Japan) operating at an excitation wavelength of 470 nm and an emission wavelength of 540 nm. Quantification of the peaks was performed using a Chromatopac Model CR-8A integrator (Shimadzu). The mobile phase was prepared by the addition of acetonitrile (400 ml) and ethanol (200 ml) to 400 ml of trifluoroacetic acid solution (0.1 v/v%) in water. The samples were eluted from the column at 25 °C at a flow rate of 1.0 ml/min.

Extraction from Plasma and Derivatization Benzene as an organic solvent for sample pretreatment was compared with *n*-hexane. Noise of blank plasma peak using benzene at the retention time of BPHP derivative was less than that using *n*-hexane. Thus, our preliminary study showed that benzene was more useful for extraction of rat plasma than *n*-hexane. Control plasma was prepared from rats. An aliquot of 200 μl of sample was rendered alkaline by the addition of NaOH (2 M, 100 μl). IS solution in water (1 μg/ml, 100 μl) was added to prepare the standard curve for BPHP. Then, the mixture was vortex-mixed for 1 min and extracted with benzene (3 ml, twice). The pooled benzene phase was evaporated to dryness. Borate buffer (0.1 M) containing ethylenediaminetetraacetic acid disodium salt (1 mM) was adjusted to pH 8.0 by the addition of NaOH (1 M). Borate buffer (300 μl) was added to the extract. NBD-F solution in acetonitrile (20 mM, 100 μl) was added and vortex-mixed. The mixture was allowed to stand for 3 min at 60 °C. Then, it was set on ice for 3 min to stop the derivatization reaction before HCl (0.05 M, 400 μl) was added. The derivatives (20 μl) were injected into the HPLC system.

Calibration Curve The solution of BPHP (1 mM) in 0.01 M HCl was added to drug-free plasma from rats. The concentration of BPHP was 0, 0.01, 0.03, 0.06, 0.1, 0.3, 0.6 or 1 μg/ml. All samples were extracted and analyzed using the procedures described above.

Animal Study Male Wistar rats were obtained from Sankyo Laboratory Animals (Toyama, Japan) and treated in accordance with the guidelines of the Institutional Animal Care and Use Committee of Hokuriku University. BPHP or BRO (each 40 μmol/kg) was intraperitoneally (i.p.) or per orally (*p.o.*) administered to rats after having been suspended in corn oil. Rats were fasted for 12 h prior to the administration, while water was freely available. Under light anesthesia with diethyl ether, blood samples (0.4 ml) were withdrawn with heparinized syringes from the jugular vein at the designated time intervals *via* a separate venous puncture. Blood samples were centrifuged (3000×*g*, 5 min) to obtain the plasma. In the same manner, drug-free pooled plasma sample were obtained from rats.

Pharmacokinetic and Statistical Analysis The area under the plasma concentration-time curve from zero to 8 h (*AUC*) of BPHP was calculated using the linear trapezoidal rule. The ratio of BRO metabolism to BPHP was estimated as $(AUC_{p.o.,BRO\rightarrow BPHP}/AUC_{p.o.,BPHP})\times 100$, where the *AUC* values of BPHP after *p.o.* administration of BPHP and BRO are abbreviated as $AUC_{p.o.,BPHP}$ and $AUC_{p.o.,BRO\rightarrow BPHP}$, respectively. The data were analyzed using Student's *t*-test to compare the unpaired mean values of the two sets of data. The criterion of a significant difference between the sets of data was taken to be $p<0.05$.

RESULTS AND DISCUSSION

For the time course study of derivatization, the reaction time was set at 2, 3, 6, 10 or 20 min. BPHP and IS (each 0.5 μg/ml) in borate buffer (pH 8.0) were derivatized as described under Experimental. The ratio of derivatization of BPHP reached a maximum at 3 min (data not shown), and tended to decrease after 6 min. Therefore, the derivatization time of 3 min was selected. Among borate buffers of pH 7.5 to 9.5, no significant difference of peak area was observed, so borate buffer of pH 8.0 was selected. The optimums at BPHP derivatization were almost consisted with those at CPHP derivatization.[13]

Figure 2 shows the chromatograms obtained from (A) plasma spiked with BPHP (0.1 μg/ml) and IS (1 μg/ml) and (B) plasma at 0.5 h after a single i.p. administration of BPHP to rats (40 μmol/kg). The retention times of the BPHP and IS derivatives were 7.7 and 11.5 min, respectively.

The standard curve of BPHP was constructed by plotting integrated peak area ratios of BPHP to IS *vs.* BPHP concentration. The plot was linear ($y=0.8691x+0.0093$) for BPHP in the concentration range from 0.01 to 1 μg/ml, $r^2=0.9959$). The lower limit of detection was 0.003 μg/ml (signal-to-noise

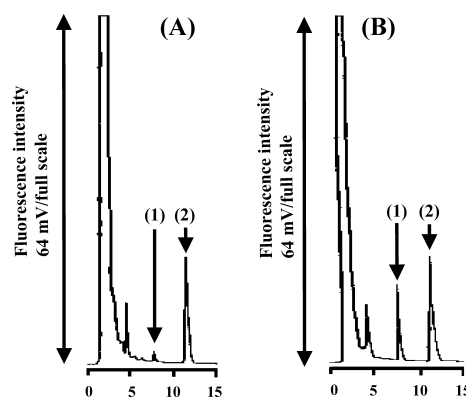

Fig. 2. Chromatograms of NBD-F Derivatives in Extracted Rat Plasma Samples

(A) Plasma spiked with BPHP (0.1 μg/ml) and IS (1 μg/ml); (B) plasma at 0.5 h after a single i.p. administration of BPHP (40 μmol/kg). The attenuation for all chromatograms is 64 mV/full scale. Peaks: 1=BPHP-NBD, 2=IS-NBD.

ratio of 3 : 1). The detection limit of BPHP was 3.3 to 6.7-fold improved compared with our previous data[14] and about 3 times better to that of CPHP.[13] A slope value of standard curve for BPHP was about twice larger than that for CPHP.[13] CPHP derivative was detected at a tailing of blank plasma peak (retention time of 6.9 min), although BPHP derivative was detected at the nearby baseline. Those will be partially related to the difference of sensitivity. This procedure is the first to be established for BPHP determination using pre-column derivatization technique.

Precision and accuracy for intra- and inter-day assays of the BPHP derivative are shown in Table 1. In the intra- and inter-day assays, the range of standard deviation of the average value of BPHP was within 5.2 to 12.0%. The recovery of BPHP ranged from 93.7 to 108.0%.

Plasma concentration–time courses of BPHP after a single i.p. or p.o. administration of BPHP and a single p.o. administration of BRO were constructed for up to 8 h (Fig. 3). In order to compare the present results with our previous data, the time courses of CPHP after CPHP or HAL administration are shown overlapped in Fig. 3. Plasma concentrations of BPHP after i.p. administration of BPHP at 0.17 to 1 h were significantly lower than those of CPHP after administration of HAL (Fig. 3A). While the time course of BPHP after p.o. administration of BPHP tended to be lower than that of CPHP after p.o. administration of CPHP (Fig. 3B), that of BPHP after p.o. administration of BRO tended to be higher than that of CPHP after p.o. administration of HAL (Fig. 3C). Since it was reported that CPHP was metabolized to 4-(4-chlorophenyl)-1,2,3,6-tetrahydropyridine,[15] 4-(4-bromophenyl)-1,2,3,6-tetrahydropyridine may be formed from

BPHP. It was discussed that BPHP might be more metabolized than CPHP as well as BRO was more susceptible than HAL to N-dealkylation.

Pharmacokinetic parameters are listed in Table 2. There was a significant difference between $AUC_{i.p.,BPHP}$ and $AUC_{i.p.,CPHP}$. The value of $AUC_{p.o.,BPHP}$ was significantly lower than that of $AUC_{p.o.,CPHP}$. The $AUC_{p.o.}/AUC_{i.p.}$ ratios of BPHP and CPHP were estimated to be 46 and 56%, respectively. The ratio of BRO metabolism to BPHP (48%) was 1.8-fold higher than that of HAL metabolism to CPHP (27%). These results indicate that BPHP is less well absorbed than CPHP from the rat intestine, while BRO is more susceptible than HAL to N-dealkylation.

Fang et al.[3] demonstrated that recombinant human cytochrome P450 (CYP) 3A4, 3A5, 1A1, 2C19, 2C8, 2C9, and 2D6 were able to catalyze the dealkylation of HAL to CPHP. CYP3A metabolizes BRO to 4-(fluorobenzoyl)propionic acid, an alternative metabolite to the N-dealkyl one, in rat hepatic microsomes.[16] Anti-rat CYP3A2 antiserum inhibited 4-(fluorobenzoyl)propionic acid formation by 80%, whereas other anti-rat CYP antisera (1A1, 1A2, 2B1, 2C11, and 2E1) had little effect. These previous results indicate that BPHP formation from BRO is at least partially due to CYP3A2 in

Table 1. Intra- and Inter-day Assay Reproducibility for Determination of BPHP

Concentration (μg/ml)	Measured (μg/ml) (Mean$\pm$S.D., $n=6$)	C.V. (%)	Recovery (%)
Intra-day assay			
0.01	0.0106$\pm$0.0011	10.4	106.0
0.1	0.0990$\pm$0.0051	5.2	99.0
1	0.948$\pm$0.083	8.8	94.8
Inter-day assay			
0.01	0.0108$\pm$0.0013	12.0	108.0
0.1	0.101$\pm$0.010	9.9	101.0
1	0.937$\pm$0.062	6.6	93.7

Table 2. Comparison between Pharmacokinetic Parameters of BPHP and CPHP in Rats

i.p. administration

Parameters	Administered compounds	
	BPHP	CPHP
$AUC_{i.p.}$ (nmol$\times$h/ml)	9.6$\pm$1.5*	12$\pm$2
(expressed as μg$\times$h/ml)	2.5$\pm$0.4	2.5$\pm$0.4

p.o. administration

Parameters	Administered compounds			
	BPHP	BRO	CPHP	HAL
$AUC_{p.o.}$ (nmol$\times$h/ml)	4.4$\pm$0.7*	2.1$\pm$0.5	6.7$\pm$1.4	1.8$\pm$0.4
(expressed as μg$\times$h/ml)	1.1$\pm$0.2	0.54$\pm$0.13	1.4$\pm$0.3	0.38$\pm$0.08
$AUC_{p.o.}/AUC_{i.p.}$ (%)	46	(—)	56	(—)
Metabolic ratio (%)	(—)	48	(—)	27

Each value represents the mean$\pm$S.D. of five rats. * Significantly different from CPHP administration at $p<0.05$. (—); not calculated.
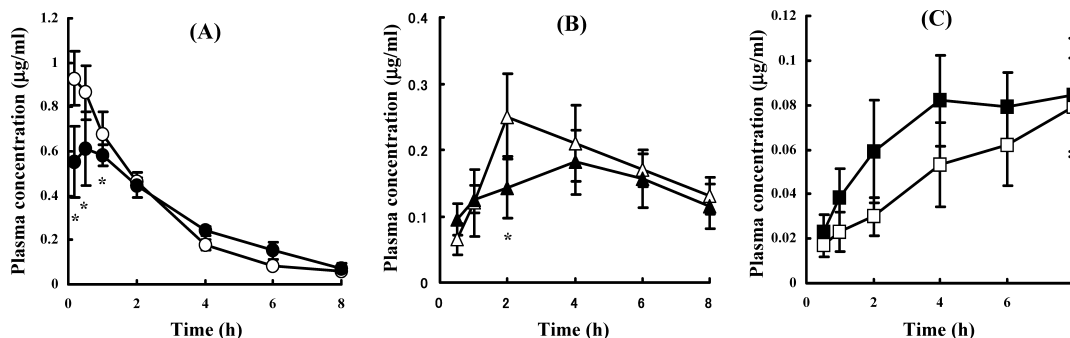

Fig. 3. Plasma Concentration–Time Courses of BPHP and CPHP in Rats

(A) Comparison between plasma-concentration time courses of BPHP (●) and CPHP (○) after a single i.p. administration of BPHP or CPHP, respectively; (B) comparison between plasma-concentration time courses of BPHP (▲) and CPHP (△) after a single p.o. administration of BPHP or CPHP, respectively; (C) comparison between plasma-concentration time courses of BPHP (■) and CPHP (□) after a single p.o. administration of BRO or HAL, respectively. Each point represents the mean$\pm$S.D. of five rats. * Significantly different from CPHP concentration in plasma at $p<0.05$.

rats. *CYP3A4* mRNA in man corresponds to *CYP3A2* mRNA in the rat.[17] It is well known that CYP3A4 or 3A2 is induced not only by glucocorticoids such as dexamethasone and prednisolone, but also by rifampicin, carbamazepine, and so on.[18—21] Treatment with dexamethasone (80 mg/kg) for 2 d affected the enzyme activity, and the elimination half-life of BRO was significantly shortened by treatment with dexamethasone.[16] The use of carbamazepine was associated with significantly lower HAL plasma levels.[22] It is considered that co-administration of BRO or HAL with enzyme inducers such as those described above will tend to result in more severe neurotoxic side effects because of increased plasma BPHP or CPHP levels, as well as a reduced pharmacological effects because of the decreased level of intact neuroleptic. Further studies are needed on the effects of CYP3A4 or 3A2 inducers on *N*-dealkylated metabolite formation from neuroleptics, as well as on neurotoxicity and pharmacological effects.

CONCLUSION

We present a sensitive method for determination of BPHP in rat plasma. This method is suitable for pharmacokinetic study of BPHP after BRO administration, and for assessing potential BPHP or CPHP toxicity in patients treated with BRO or HAL.

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