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Studies on Analgesic Agents. XIII.[1] Metabolic Fate of 1-Butyryl-4-cinnamylpiperazine Hydrochloride(BCP-HCl) in Rats

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The metabolic fate of a new analgesic agent, 1-*n*-butyryl-4-cinnamylpiperazine hydrochloride (BCP-HCl) in the rat was investigated *in vivo* and *in vitro*.

The following four urinary metabolites were detected in addition to the unchanged drug by both the thin-layer(TLC) and gas-liquid chromatography(GLC); 1-*n*-butyrylpiperazine(I), 1-*n*-butyryl-4-(4′-hydroxycinnamyl)piperazine(III), 1-(4′-hydroxycinnamyl)-piperazine(IV) and 1-cinnamylpiperazine(V). These were isolated and identified physicochemically (ultraviolet, infrared and mass spectrometry and mixed melting point test). In addition to the above metabolites, piperazine hexahydrate(II) and two unidentified products, UK-1 and UK-2 were detected in the urine by the TLC but not by the GLC.

In the bile BCP, I, III, and IV were detected in addition to the glucuronide and sulfate conjugates of III and IV. One unknown metabolite corresponding to the urinary UK-1 was also detected by the TLC of the biliary metabolites hydrolyzed with β-glucuronidase or arylsulfatase.

In vitro incubation of BCP-HCl with the post-mitochondrial fraction of the rat liver resulted in the formation of the four metabolites, I, III, IV and V.

In the previous paper, Irikura, *et al.*[3] have reported the analgesic action of 1-*n*-butyryl-4-cinnamylpiperazine hydrochloride (BCP-HCl). However, nothing has yet been reported on the metabolism of the drug. In this paper, we have studied the metabolic fate of BCP-HCl in the rat by analyzing the urinary and biliary metabolites as well as metabolites produced in the *in vitro* system using the post-mitochondrial fraction of rat livers. Both thin-layer (TLC) and gas-liquid chromatography (GLC) were used for detecting and isolating the metabolites. Some of the metabolites were physicochemically identified.

Material and Method[4]

Chemicals——The authentic chemicals; BCP-HCl, mp 205—207°; 1-*n*-butyrylpiperazine(I), bp 117—121° (3 mmHg); 1-cinnamylpiperazine(V) dihydrochloride, mp 205—210° (decomp.); 1,4-diacetyl piperazine, mp 140—142° and 1-phenethyl-4-propionylpiperazine hydrochloride, mp 204—205°, were synthesized as described in the previous paper.[5] Piperazine hexahydrate(II) of the reagent grade, mp 43—45°, was purchased from a local dealer.

1-*n*-Butyryl-4-(4′-hydroxycinnamyl)piperazine(III) was synthesized as follows. 4-Ethoxycarbonyloxy cinnamic acid[6] was converted to 4-ethoxycarbonyloxy cinnamyl alcohol, mp 60—62°, by a slightly modified method of Ishizumi, *et al.*[7] The alcohol was then converted to 4-ethoxycarbonyloxy cinnamyl chloride by the reaction with thionyl chloride. A mixture of 4-ethoxycarbonyloxy cinnamyl chloride (3.5 g), 1-*n*-butyrylpiperazine (2.4 g) and triethylamine (2 ml) in benzene (100 ml) was gently heated at about 80°

1) Part XII: T. Irikura, H. Ohkubo, T. Hirayama, S. Imai, and M. Yamauchi, *Oyo Yakuri*, **6**, 635 (1972).
2) Location: *a) Hongo, Bunkyo-ku, Tokyo*; *b) Ukima, Kita-ku, Tokyo.*
3) T. Irikura, K. Nishino, N. Ito, M. Ito, and H. Ohkubo, *Japan. J. Pharmacol.*, **20**, 287 (1970).
4) All the melting points were measured on a micro hot stage and uncorrected.
5) T. Irikura, K. Masuzawa, K. Nishino, M. Kitagawa, H. Uchida, N. Ichinoseki, and M. Ito, *J. Med. Chem.*, **11**, 801 (1968).
6) J. Shinoda and S. Sato, *Yakugaku Zasshi*, **48**, 933 (1928).
7) K. Ishizumi, K. Koga, and S. Yamada, *Chem. Pharm. Bull.* (Tokyo), **16**, 492 (1968).

for 6 hr. After cooling, the triethylamine hydrochloride which had separated was filtered off. The benzene layer was washed twice with 50 ml of H_2O, dried over Na_2SO_4, and evaporated to yield an oily residue (3 g). A suspension of this residue (3 g) in 10% NaOH aqueous solution (30 ml) was warmed at 40° for 30 min with stirring. The reaction mixture was made acidic with AcOH (pH 6). The resultant solution was extracted three times with 70 ml of $CHCl_3$. The combined extracts were washed twice with H_2O, dried over Na_2SO_4, and evaporated to yield 1.8 g (70%) of III as a syrup. Hydrochloride was recrystallized from CH_3CN to give colorless needles, mp 218—221° (decomp.). *Anal.* Calcd. for $C_{17}H_{24}O_2N_2 \cdot HCl$: C, 62.85; H, 7.76; N, 8.62. Found: C, 62.63; H, 7.65; N, 8.50.

1-(4′-Hydroxycinnamyl)piperazine(IV) was synthesized as follows. 1-(4′-Ethoxycarbonyloxy cinnamyl)-4-formylpiperazine (15.5 g) was prepared from a mixture of 4-ethoxycarbonyloxy cinnamyl chloride (13.8 g), 1-formylpiperazine[8] (10 g) and triethylamine (10 ml) in benzene (100 ml) by the same method as above. A suspension of this product (10 g) in 20% aqueous KOH (50 ml) was allowed to stand at room temperature for 3 days with stirring. The resultant solution was then made acidic with AcOH (pH 6), and the separated syrup was extracted with three portions of 100 ml of MeOH–$CHCl_3$ (1:9, v/v). The combined extracts were dried over Na_2SO_4 and evaporated to yield brown crystals. The crystals were washed with 100 ml of cold $CHCl_3$ and recrystallized from CH_3CN to give 4.5 g (66%) of IV as colorless needles, mp 169—172°. Dihydrochloride monohydrate of IV was obtained as colorless crystals, mp 200—204° (decomp.). *Anal.* Calcd. for $C_{13}H_{18}ON_2 \cdot 2HCl \cdot H_2O$: C, 50.49; H, 7.17; N, 9.06. Found: C, 50.19; H, 7.40; N, 9.02.

1-*n*-Butyryl-4-(4′-methoxycinnamyl)piperazine (methyl derivative of III) was prepared by the manner similar to that described for the synthesis of III. A portion of the product was recrystallized from MeOH as a picrate, mp 120—122°. *Anal.* Calcd. for $C_{18}H_{26}O_2N_2 \cdot C_6H_3O_7N_3$: C, 54.23; H, 5.50; N, 13.18. Found: C, 54.45; H, 5.64; N, 12.95.

1-(4′-Methoxycinnamyl)piperazine (methyl derivative of IV) was prepared by the manner similar to that described for the synthesis of IV. Picrate: Yellowish crystals, mp 218—223° (decomp.). *Anal.* Calcd. for $C_{15}H_{22}ON_2 \cdot 2(C_6H_3O_7N_3)$: C, 45.22; H, 3.80; N, 16.23. Found: C, 45.50; H, 3.78; N, 15.96.

1,4-Dicinnamyl piperazine was synthesized by mixing cinnamyl chloride, piperazine hexahydrate and triethylamine in benzene at about 80° for 6 hr. The product was recrystallized from MeOH as colorless needles, mp 126—127°. *Anal.* Calcd. for $C_{22}H_{26}N_2$: C, 82.97; H, 8.23; N, 8.80. Found: C, 82.70; H, 8.20; N, 8.56.

Enzymes——β-Glucuronidase (specific activity 5.2 U/ml) and arylsulfatase (specific activity 2.6 U/ml) from Helix pomatia were purchased from Boehringer Mannheim Japan Co., Ltd.

Biochemicals——NADP and glucose-6-phosphate were purchased from Boehringer Mannheim Japan Co., Ltd.

Animal Care——Wistar male rats weighing about 250 g received an oral administration of BCP-HCl dissolved in 0.9% NaCl solution at the dose of 125 mg per kg body weight. The animals were housed individually in metabolic cages allowing clean separation of the urine from the feces. The semi-synthetic pellet food (CLEA Japan, CE-2) and water were given *ad libitum*. The urine was collected during the first 24 hr period after administration of the drug into ice-cold cylinder containing a few drops of toluene. To collect the bile, rats were anesthetized lightly with ether and a polyethylene tube (1 mm × 30 cm) was cannulated into the bile duct. Rats then received an oral administration of the drug and the first 24 hr bile was collected while the animals were kept in the Bollman's restraining cages[9] without food and water.

Extraction of Metabolites from Urine and Bile——Urine or bile thus collected was adjusted to pH 11 with 5N NaOH and unconjugated metabolites in them were extracted repeatedly with ethyl acetate. The aqueous phase, which was supposed to contain some conjugated metabolites, such as glucuronide or sulfate conjugates, was neutralized with HCl and then subjected to the enzymatic hydrolysis. For the hydrolysis of glucuronides, 40 ml of the neutralized aqueous phase was mixed with 40 ml of 0.2M acetate buffer, pH 4.5 and 1 ml of β-glucuronidase solution (5.2 U) and the mixture was incubated at 37° for 24 hr in the presence of a few drops of toluene. As blank experiments, β-glucuronidase-free mixtures were incubated similarly. For the hydrolysis of sulfate conjugates another 40 ml aliquot of the aqueous phase was mixed with 40 ml of 0.2M acetate buffer, pH 6.2 and 1 ml of arylsulfatase solution (2.6 U) and the mixture was incubated at 37° for 24 hr in the presence of toluene. As blank experiments, arylsulfatase-free mixtures were incubated similarly. After the incubation metabolites liberated from the conjugates were extracted with ethyl acetate as described above.

***In Vitro* System for BCP-HCl Metabolism**——Rats (Wistar, male) weighing 150 g were killed by decapitation and the livers were perfused *in situ* with ice-cold 1.15% KCl. The liver was excised and homogenized with 4 volumes of ice-cold 1.15% KCl in a Potter-Elvehjem glass homogenizer. The homogenate was centrifuged at 9000 × *g* for 20 min. The incubation mixture contained 120 ml of the 9000 × *g* supernatant (equivalent to 24 g of wet liver), 2 mmoles of $MgCl_2$ in 8 ml of H_2O, 0.8 mmole of glucose-6-phosphate in

8) K. Fujii, K. Tomino, and H. Watanabe, *Yakugaku Zasshi*, **74**, 1049 (1954).
9) J.L. Bollman, J.C. Cain, and J.H. Grindlay, *J. Lab. Clin. Med.*, **33**, 1349 (1948).

4 ml of H_2O, 16 μmoles of NADP in 4 ml of H_2O, 148.8 μmoles of BCP-HCl in 8 ml of H_2O, 56 ml of 0.2M phosphate buffer, pH 7.4 in the final volume of 200 ml. The whole mixture was incubated at 37° for 2 hr under gentle shaking. As the controls, the mixture without either BCP-HCl or the 9000×*g* supernatant was incubated similarly. Extraction of metabolites thus generated in the *in vitro* system was carried out with ethyl acetate in the same way as described above.

Further Fractionation of Metabolites extracted with Ethyl Acetate——Metabolites extracted with ethyl acetate from the urine, the bile or the *in vitro* metabolic system were subjected to further fractionation according to the solubilities as summarized in Chart 1 and the metabolites were separated into the two fractions, Fr. 1 and Fr. 2. Fr. 1 comprises the metabolites extractable with benzene from the alkaline aqueous solution and Fr. 2 comprises those unextracted with benzene but extracted with ethyl acetate. The fractions containing metabolites which were released from conjugates by β-glucuronidase and arylsulfatase were referred to as Fr. 3 and Fr. 4, respectively. These products were not extracted with benzene from alkaline solution and considered to be phenolic derivatives.

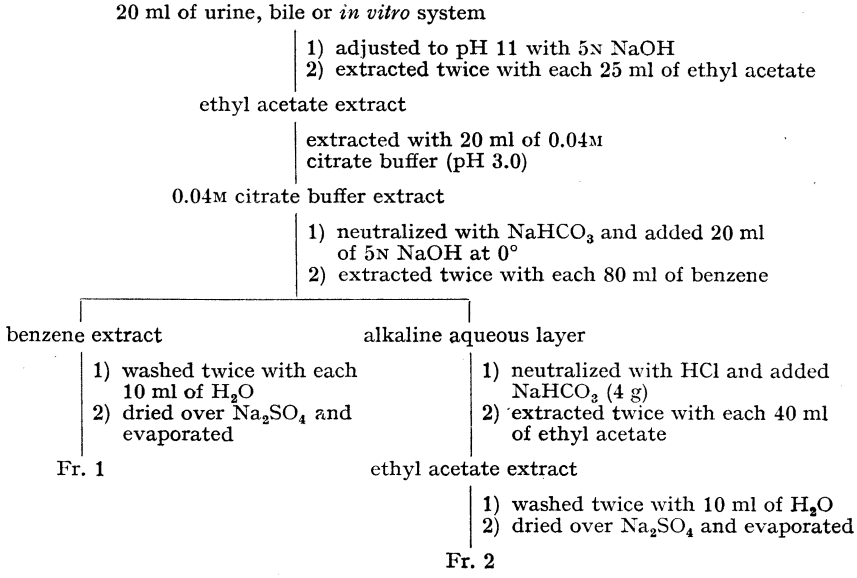

Chart 1. Procedures for the Extraction and Fractionation of BCP-HCl Metabolites (unconjugated)

Thin-Layer Chromatography (TLC)——Each of the fractions thus separated was subjected to ascending monodimensional TLC along with some authentic compounds as references. The chromatographic plate (5×20 cm) with silica gel (Kieselgel F_{254}, 250 μ thick, Merck) and the three different solvent systems, A, B and C were used; (A) *n*-BuOH–AcOH–H_2O (4:1:2, v/v), (B) $CHCl_3$–MeOH–4% NH_4OH (8:8:1, v/v) and (C) MeOH–4% NH_4OH (5:1, v/v). Detection of spots on the chromatograms was carried out with the five different procedures; *i.e.* UV irradiation (253.6 mμ), exposure to I_2 vapor and spraying of 1% 2,6-dichloro-*p*-quinone[10] in acetone, iodoplatinate reagent[11] and diazotized sulfanilic acid.[12]

Gas-Liquid Chromatography (GLC)——A Shimadzu gas chromatograph (GC-1C) was used throughout the present study. The column (glass, 1.875 m length×4 mm diameter) was filled with 1.5% methyl silicone (SE-30)-coated Chromosorb W (60—80 mesh). The temperature at the inlet was the same as that at the column and was conditioned to 120°, 170°, 180° and 230° depending on the nature of metabolites, while it was 300° at the detector. Nitrogen was used as carrier gas at the flow rate of 50 ml/min. For the identification purpose, a hydrogen flame ionization detector (FID) was used, while a thermal conductivity detector (TCD) was used for the isolation of metabolites. Retention times of the authentic samples were measured separately. Compounds such as (1) 1,4-bis(acetyl)piperazine, (2) 1-phenethyl-4-propionylpiperazine and (3) 1,4-bis(cinnamyl)piperazine were sometimes added to the metabolite samples as internal standards. In case of collecting a metabolite (II), a 10 cm long glass tube was attached to the column outlet to condense the metabolite.

10) H. Wachsmuth and L.v. Koeckhoven, *J. Pharm. Berg.*, **17**, 220 (1962).
11) R. Kuntzman, A. Klutch, I. Tsai, and J.J. Burns, *J. Pharmacol. Exptl. Thrap.*, **149**, 29 (1965).
12) F. Feigl, "Spot Test in Organic Analysis," Maruzen, Tokyo, 1965, p. 140.

Methylation of Metabolites for GLC——Samples such as Fr. 2, Fr. 3 and Fr. 4 contained some phenolic metabolites, which were unsuitable for direct GLC and therefore methylated prior to GLC. This methylation was carried out at 4° for 15 hr in 5 ml of 15% diazomethane in ether. After the reaction, 5% AcOH in ether was added drop by drop until yellowish color of excessive diazomethane was disappeared, and the solvents were evaporated off. Water (4 ml) was added to the residue and the solution was washed with 30 ml of ethyl acetate. The aqueous phase was made alkaline with 4 ml of 5N NaOH and extracted with 30 ml of ethyl acetate. The ethyl acetate extract was washed with 5 ml of H_2O and dried over Na_2SO_4. After evaporation to dryness, the residue was dissolved in a small volume of MeOH and subjected to GLC. Fr. 1 from the urine, bile or *in vitro* system could be applied to chromatography wihout methylation.

Result

1) Urinary Metabolites (unconjugated)

a) TLC of the Unconjugated Urinary Metabolites——Unconjugated metabolites extracted with ethyl acetate from the alkaline urine (pH 11) were separated into Fr. 1 and 2 as described above. Fr. 1 and 2 were applied onto the silica gel thin-layer chromato-plate together with

Table I. Summary of BCP-HCl Metabolites detected by Thin-Layer Chromatography

Sample[a]	Identity	*Rf* values in solvent systems[b] A	B	C	Detection of spots with: UV light of 253.6mμ (black)	2,6-Dichloro-*p*-quinone (purple)	Iodoplatinate (blue)	Diazotized sulfanilic acid (yellow)	I_2 vapor (yellow)
Authentic compounds	BCP	0.63	0.85	0.67	+	−	+	−	+
	I	0.34	0.40	0.35	−	+	+	−	+
	II	0.11	0.13	0.10	−	+	+	−	+
	III	0.60	0.65	0.67	+	−	+	+	+
	IV	0.32	0.27	0.22	+	+	+	+	+
	V	0.39	0.39	0.22	+	+	+	−	+
Urinary metabolites in Fr. 1	BCP	0.63	0.85	0.67	+	−	+	−	+
	I	0.34	0.40	0.35	−	+	+	−	+
	V	0.39	0.39	0.22	+	+	+	−	+
Urinary metabolites in Fr. 2	II	0.11	0.13	0.10	−	+	+	−	+
	III	0.60	0.65	0.67	+	−	+	+	+
	IV	0.32	0.27	0.22	+	+	+	+	+
	UK-1[c]	0.40	—[d]	—	+	+	+	+	+
	UK-2[c]	0.26	—	—	+	+	+	+	+
Biliary metabolites in Fr. 1	BCP	0.63	0.85	0.67	+	−	+	−	+
	I	0.34	0.40	0.35	−	+	+	−	+
Biliary metabolites in Fr. 2	III	0.60	0.65	0.67	+	−	+	+	+
	IV	0.32	0.27	0.22	+	+	+	+	+
Biliary metabolites in Fr. 3[e]	III	0.60	0.65	0.67	+	−	+	+	+
	IV	0.32	0.27	0.22	+	+	+	+	+
	UK-1	0.40	—	—	+	+	+	+	+
Biliary metabolites in Fr. 4[e]	III	0.60	0.65	0.67	+	−	+	+	+
	IV	0.32	0.27	0.22	+	+	+	+	+
	UK-1	0.40	—	—	+	+	+	+	+
In vitro metabolites in Fr. 1	BCP	0.63	0.85	0.67	+	−	+	−	+
	I	0.34	0.40	0.35	−	+	+	−	+
	V	0.39	0.39	0.22	+	+	+	−	+
In vitro metabolites in Fr. 2	III	0.60	0.65	0.67	+	−	+	+	+
	IV	0.32	0.27	0.22	+	+	+	+	+

a) Extraction and fractionation of metabolites are described in Materrial and Method.
b) solvent systems: A; *n*-BuOH-AcOH-H_2O (4:1:2, v/v), B; $CHCl_3$-MeOH-4% NH_4OH (8:8:1, v/v), C; MeOH-4%NH_4OH (5:1, v/v)
c) unknown metabolites
d) not detected in the solvent systems of B and C
e) fractions for the analysis of conjugated metabolites

some authentic compounds such as BCP, I, II, III, IV and V. The solvent systems used in the present study were A, B and C (see Material and Method) which had been proved to nicely separate these authentic compounds on the chromatograms. Among the five different detection procedures, UV (253.6 mμ) irradiation is suitable for detection of compounds having aromatic ring(s) such as BCP, III, IV and V. Phenolic compounds like III and IV were specifically stained with diazotized sulfanilic acid (yellow) and compounds like I, II, IV and V having at least one unsubstituted piperazine nitrogen (the secondary amino group) were stained specifically with 2,6-dichloro-*p*-quinone(purple). All the authentic compounds were stained with iodine vapor(yellow) as well as with iodoplatinate (blue).

As summarized in Table I, the urinary Fr. 1 gave three spots which corresponded to BCP, I and V, while the urinary Fr. 2 gave five spots which corresponded to II, III and IV in addition to the two unknown spots, UK-1 and 2. It should be noted here that none of these eight spots was detected on the chromatograms of control Fr. 1 and 2 samples (from rats without BCP-HCl administration). UK-1 and 2 were detected only when the solvent system A was used. UK-1 reextracted with $CHCl_3$–MeOH (9:1, v/v) from the chromatogram yielded IV, on rechromatography with the solvent system A. This suggests the possibility that UK-1 may be a labile derivative of IV.

b) GLC of the Unconjugated Urinary Metabolites——Result of GLC of urinary Fr. 1 and 2 are summarized in Table II. The four different column temperatures were used for

Table II. Summary of BCP-HCl Metabolites by Gas–Liquid Chromatography

Sample[a]		Identity	Retention times (min) under the column temperature of			
			120°	170°	180°	230°
Authentic compounds		BCP	—[b]	—	28.4	—
		I	6.2	—	—	—
		III′[c]	—	—	—	9.6
		IV′[c]	—	12.7	—	—
		V	—	—	3.5	—
Internal standards[d]		1	19.8	—	—	—
		2	—	14.9	10.9	—
		3	—	—	—	13.6
Urinary metabolites	in Fr. 1	I	6.2	—	—	—
		V, BCP	—	—	3.5, 28.4	—
	in Fr. 2	IV′	—	12.7	—	—
		III′	—	—	—	9.6
Biliary metabolites	in Fr. 1	I	6.2	—	—	—
		BCP	—	—	28.4	—
	in Fr. 2	IV′	—	12.7	—	—
		III′	—	—	—	9.6
	in Fr. 3[e]	IV′	—	12.7	—	—
		III′	—	—	—	9.6
	in Fr. 4[e]	IV′	—	12.7	—	—
		III′	—	—	—	9.6
In vitro metabolites	in Fr. 1	I	6.2	—	—	—
		V, BCP	—	—	3.5, 28.4	—
	in Fr. 2	IV′	—	12.7	—	—
		III′	—	—	—	9.6

a) Extraction and fractionation of metabolites are described in Material and Method.
b) not examined
c) III and IV were methylated to form III′ and IV′ for gas chromatographic analysis, III′: 1-*n*-butyryl-4-(4′-methoxycinnamyl)piperazine, IV′: 1-(4′-methoxycinnamyl)piperazine
d) 1: 1,4- diacetyl piperazine, 2: 1-phenethyl-4-propionylpiperazine, 3: 1,4-dicinnamylpiperazine
e) fractions for the analysis of conjugated metabolites

selective separation of the authentic compounds such as BCP, 1-*n*-butyrylpiperazine(I), 1-*n*-butyryl-4-(4′-methoxycinnamyl)piperazine(III′), 1-(4′-methoxycinnamyl)piperazine(IV′) and V.

GLC of the urinary Fr. 1 revealed the presence of compounds having retention times (t_R) similar to those of BCP (28.4 min at 180°), I (6.2 min at 120°) and V (3.5 min at 180°). In case of GLC of the urinary Fr. 2, which was methylated with diazomethane, two major peaks similar to III′ (9.6 min at 230°) and IV′ (12.7 min at 170°) were detected. None of these five peaks (BCP, I, III′, IV′ and V) was detected by GLC of the control urinary samples. Compound II(piperazine), which was detected in the urinary Fr. 2 by the TLC, was not detected by the GLC. Probably this metabolite was lost during the methylation and reextraction process of Fr. 2. At the same time, neither UK-1 nor 2 was detected by the GLC.

c) Isolation and Identification of Some of the Urinary Metabolites——The first 24 hr urine was collected and pooled from 50 experimental rats administered with 125 mg BCP-HCl per kg body weight and the metabolites extracted with ethyl acetate from the alkaline urine were fractionated into Fr. 1 and 2 as described above. Fr. 1 was subjected to the TLC with the solvent system of C and the spots of *Rf* 0.67, 0.35 and 0.22 were extracted with $CHCl_3$–MeOH (9:1, v/v). The pooled extract of *Rf* 0.67 was evaporated and the residue was dissolved in a small volume of EtOH and mixed with an equal volume of 10% HCl in EtOH to yield crystals, which were then recrystallized from EtOH–ether (9:1, v/v). The substance showed the following physicochemical parameters which coincided with those of authentic BCP-HCl; mp 203—206°, UV ($\lambda_{max}^{1N\ HCl}$): 253 mμ; IR (ν_{max}^{KBr}): 950, 1640, 2400—2840 cm^{-1}; and mass spectra (*m/e*): 272 (M^+-HCl), 201, 155, 117 and 85. When the spot of *Rf* 0.22 was extracted and recrystallized in the same manner as described above, crystals having mp 200—205° (decomp.) was obtained. UV ($\lambda_{max}^{1N\ HCl}$): 253 mμ; IR (ν_{KBr}^{max}): 940, 1600, 2300—3000 and 3450 cm^{-1}; and mass spectra (*m/e*): 202 (M^+-2HCl), 117 and 85, were observed for this substance, confirming the identity to 1-cinnamylpiperazine (V) dihydrochloride. The substance of *Rf* 0.35 was dissolved in 0.1 ml of MeOH and its 10 μl aliquots were applied to the GLC (column temperature of 120°) at 30 min intervals to collect the fraction of t_R 6.2 min for one min (from 6 min to 7 min after charging the sample). The pooled sample in a glass tube thus collected was washed out with MeOH. The solvent was evaporated and the oily residue was subjected to infrared (IR) and mass spectroscopy. The resulting IR (see Chart 2) and mass spectra [(*m/e*): 156 (M^+), 85] showed the identity to 1-*n*-butyrylpiperazine (I).

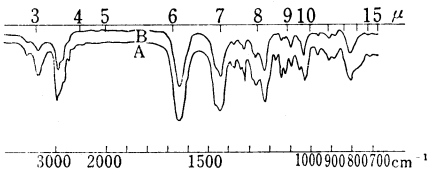

Chart 2. IR Absorption Spectra of 1-*n*-Butyrylpiperazine (I) and a Metabolite having a Retention Time identical to that of I in the Gas–Liquid Chromatography

A: 1-*n*-butyrylpiperazine (I), B: the metabolite isolated by gas–liquid chromatography IR spectroscopy was carried out by the liquid film method.

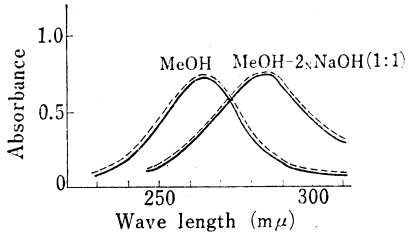

Chart 3. Ultaviolet Absorption Spectra of 1-(4′-Hydroxycinnamyl)-piperazine (IV) and a Metabolite having a *Rf* Value identical to that of IV on the Thin-Layer Chromatogram

The dotted curve is for 1-(4′-hydroxycinnamyl)-piperazine and the solid curve for the metabolite. Concentration of each compound is about 10 μg/ml.

Fr. 2, which is supposed to contain phenolic metabolites, was subjected to the TLC with the solvent system of A and the spots of *Rf* 0.32 and 0.60 were extracted with $CHCl_3$–MeOH (9:1, v/v). The compound of *Rf* 0.32 was rechromatographed with the solvent system of C, and the resulting spot of *Rf* 0.22 was extracted with $CHCl_3$–MeOH (9:1, v/v). The solvent was evaporated off and the residue was recrystallized from ethyl acetate and then from MeOH to yield crystals, mp 167—170°. The ultraviolet (UV) and IR spectra are illustrated in Chart 3 and 4, respectively. The mass spectrum showed a molecular ion at (*m/e*) 218 (M^+), and prominent peaks at (*m/e*) 133 and 85. All these data confirmed the identity of this substance to 1-(4′-hydroxycinnamyl)piperazine (IV). The compound of *Rf* 0.60 (solvent A) was extracted and acidified with HCl and recrystallized from ether–EtOH (9:1, v/v) and then from EtOH to crystals, mp 215—218° (decomp.). This was identified to 1-*n*-butyryl-4-(4′-hydroxycinnamyl)piperazine (III) hydrochloride by IR spectrum shown in Chart 5 as well as mass spectra having prominent peaks at (*m/e*) 288 (M^+-HCl), 217, 133 and 85. The UV spectrum of this metabolite was the same as that of III (λ_{max}^{MeOH} 265 mμ and $\lambda_{max}^{MeOH\text{-}2N\ NaOH(1:1)}$ 287 mμ), and the spectral features were exactly similar to those in Chart 3.

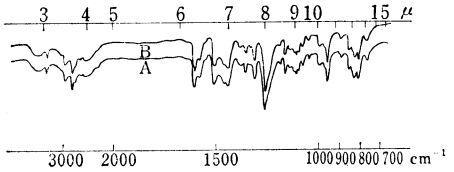

Chart 4. IR Absorption Spectra of 1-(4′-Hydroxycinnamyl)piperazine (IV) and of a Metabolite having the *Rf* Value Same with IV on the Thin-Layer Chromatogram

A: 1-(4′-hydroxycinnamyl)piperazine, B: the metabolite IR spectroscopy was carried out by the KBr disk method.

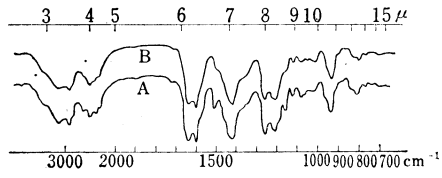

Chart 5. IR Absorption Spectra of 1-*n*-Butyryl-4-(4′-hydroxycinnamyl)piperazine (III) and a Metabolite having the *Rf* Value Same with III on the Thin-Layer Chromatogram

A: 1-*n*-butyryl-4-(4′-hydroxycinnamyl)piperazine hydrochloride, B: hydrochloride of the metabolite IR spectroscopy was carried out by the KBr disk method.

2) Biliary Metabolites (unconjugated)

Biliary metabolites were extracted with ethyl acetate from the alkaline bile (pH 11) from BCP-HCl-administered rats and they were fractionated into corresponding Fr. 1 and 2. The TLC of the biliary Fr. 1 suggested the presence of BCP and I as evidenced in Table I. A spot corresponding to V, which was detected in the urinary Fr. 1 by the TLC, was not observed. Similarly, both III and IV were detected in the biliary Fr. 2 by the TLC. Compound II and the two unknown metabolites, UK-1 and 2, were not detected in the biliary Fr. 2 in contrast to the urinary counterpart.

Results of GLC of the biliary metabolites are summarized in Table II. The biliary Fr. 1 showed two peaks having the retention times similar to those of BCP and I in accord with the results of TLC of Fr. 1. The methylated biliary Fr. 2 gave two peaks corresponding to III′ (methylated III) and IV′ (methylated IV) also in accord with the thin-layer chromatographic results. It should be noted that none of these spots or peaks was detected in the chromatograms of Fr. 1 and 2 from the control bile.

3) Conjugated Metabolites in the Bile

After the extraction of unconjugated metabolites with ethyl acetate, the alkaline urine or bile was neutralized and treated separately with either β-glucuronidase or arylsulfatase as described in Material and Method. Free metabolites yielded from the conjugates, if any, were then extracted with ethyl acetate. The biliary Fr. 3 (the ethyl acetate extract after β-glucuronidase treatment) and Fr. 4 (the ethyl acetate extract after arylsulfatase treatment) thus prepared were then subjected to TLC. As evidenced in Table I, two compounds

corresponded to the phenolic authentic compounds, III and IV. In addition, one spot (TLC) corresponding to urinary UK-1 was detected in the biliary Fr. 3 and 4 when the solvent system A was applied. Similary, as shown in Table II, two peaks (GLC) corresponded to the authentic compounds, III′ and IV′. In contrast to the biliary Fr. 3 and 4, the urinary Fr. 3 and 4 treated in the same way, did not show any of these metabolites, suggesting the absence of conjugated metabolites in the urine.

4) The *in Vitro* Metabolites

Fr. 1 and 2 were prepared from the *in vitro* incubation mixture as described in Material and Method. As summarized in Table I, the three spots corresponding to BCP, I and V were detected on the TLC of Fr. 1, while the two spots corresponding to III and IV were detected on that of Fr. 2. The results of GLC of Fr. 1 and methylated Fr. 2 are summarized in Table II, which were in accord with the results obtained by TLC. The chromatographic analysis of metabolites produced in the *in vitro* system coincided with the results obtained for the urinary metabolites except for that spots of piperazine (II) and the two unknown, UK-1 and 2, were not detected in the *in vitro* system.

Fr. 1 samples were prepared from the complete, the subtsrate-omitted and the enzyme ($9000\times g$ supernatant)-omitted incubation systems, respectively. An aliquot of each Fr. 1 sample was evaporated and the residue was dissolved in 0.2 ml of 10% $NaHCO_3$. It was then allowed to react with 0.2 ml of 5% 2,4-dinitrofluorobenzene (DNFB) in EtOH according to the method of Sanger.[13] After the reaction, solvents were evaporated and the residue was dissolved in 3 ml of H_2O and extracted 3 times with 15 ml of ether. The ethereal extract was evaporated and the residue was dissolved in a small volume of MeOH and applied on a silica gel thin-layer plate along with dinitrophenylated I and V. The solvent ($CHCl_3$–EtOH, 40: 0.3, v/v) was run ascendingly twice; to 10 cm distance at the first run and to 13.5 cm distance at the second run. Two spots which showed the same *Rf* values with DNP-I (*Rf* 0.39) and DNP-V (*Rf* 0.48), respectively, were observed in dinitrophenylated Fr. 1 from the complete incubation system but none of them was observed in the corresponding samples prepared from the substrate-omitted and enzyme-omitted systems.

The two *in vitro* metabolites in Fr. 2 indicated the mobility same to III and IV on TLC. The spots were extracted separately with $CHCl_3$–MeOH (9:1, v/v) and measured UV absorption spectra. Each extract revealed the absorption maximum at 265 mμ in MeOH and at 287 mμ in MeOH–2N NaOH (1:1, v/v). Such spectral features are exactly identical to those of authentic III and IV.

Discussion

In this paper we have studied on the metabolites of 1-*n*-butyryl-4-cinnamylpiperazine hydrochloride (BCP-HCl) in the urine and the bile of rats as well as on the metabolites produced in the *in vitro* system using the rat liver post-mitochondrial supernatant. Both TLC and GLC were used for the detection and isolation of metabolites, and several major metabolites thus isolated were identified by comparing their chemical and physicochemical properties with some authentic compounds.

As the results, the following seven metabolites, 1-*n*-butyrylpiperazine (I), piperazine hexahydrate (II), 1-*n*-butyryl-4-(4′-hydroxycinnamyl)piperazine (III), 1-(4′-hydroxycinnamyl)-piperazine (IV), 1-cinnamylpiperazine (V), UK-1 and 2 were detected as urinary metabolites of BCP-HCl and identified as such except the last two compounds. In the bile, I, III and IV, in addition to the glucuronide and sulfate conjugates of III, IV and UK-1 were detected. The metabolites produced in the *in vitro* system were I, III, IV and V. Although the chemical entities of UK-1 and 2 have not yet been clearly elucidated, the following findings seem

13) F. Sanger, *Biochem. J.*, 39, 507 (1945).

to be useful in speculating their chemical entities. The fact that both UK-1 and 2 react with 2,6-dichloro-*p*-quinone as well as with diazotized sulfanilic acid suggests that these metabolites may possess at least one secondary amino group (probably in a form of deacylated piperazine ring) and the phenolic nature at the same time. The fact that UK-1 was partially converted with ease to 1-(4′-hydroxycinnamyl)piperazine (IV) and the fact that both UK-1 and 2 could be detected by TLC using the solvent system A but not the solvent systems of B and C which contained ammonia suggested that UK-1 and 2 were rather labile metabolites and easily converted to the known metabolites such as IV and II, which were present in the urinary Fr. 2 and could commonly be detected with any solvent system. From these experimental findings we speculated the chemical entities of UK-1 and 2 as follows; 1-(4′-hydroxycinnamyl)piperazine-N^1-oxide (N-oxide of IV) for UK-1 and the reaction product of *p*-hydroxycinnamaldehyde and piperazine (II) for UK-2. The metabolic fate of BCP-HCl in the rat may be summarized in the metabolic map illustrated in Chart 6 with some reservation as to the chemical entities of UK-1 and 2.

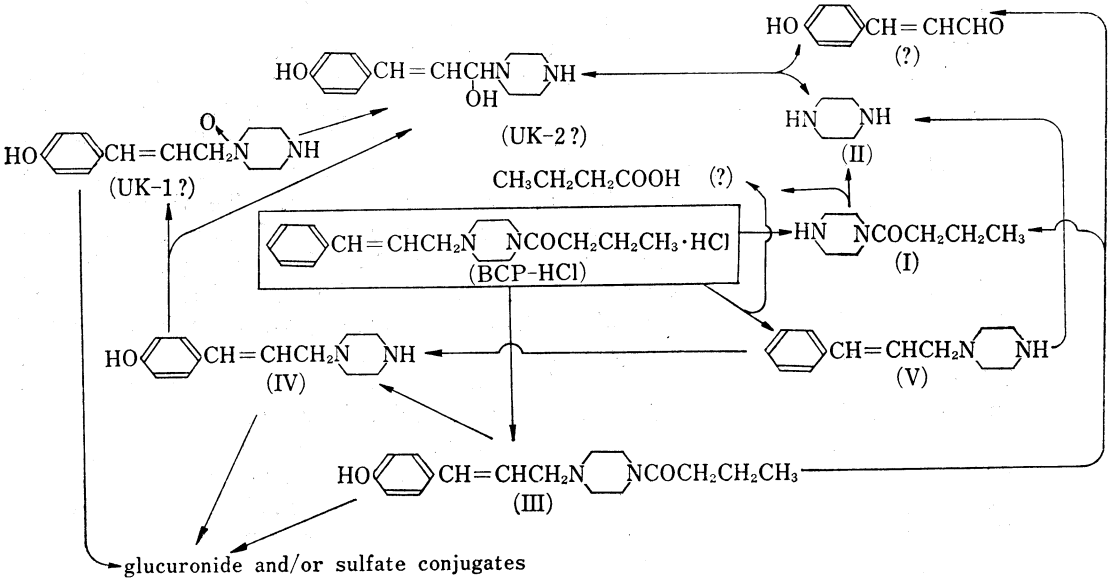

Chart 6. Proposed Metabolic Pathways of BCP-HCl in the Rat

The fact that the rat liver post-mitochondrial system can generate I, III, IV and V from BCP-HCl seems to be in accord with the concept that the rat liver enzymes are responsible for N-dealkylation (microsomal enzymes requiring NADPH and oxygen), aromatic ring hydroxylation (microsomal enzymes requiring NADPH and oxygen) as well as hydrolytic deacylation (amidase) and seems to support that the liver may be a major site for the BCP-metabolisms.

As to a chemical congener of BCP, cinnarizine,[14] it has been reported that this drug is N-dealkylated *in vivo* to yield cinnamaldehyde, which is then further oxidized and finally excreted in urine as benzoic acid and hippuric acid.

In this paper the major purpose was to isolated and identity the metabolites of BCP-HCl in the rat, which contain a piperazine ring. Quantitative analyses of these metabolites are now in progress in our laboratory using the GLC.

Acknowledgement The authors thank Mr. H. Saito and Mr. T. Umezawa (Kyorin Chemical Laboratory) for the physicochemical analysis.

14) W. Soudijn and I.v. Wijngaarden, *Life Sci.*, **7**, 231 (1968).