THE STUDY ON THE BIOLOGICAL FATE OF PARABEN AT THE DOSE OF PRACTICAL USAGE IN RAT. I. THE METABOLISM AND EXCRETION OF ETHYL *p*-HYDROXYBENZOATE (ETHYL PARABEN) AND *p*-HYDROXYBENZOIC ACID*

HIROSHI KIWADA, **¹ SHOJI AWAZU, **² AND MANABU HANANO

Faculty of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo Bunkyo-ku, Tokyo 113, Japan

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The biological fates of ethylparaben (EP) which has been widely used as a preservatives for the pharmaceutical preparations and foods, and *p*-hydroxybenzoic acid (HB) which is the parent compound of EP, were investigated at the dose of practical usage (2 mg/kg) in rats. Although both EP and HB were metabolized to glycine conjugate (M1), ester type glucuronide (M3) and sulfate (M4) of HB and excreted in the urine and bile, the excretion ratios were different as compared with the results of higher dose experiments carried out by other authors, and a route dependency was also found in the rate of excretion in the bile.

The excretion data obtained in this study are shown as follows: In intravenous administration of EP, the excreted total activity was 91.3% per dose in the urine and 5.97% in the bile, and percentages per dose of the major metabolites excreted in the urine were 8.14% of HB, 39.6% of M1, 29.5% of M3 and 6.48% of M4. In intraduodenal administration of EP, the excreted total activity (83.5%) and the activity excreted as HB (3.51%) decreased compared with the intravenous administration.

In the HB administration, the route dependency of the total activity was not found, but a decrease in the excretion of HB in the intraduodenal administration was found.

The excretion of total radioactivity almost ceased by 5 hr and the biological half-lives obtained from the β -phase of the sigma-minus plots of the metabolites excreted in the urine were 40—70 min.

The result obtained in this study differ from those of other authors at the high dose experiments.

Keywords—biological fate; metabolism; excretion; paraben; ethyl *p*-hydroxybenzoate; *p*-hydroxybenzoic acid

Alkyl *p*-hydroxybenzoate (paraben) has been widely used as a preservative for pharmaceutical preparations, foods, and cosmetics.

Matthews *et al.* showed that parabens were safe preservatives based on the study in the acute and clinical toxicity.¹⁾ On the biological fates, Tsukamoto *et al.* demonstrated that *p*-hydroxy-

benzoic acid (free acid, HB), *p*-hydroxyhippuric acid (glycine conjugate of HB, M1), *p*-carboxyphenyl glucuronide (ether type glucuronide of HB, M2), *p*-hydroxybenzoyl glucuronide (ester type glucuronide of HB, M3) and *p*-carboxyphenyl sulfate (ether sulfate of HB, M4) were excreted in the urine of the rabbit after oral administration of

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** Present address: 1) *Faculty of Pharmaceutical Sciences, Science University of Tokyo, 12, Ichigaya Funagawara-machi Shinjuku-ku, Tokyo 162, Japan*; 2) *Tokyo College of Pharmacy, Horinouchi Hachioji, Tokyo 192-03, Japan*.

ethylparaben (EP) at a dose of 400—800 mg/kg.²⁻⁴⁾ And Derache *et al.* demonstrated that HB, M1, M4 and glucuronide of HB were excreted in the urine of rats after oral administration of HB at the dose of 100 mg/head, and also presented the total blood concentration curve of various parabens at the same dose level.⁵⁾

Since the safety of various additives has been questioned recently, it has become important to obtain a more detailed information on the biological fates at the dose of practical usage.

In this study, the carbonyl-¹⁴C- labeled HB or EP synthesized from the labeled HB was injected into the femoral vein or the duodenum of rats at the dose of 2 mg/kg which is the practical dose of usage in man, and the excretion of its metabolites in the urine and bile was determined with time.

EXPERIMENTAL

Materials —i) ¹⁴C-HB: Carbonyl labeled ¹⁴C-HB was purchased from Daiichi Pure Chemicals Co., Ltd., of which specific activity was 3.13 mCi/mmol and radiochemical purity was 99%. This was used without further purification or dilution with nonlabeled HB.

ii) ¹⁴C-EP: ¹⁴C-EP was synthesized in a micro-scale from ¹⁴C-H according to the method reported by Cavill *et al.*⁶⁾ as follows. In 0.5 ml of ethanol, 24.8 mg of labeled HB was dissolved and a drop of conc. H₂SO₄ was added. The mixture was refluxed on an oil bath at 140° for 8 hr and was neutralized with ammonia in a cold bath. After the excess of ethanol was evaporated, a small volume of water was added to the residue and the product was extracted with benzene. After the benzene was evaporated, the colourless fine needles were recrystallized from H₂O-EtOH mixture. This product was confirmed by melting point, 116° (lit.⁶⁾ 115°, auth. 116—117°) and infrared (IR) spectrum, comparing with authentic sample, ethyl *p*-hydroxybenzoate (Wako Pure Chemical Ind. Ltd., G.R.).

Method of Determination —Samples of urine and bile were diluted to 10 ml with water. Liquid scintillator, 15 ml, (0.4 mg of POPOP, 6.5 g orof DPO and 75 g of naphthalene/1000 ml of dioxane)

was added to 0.1 ml of the diluted urine sample and to 0.2 ml of the bile sample respectively. The total radioactivity of each sample was counted with a liquid scintillation counter (Packard 3214).

Benzene at volumes of 2.5 ml and 5.0 ml were added to another 0.5 ml of diluted urine sample and 1.0 ml of diluted bile sample respectively. The mixture were shaken for 15 min and centrifuged for 10 min at 3000 rpm in order to separate benzene from aqueous layer. The liquid scintillator, 15 ml, was added to 2.0 ml of benzene layer of each sample, and the radioactivity of unmetabolized paraben was counted. This benzene extraction procedure was not carried out in the sample from the rat receiving HB. EtOH (5—7 ml) was added to the aqueous layer, shaken for 15 min, and centrifuged for 10 min at 3000 rpm for removal of protein and extraction of metabolites. The extract was concentrated by evaporation at 45°, dissolved again in several drops of EtOH, and charged on the thin-layer chromatography (TLC) plate of cellulose (Merck No.5728). TLC was developed in (EtOH: AcOH: H₂O = 4:1:5) and air-dried at room temperature. An X-ray film (Fuji KX-Medical) was exposed on this TLC plate for the autoradiography. After 1 week, the TLC plate including the spots of the autoradiogram was scraped. To the scraped cellulose powder several drops of EtOH and 15 ml of liquid scintillator were added and the radioactivities of the metabolites counted.

The metabolites were determined as follows. The urine of rabbit after administration of EP (5 g), suspended with 1 g of CMC (carboxymethylcellulose) in water, was treated by the method of Tsukamoto *et al.*^{4,5)} and the resultant paper chromatogram was transcribed to the cellulose thin-layer chromatogram obtained by the present method from the same urine.

Experimental Procedure —Male albino Wistar rat (210—240 g) anesthetized with ether was cannulated in the upper and down ward bile-duct, femoral vein, femoral artery, and bladder. The operated rat was set in the Bollman cage and infused into the downward bile duct with the bile collected to another rat at the infusion velocity of 0.7 ml/hr over night.

The solution for administration was prepared as follows. ^{14}C -HB was dissolved in saline solution at the concentration of 0.05% and ^{14}C -EP was dissolved in saline solution containing small amount of EtOH as a preservative. After these solutions were administered into the femoral vein or into the duodenum through the bile-duct at the dose of 4.0 ml/kg, the urine and bile samples were collected at appropriate time intervals for 5 hr through the polyethylene tubing set into the bladder and bile duct, respectively.

Data Analysis—A cpm (counts per minute) value obtained from each sample was converted to the dpm (disintegrations per minute) value with the quenching curve obtained in each treatment of the sample with the auto external standard method and then to the excreted amount per dose. The cumulative percent excretion per dose of each fraction for 5 hr, and then from these cumulative values the sigma-minus values were calculated. The nonlinear least square method was applied for curve fitting of two-compartment kinetic analysis of the excretion in the urine. These calculations

were carried out by the TSS system of HITAC 8800/8700 digital computer in the computer center of the University of Tokyo. The routine programs for these calculations were written in Fortran by the authors.⁷⁾

RESULTS

Excretion of EP and Its Metabolites

In urinary excretion, the time-courses of the cumulative percent per dose for the total and the major metabolites of EP after the intravenous administration of EP are shown in Fig. 1-a. Since the excretion almost ceased by 5 hr as shown in Fig. 1, the excreted percent per dose in the urine and bile for 5 hr are listed in Table I. Little unmetabolized EP was detected in both samples of urine and bile, and M2 was hardly detected because the spot of M2 on the autoradiogram was broad and weak after 1 week exposure. The same course and percentages after duodenal administration of EP are shown in Fig. 1-b and listed in Table I, respectively. The activity excreted in the urine was 83.5% per dose which was smaller than that following the in-

FIG. 1. Urinary Excretion Time Courses of the Total and Main Metabolites after the Administration of EP (Ethyl *p*-Hydroxybenzoate)

○ : total, ● : HB (*p*-hydroxybenzoic acid), ◇ : M1 (*p*-hydroxyhippuric acid) ◆ : M3 (*p*-hydroxybenzoyl glucuronide), △ : M4 (*p*-carboxyphenyl sulfate).

Plots are the mean values and vertical bars show the range of two rats.

All lines are drawn by visual approximation.

a : intravenous administration, b : intraduodenal administration.

TABLE I. *The Excreted Percents per Dose of the Main Metabolites in Urine and Bile 5 hr after the Administration of EP*

Excreted form	Excreted percent per dose			
	Intravenous administration		Intraduodenal administration	
	Urine	Bile	Urine	Bile
Total	91.34±0.71	5.97±1.77	83.45±2.00	12.83±0.50
EP	0.03±0.00	—	0.01±0.00	—
HB	8.16±0.93	0.45±0.12	3.51±0.36	0.53±0.10
M1	39.31±2.95	3.71±1.08	37.58±3.77	6.42±1.24
M3	29.47±2.01	0.31±0.09	31.61±4.57	0.65±0.05
M4	6.47±0.48	0.43±0.19	8.12±0.73	0.52±0.02

Total: EP+HB+M1+M3+M4+remained radioactivity on the TLC as the unknown metabolites and others.

EP, HB, M1, M3 and M4: see text.

Results are expressed as the mean value ± range of two rats.

FIG. 2 *Urinary Excretion Time Courses of Total and Main Metabolites after the Administration of EP*
 ○ : total, ● : HB, ◇ : M1, ◆ : M3, △ : M4, (see in Fig. 1).

Plots are the mean values and vertical bars show the range of two rats.

All lines are drawn by visual approximation.

a: intravenous administration, b: intraduodenal administration.

travenous administration, while the excretion in the bile was higher after the intraduodenal than the intravenous administration.

Excretion of HB and Its Metabolites

The time-courses of the cumulative percent per dose excreted in the urine for the total and the major metabolites after the intravenous administration of HB are shown in Fig. 2-a. The excreted percent per dose in the urine and bile up to 5

hr are listed in Table II. Fig. 2-b shows the excretion in the urine after the intraduodenal administration of HB. The excreted percent per dose in the urine and bile up to 5 hr are also listed in Table II. The route dependency in excretion was not recognized in the experiments with HB.

Kinetic Analysis

The semi-logarithmic plots of the activities per dose to be excreted against time after the ad-

TABLE II. *The Excreted Percents per Dose of the Main Metabolites in Urine and Bile 5 hr after the Administration of HB*

Excreted form	Excreted percent per dose			
	Intravenous administration Urine	Bile	Intraduodenum administration Urine	Bile
Total	95.41 ± 3.05	4.77 ± 1.87	95.73 ± 1.98	3.86 ± 0.17
HB	15.19 ± 4.47	0.36 ± 0.02	3.04 ± 1.78	0.36 ± 0.02
M1	48.79 ± 0.68	3.29 ± 1.48	51.53 ± 1.42	2.35 ± 0.26
M3	18.65 ± 0.57	0.24 ± 0.07	24.22 ± 2.54	0.25 ± 0.04
M4	8.55 ± 0.16	0.15 ± 0.04	9.49 ± 4.04	0.13 ± 0.03

Total: HB+M1+M3+M4+remained radioactivity on the TLC as the unknown metabolites and others.
HB, M1, M3 and M4: see text.

Results are expressed as the mean value + range of two rats.

FIG. 3. *Semilogarithmic Plots of the Main Metabolites to be excreted in Urine after the Administration of EP*

● : HB, ◇ : M1, ○ : M3, △ : M4, (see in Fig. 1).

Plots are mean values and all lines are drawn by visual approximation.

a: intravenous administration, b: intraduodenal administration.

ministration of EP or HB, namely the sigma-minus plot, are shown in Fig. 3, 4, for the data shown in Fig. 1, 2, respectively. The biological half-lives obtained from the β -phase of the sigma-minus plot are listed in Table III, and the elimination rate constants obtained from the two compartment open model analysis of the sigma-minus plot are listed in Table IV.

DISCUSSION

The excretion of EP in the urine and bile was negligible, while HB and its metabolites was ex-

creted rapidly after EP was administered intravenously at the dose of practical usage. The excretion pattern was similar to that following the HB administration. This fact suggests that the hydrolysis of EP is very rapid, and that HB, the hydrolyzed product of EP, is rapidly metabolized to M1, M3 and M4, and successively excreted in the urine.

Both the excretion rate and metabolic ratio, however, was dose dependent, and differ from the results at the higher dose reported by Tsukamoto *et al.* and Derache *et al.*

FIG. 4. Semilogarithmic Plots of the Main Metabolites to be Excreted in Urine after the Administration of EP

● : HB, ◇ : M1, ○ : M3, △ : M4, (see in Fig. 1)

Plots are mean values and all lines are drawn by visual approximation.

a: intravenous administration, b: intraduodenal administration.

TABLE III. The Biological Halflives of the Main Metabolites (min)

Excreted form	Administered form			
	EP-IV	EP-ID	HB-IV	HB-ID
HB	67.37±2.89	54.30±2.12	64.22±5.22	70.37±17.27
M1	57.67±7.52	56.92±5.34	41.10±1.59	46.06± 2.34
M3	48.97±5.25	52.87±0.23	57.94±8.20	42.56± 1.80
M4	45.94±0.79	44.97±0.17	44.42±2.72	47.44± 3.72

EP-IV: intravenous administration of EP.

EP-ID: intraduodenal administration of EP.

HB-IV: intravenous administration of HB.

HB-ID: intraduodenal administration of HB.

EP, HB, M1, M3 and M4: see text.

Results are expressed as the mean value ± range of two rats.

Tsukamoto *et al.* reported that 36 % of HB, 20 % of M1, 8 % of M2, 12 % of M3 and 9 % of M4 were excreted in 24 hr urine of rabbit after the oral administration of EP at a dose of 400–800 mg/kg, and that, after the administration of HB at the same dose, 55 % of HB, 18 % of M1, 7 % of M2, 6 % of M3 and 5 % of M4 were excreted.⁵⁾ Derache *et al.* reported that, 40 % of HB, 23.5 % of M1, 23 % of glucuronide of HB, 5 % of M4 and 5.5% of unknown metabolite per dose were ex-

creted in 24 hr urine of rat after oral administration of HB at the dose of 100 mg/head.³⁾

In this experiment at the dose of 2 mg/kg, the excretion in the urine almost ceased by 5 hr after intraduodenal administration in rat and 3.51 % of HB, 37.6 % of M1, 31.7 % of M3, 8.12 % of M4 and 6.08 % of unknown metabolite per dose were excreted after EP administration, and 3.04 % of HB, 51.5 % of M1, 24.2 % of M3, 9.49 % of M4 and 6.44 % of unknown metabolite were excreted

TABLE IV. *The Elimination Rate Constants of the Main Metabolites (hr⁻¹)*

Excreted form	Administered form			
	EP-IV	EP-ID	HB-IV	HB-ID
HB	4.47±0.51	5.60±1.28	5.61±0.37	6.37±1.08
M1	4.31±0.29	3.56±1.99	5.50±0.31	6.12±0.70
M3	3.60±0.32	3.06±0.09	4.00±0.39	4.04±0.12
M4	1.55±0.15	2.93±0.30	2.58±0.72	2.73±0.21

EP-IV: intravenous administration of EP, EP-ID: intraduodenal administration of EP, HB-IV: intravenous administration of HB, HB-ID: intraduodenal administration of HB, EP, HB, M1, M3 and M4: see text.

Result are expressed as the calculated rate constant±standard error obtained from the least square analysis of the sigma-minus plots of mean values in each experiment as shown in Fig. 3 and 4.

TABLE V. *The Comparison of the Metabolic Ratio between High Dose and Practical Dose*

Author	Derache	Tsukamoto		Present	
Animal	Rat	Rabbit		Rat	
Dose	100mg/head	400—800mg/kg		2mg/kg	
Time	24 hr	24 hr		5 hr	
Drug	HB	HB	EP	HB	EP
Metabolite	Excretion ratio (% per dose)				
Free	40	55	36	3.0	3.5
Gly	23.5	18	20	51.5	37.6
Glu (eth)	23	7	8	—	—
Glu (est)	—	6	12	24.2	31.6
Sul	5	5	9	9.5	8.1
Unknown	5.5	—	—	6.4	6.1
Total	97	91	85	95.7	83.5

Free: unconjugated *p*-hydroxybenzoic acid (HB), Gly: glycine conjugate of HB (M1), Glu (eth): ether glucuronide of HB (M2), Glu (est): ester glucuronide of HB (M3), Sul: ether sulfate of HB (M4).

in the urine after HB administration. As described previously M2 might be excreted appreciably, though the spot of M2 was not detected on the autoradiogram, because the spot was broad. These comparisons are shown in Table V. The unconjugated HB excreted in the urine in the present study was less than that found in the previous study. This shows the saturation of the conjugating capacity for HB. Levy *et al.*^{8,9)} reported that man has a limited capacity for the glycine and glucuronic acid conjugation of salicylic acid at the therapeutic dose range of salicylate, and the excretion of the unchanged salicylic acid increased in

a higher dose. It would be considerable that rat also has a limited capacity in the conjugation of HB at a higher dose. The excretion of total radioactivity almost ceased by 5 hr in this study as shown in Fig. 1 and 2. In the study of Tsukamoto *et al.*¹⁰⁾ 76 %, 82 % and 86 % per dose was excreted in the urine till 9, 24 and 48 hr after the administration of EP at the dose of 400 mg/kg respectively, and similar results have been obtained in the case of HB administration. Although this delay of excretion reported could be partially explained by the gastric emptying time or the entero-hepatic circulation, it may be speculation that rat also has the limited

capacity for the excretion of HB and its conjugates like salicylic acid in man as reported by Levy *et al.*

The variation in the excretion by the route of administration was found in the case of EP administration in this study. In the intravenous administration, the total activity, 91.3 % per dose was excreted in the urine and 5.97 % of dose was excreted in the bile. In contrast, 83.5 % was found in the urine and 12.8 % in the bile by intraduodenal administration.

The excretion ratio of HB after EP administration decreased from 8.14 % per dose in the intravenous administration to 3.51 % per dose in the intraduodenal administration. The route dependency of the ratio between the urinary and biliary excretion after HB administration was not found but of the ratio of free HB to the dose in the urinary excretion. These results in excretion *i.e.* the route dependency on EP administration and the difference between HB and EP administration, suggest that EP was hydrolyzed and partially conjugated in the liver cell before the release of HB in the blood for the first pass, and excreted in the bile, and that the rate of uptake into the metabolic site of EP was more rapid than that of HB, and rate of release of HB into the blood was slower than that of conjugates.

This mechanism can explain the increase in the biliary excretion, and the decrease in the excretion ratio of HB in the urine after the intraduodenal administration. An example for the change in the excreted ratio of the metabolite between the direct precursor and the pro-precursor or by the route of administration of the pro-precursor as found in this study has been reported by Shomura *et al.*¹¹⁾ with a macrolide antibiotic and its metabolite. As to the results of kinetic analysis, the biological half-lives of HB were slightly longer than other conjugates in every experiments. But differences in the half-lives among the chemical forms or the route of administration were not found. This is derived from the calculated half-lives of the β -phase of the sigma-minus plots, because the difference of the excretion ratio is attributed to the difference of the biological fate in the early step at the higher concentration. And the elimination constant obtained from the

least square analysis of the sigma-minus plots according to the two-compartment model, showed small values in only M4 in every experiment. But it did not show the difference between the administration as described above. Therefore, it indicates that the absorption of EP or HB and the hydrolysis of EP are very rapid and that the quantity differences among the chemical forms or the routes of administration as mentioned above are attributed to the biological fate in the very early stages such as absorption or hydrolysis at a high concentration, and significant kinetic differences were not found in the later stages of steady states.

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