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107. **Hisao Tsukamoto***1 **and Seisuke Terada***2 : Metabolism of Drugs. XLVII.*3 Metabolic Fate of *p*-Hydroxybenzoic Acid and its Derivatives in Rabbit. (4).

(*Institute of Pharmaceutical Sciences, Faculty of Medicine, Kyushu University**1 *and Hygienic Research Laboratory of Nagasaki Prefecture**2)

Alkyl *p*-hydroxybenzoates are widely used as a preservative for drugs, foods, and cosmetics. Nevertheless, there is only a few literature on the metabolism and excretion of these materials.

p-Hydroxybenzoic acid and its alkyl esters which possess two functional groups might undergo various metabolic changes in the animal body. Particularly in the ester, a functional carboxyl group could be formed on hydrolysis *in vivo.*[1,2]

It has been reported by some workers that the metabolic products of *p*-hydroxybenzoic acid detected and isolated from the urine of animals or man receiving this drug were *p*-carboxyphenyl sulfate (sulfuric acid conjugate)[3,4] the free acid, *p*-hydroxyhippuric acid (glycine conjugate),[5] *p*-carboxyphenyl glucuronide (ether-type glucuronide), and *p*-hydroxybenzoyl glucuronide (ester-type glucuronide).[6]

The authors reported previously that the above five metabolites also occurred in the urine of rabbits administered orally methyl *p*-hydroxybenzoate, but the excretion rate of the free or conjugated *p*-hydroxybenzoic acid differed when given *p*-hydroxybenzoic acid, though the result could not be quantified.[7]

Metabolism of *p*-hydroxybenzoic acid has already been observed quantitatively by several workers. Schotten[8] found that after feeding man with *p*-hydroxybenzoic acid, 16% of its dose was excreted in the urine during 24 hours, as *p*-hydroxyhippuric acid and 35% unchanged. Hartles and Williams,[4] and Bray, *et al.*,[9] reported that, in a rabbit, *p*-hydroxybenzoic acid was largely unchanged and converted into the glucuronide (10~20%), ethereal sulfate (5~7%), and glycine conjugate (about 20%).

On the other hand, Jones, *et al.*[2] described the result of quantitative experiment on the metabolic fate of alkyl *p*-hydroxybenzoates and stated that after 1.0 g./kg. of these esters was given orally to dogs, most of the material was metabolized and excreted during 6~24 hours and that the recoveries from the urine of total material ranged 50~90%.

Biological interest in the fate of *p*-hydroxybenzoic acid and its esters has been reported but individual excretion rate of the above-mentioned five metabolites in rabbit has not yet been observed in detail.

The present investigation is a comparative study on the metabolism of various alkyl *p*-hydroxybenzoates and of *p*-hydroxybenzoic acid, which is the major metabolic product of its esters. Metabolism of the esters is very rapid and excretion of the free

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*1 Katakasu, Fukuoka (塚元久雄).

*2 Nakagawa-cho, Nagasaki (寺田精介).

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acid in the urine after administration of the esters is much less than that in the case of *p*-hydroxybenzoic acid, but output of the acids conjugated with glucuronic acid or glycine is much larger.

Experimental

Materials——Methyl *p*-hydroxybenzoate, m.p. 127°, ethyl *p*-hydroxybenzoate, m.p. 117°, propyl *p*-hydroxybenzoate, m.p. 96.5°, and butyl *p*-hydroxybenzoate, m.p. 71° were purchased, isopropyl *p*-hydroxybenzoate, m.p. 86°, isobutyl *p*-hydroxybenzoate, m.p. 77°, and *sec*-butyl *p*-hydroxybenzoate, m.p. 61°, were supplied by the courtesy of Ueno Seiyaku Co., and *p*-hydroxybenzoic acid, m.p. 214° was prepared by alkaline hydrolysis of its methyl ester.

Animal, Diet, and Dosage——Male rabbits (2.25～3.50 kg. body wt.) kept on a fixed diet as previously described,⁷⁾ were used. The materials were administered by a stomach tube as a 12% solution in the form of Na salt and 2 dose levels were used, 0.4 and 0.8 g./kg.

Procedure——The sample solution containing 50～300 γ of *p*-hydroxybenzoic acid in 10 ml. was placed in a glass-stoppered tube and added with 1 ml. of Millon's reagent with heating in a boiling water bath.¹⁰⁾ The tube was immersed in a boiling water bath for 10 min. and added with 8 ml. of H_2O immediately, and then cooled under running H_2O. The reaction mixture was diluted to 20.0 ml. with H_2O, mixed well, and absorbance measured at 510 mμ against a reagent blank within 5 min. Simultaneously, the absorbance was controlled with the use of a solution of *p*-hydroxybenzoic acid of known concentration.

Estimation of Free and Conjugated *p*-Hydroxybenzoic Acid——The method for the separation described by Bray, *et al.*⁹⁾ was applied in this work. Urine (10 ml.), acidified with $2N\,H_2SO_4$ (2 ml.), was extracted with three 15 ml. portions of Et_2O. The extract was evaporated to dryness under a reduced pressure, the residue was extracted successively with three 10 ml. portions of boiling toluene, and the toluene solution was evaporated to dryness under a reduced pressure. The solid residue was dissolved in H_2O and an aliquot of this solution was used for the determination of free *p*-hydroxybenzoic acid by the above procedure.

The residue (toluene-insoluble part) was combined with the aqueous solution remaining after the above Et_2O extraction. To this solution, 8 ml. of $10N\,H_2SO_4$ was added and the mixture was hydrolyzed by boiling for 60 min. The hydrolyzate was extracted with three 15 ml. portions of Et_2O, the extract was dried over anhyd. Na_2SO_4, and evaporated to dryness. The residue was dissolved in H_2O and an aliquot of this solution was used for the determination of conjugated *p*-hydroxybenzoic acid by the above procedure.

Control experiments, in which a mixture of known amounts of *p*-hydroxybenzoic acid (10 mg.), *p*-hydroxyhippuric acid (corresponding to 5 mg. as *p*-hydroxybenzoic acid), *p*-carboxyphenyl glucuronide (2.5 mg., ditto) and *p*-carboxyphenyl sulfate (2.5 mg., ditto) were extracted from normal rabbit urine, gave average recoveries corresponding to 97% of free *p*-hydroxybenzoic acid and 96% of the conjugated acid.

Estimation of Individual Metabolic Product in the Urine——It has previously been shown⁶⁾⁷⁾ that the urine of rabbits receiving *p*-hydroxybenzoic acid and its ester, gave five spots on the paper chromatogram using the solvent system of BuOH-AcOH-H_2O, and that the spots of Rf 0.88, 0.74, 0.57, 0.48, and 0.38 are those of *p*-hydroxybenzoic acid, *p*-hydroxyhippuric acid, *p*-carboxyphenyl glucuronide, *p*-hydroxybenzoyl glucuronide, and *p*-carboxyphenyl sulfate, respectively. Because of good separation, this technique was used for the estimation of each individual metabolite. The 24 hr. urine adjusted to pH 5 with AcOH, was evaporated at 35° under a reduced pressure to one-half the original volume, mixed with an equal part of EtOH, and centrifuged. An aliquot of the supernatant (0.05～0.10 ml.) was chromatographed on several sheets of filter paper (Toyo Roshi No. 50, 2 × 40 cm., solvent BuOH-AcOH-H_2O=4:1:5). Each zone of metabolites, whose position was tested with color reagent, was cut out and eluted with three 10 ml. portions of hot $0.2N$ NaOH. The eluate was evaporated on a boiling water bath to about 10 ml. and 8 ml. of $10N\,H_2SO_4$ added. The solution was hydrolyzed by boiling for 60 min., except that of Rf 0.88 fraction. The acidic solution was extracted with three 15 ml. portions of Et_2O. Et_2O solution was dried over anhyd. Na_2SO_4 and evaporated to dryness. The solid residue was dissolved in H_2O and subjected to quantitative analysis by the above procedure.

Control experiments, in which mixtures of known amounts of the various metabolites were added to normal rabbit urine, showed average recoveries corresponding to 97% of the free acid, 89% of glycine conjugate, 90% of glucuronic acid conjugate (the ether-type), and 86% of H_2SO_4 conjugate.

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Results and Discussion

It has previously been confirmed by paper chromatography that the unchanged ester was present only in a small amount during 24 hours in the urine of rabbit orally administered methyl *p*-hydroxybenzoate.[1] In the present investigation, in order to mark quantitatively the excretory unchanged ester, an aliquot of 24 hour urine, acidified with diluted sulfuric acid, was extracted with ether. The extract was treated with sodium hydrogencarbonate solution to remove free *p*-hydroxybenzoic acid and majority of other acidic substances, and then dried over anhydrous sodium sulfate. After removal of impurities with activated alumina, the ether solution was filtered and the filtrate was evaporated to dryness. The unchanged ester thus obtained was dissolved in ethanol and its absorbance measured at 252 mμ. When the unchanged ester in the urine was analyzed by this method, present recoveries were only about 0.2~0.9% of the dose of various alkyl esters. It may, therefore, be considered that alkyl *p*-hydroxybenzoates are easily decomposed *in vivo* and essentially all of those was converted into the free and conjugated *p*-hydroxybenzoic acid.

Table I, gives summarized results the free and conjugated *p*-hydroxybenzoic acids, found in the urine after oral administration of various alkyl *p*-hydroxybenzoates and

Table I. Excretion Rate of Metabolites in Urine of Rabbits receiving *p*-Hydroxybenzoic Acid and its Alkyl Esters

Drug	Rabbit body wt. (kg.)	Dose (mg./kg.)	Time (hr.)	*p*-Hydroxybenzoic acid			Metabolic rate (%)
				Free (mg.)	Conjugated (mg.)	Total (mg.)	
Methyl *p*-hydroxybenzoate	2.30	400	0~6	295	280	575	69
			6~10	45	45	665	80
			10~24	23	12	700	84
	2.80	800	0~6	504	647	1151	57
			6~10	242	256	1649	81
			10~24	47	98	1794	88
Ethyl *p*-hydroxybenzoate	2.30	400	0~9	293	289	582	76
			9~24	13	33	628	82
			24~48	10	21	659	86
	2.50	800	0~9	454	539	993	90
			9~24	115	194	1302	78
			24~48	40	70	1412	85
Propyl *p*-hydroxybenzoate	2.60	400	0~9	202	358	560	70
			9~24	43	73	676	85
			24~48	13	12	701	88
	2.80	800	0~9	413	552	965	56
			9~24	139	192	1296	76
			24~48	33	67	1396	81
Butyl *p*-hydroxybenzoate	2.80	400	0~9	172	268	440	55
			9~24	66	141	647	81
			24~48	23	31	701	88
	3.20	800	0~9	382	525	907	50
			9~24	186	279	1372	75
			24~48	32	46	1450	80
p-Hydroxybenzoic acid	2.25	400	0~6	444	225	669	74
			6~10	56	72	797	89
			10~24	23	7	827	92
	2.25	800	0~6	824	319	1143	63
			6~10	227	121	1491	83
			10~24	31	22	1544	86

p-hydroxybenzoic acid, estimated at timely intervals. In the case of *p*-hydroxybenzoic acid, some 90% of the dose may be excreted in the urine as a metabolites during 24 hours, while about 70~90% of alkyl esters appeared as the excretory products. The present results approximately agree with observations made by Bray, *et al.*,[9] and by Jones, *et al.*[2] However, when the alkyl esters were administered, excretion was rather slower than that of *p*-hydroxybenzoic acid and became slightly slower with increase in the carbon number of alkyl group of the esters. Moreover, in the case of *p*-hydroxybenzoic acid, the output of unchanged acid was usually greater than that of the conjugated acid, while in the alkyl esters, the output of conjugated acid was considerably increased and was occasionally equal to or greater than that of the free acid.

Table II. Excretion Rate of Major Metabolites in 24 hour Urine of Rabbits receiving *p*-Hydroxybenzoic Acid and its Alkyl Esters

Drug	Rabbit body wt. (kg.)	Dose (mg./kg.)	*p*-Hydroxybenzoic acid (%)					Metabolic ratio (%)
			Free	Conjugated with				
				Glycine	Glucuronic acid (ester-type)	Glucuronic acid (ether-type)	Sulfuric acid	
Methyl *p*-hydroxybenzoate	2.60	800	43	7	4	11	9	74
	2.65	800	33	16	8	21	7	85
	2.25	400	45	14	9	14	9	91
	2.50	400	34	22	8	16	14	94
	means		39	15	7	15	10	86
Ethyl *p*-hydroxybenzoate	2.50	800	32	14	8	13	12	79
	2.50	800	47	17	7	19	4	94
	2.60	400	37	22	7	9	13	88
	2.60	400	29	27	9	9	6	80
	means		36	20	8	12	9	85
Propyl *p*-hydroxybenzoate	2.30	800	26	26	7	10	4	74
	3.10	800	28	30	5	12	9	84
	2.50	400	32	14	8	16	10	80
	3.20	400	33	27	6	14	5	85
	means		30	24	7	13	7	81
Isopropyl *p*-hydroxybenzoate	3.20	800	29	29	4	17	5	84
	3.10	800	21	31	7	20	4	83
	2.90	400	32	19	9	24	7	91
	3.50	400	23	37	4	10	12	86
	means		26	29	6	18	7	86
Butyl *p*-hydroxybenzoate	2.80	800	37	18	4	8	10	77
	2.60	800	29	23	7	9	4	72
	2.80	400	24	28	5	16	10	83
	3.00	400	34	27	6	12	5	84
	means		31	24	6	11	7	79
Isobutyl *p*-hydroxybenzoate	3.00	800	33	16	8	14	8	79
	3.40	800	26	31	6	8	6	77
	2.70	400	25	23	9	9	17	83
	3.40	400	32	22	6	10	15	85
	means		29	23	7	10	12	81
sec-Butyl *p*-hydroxybenzoate	3.10	800	24	28	5	10	8	75
	3.40	800	28	20	5	12	10	75
	3.10	400	28	30	5	9	9	81
	3.40	400	19	38	3	8	14	82
	means		25	29	4	10	10	78
p-Hydroxybenzoic acid	2.25	800	55	17	9	2	4	87
	2.30	800	63	8	6	5	6	88
	2.60	400	58	18	7	5	5	93
	2.70	400	45	28	7	10	4	94
	means		55	18	7	6	5	91

The individual metabolic product, excreted in 24 hour urine after oral administration of *p*-hydroxybenzoic acid and its alkyl esters, was separated by paper chromatography and estimated by spectrophotometric method using Millon's reagent. The separation of metabolites by this technique was satisfactory for the present purpose because control experiments with known materials showed average recovery of about 90%. It was found that, after the administration of *p*-hydroxybenzoic acid 55% of the dose was excreted in the urine during 24 hours as the unchanged acid, 18% as conjugated with glycine, 7% as the ester-type glucuronide, 6% as the ether-type glucuronide, and 5% as conjugate with sulfuric acid. When the alkyl ester was administered, it was converted into the free acid (25~39%), glycine conjugate (15~29%), ester-type glucuronide (5~8%), ether-type glucuronide (10~18%), and the sulfate (7~12%).

These results are summarized in Table II, from which it is observed that the excretion of free acid is much less in the case of the alkyl esters than that *p*-hydroxybenzoic acid and that this tendency becomes marked with increase of carbon number in the esters.

When the alkyl esters were given, glucuronic acid conjugation occurrs marked as compared to the administration of *p*-hydroxybenzoic acid, and above all, a difference in the ether-type conjugation is especially great. In the case of alkyl esters, output of the acid conjugated with glycine is occasionally equal to or greater than that of *p*-hydroxybenzoic acid.

The major metabolic products of alkyl *p*-hydroxybenzoates were tested except *p*-hydroxybenzoyl glucuronide for the preventive effect on the growth of *Staphylococcus aureus* 209-p, *Pseudomonas aeruginosa, Escherichia coli* 0111, *Aspergillu oryzae*, and *Saccharomyces sake.* Methyl *p*-hydroxybenzoate was used as the control material. Their activity was excessively weak and was less than 1/10 of that to methyl *p*-hydroxybenzoate.

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Summary

Metabolism of various alkyl *p*-hydroxybenzoates was investigated in rabbits using oral dose levels of 0.4 and 0.8 g./kg., and compared with than of *p*-hydroxybenzoic acid, which is a major metabolite of their esters. Metabolism of the esters was very rapid and excretion of the free *p*-hydroxybenzoic acid in the urine after administration was much less than that of *p*-hydroxybenzoic acid but output of the acid conjugated with glycine and glucuronic acid was much larger. When various alkyl esters were administered, those were converted into the free acid (25~39%), glycine conjugate (15~29%), ester-type glucuronide (5~8%), ether-type glucuronide (10~18%), and the sulfate (7~12%) during 24 hours.

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