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Distribution of Butylated Hydroxyanisole and Its Conjugates in the Tissues of Rats

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The distribution of butylated hydroxyanisole (BHA) and its conjugates was investigated in the liver, kidney, pancreas, plasma and urine 1 and 3 hr after oral administration of ^{3}H-labeled BHA to rats. The total radioactivities in the tissues were in the order kidney>liver>plasma>pancreas. BHA-S (sulfate-conjugate) and BHA-G (glucuronide-conjugate) could be separated clearly by paper electrophoresis. The radioactivities of BHA and its conjugates separated by electrophoresis were determined and the proportions of BHA, BHA-G and BHA-S were calculated. Unchanged BHA was mainly found in the liver and pancreas. The proportion of BHA-G increased in the order urine>kidney>plasma>pancreas>liver. On the other hand, that of BHA-S increased in the order plasma>kidney>pancreas>liver>urine. BHA-G was the major metabolite in urine and BHA-S was only a minor metabolite, but a considerable amount of BHA-S was found in plasma. Therefore, we investigated whether BHA-S administered intraperitoneally or intravenously is converted into the corresponding glucuronide to be excreted in urine. The results, however, showed that no significant amount of BHA-S was converted into BHA-G under the conditions used.

Keywords——butylated hydroxyanisole (BHA); BHA-glucuronide (BHA-G); BHA-sulfate (BHA-S); distribution; paper electrophoresis; rat

Several studies on the metabolic fate and excretion rate of BHA have been reported in man,[1,2] dogs,[1] rabbits,[3] and rats.[1,4] When BHA was orally administered to animals other than dogs, 80—90% of the dose was recovered in urine ,as the glucuronide (60—70%), ethereal sulfate (10—20%) and unchanged BHA (5%). On the other hand, in dogs, 30% of the administered dose was excreted in urine and 50—60% in feces, and the main urinary metabolites consisted of glucuronide (6%) and ethereal sulfate (23%). However, no work appears to have been done on the detailed distribution patterns of BHA and its metabolites in the tissues.

In the present study, whole-body autoradiography was carried out in rats by the method of Ullberg[5] using ^{3}H-labeled BHA.

High radioactivities were recovered in the liver, kidney and pancreas at 1 and 3 hr. Therefore, the chemical forms and distribution ratios of BHA and its metabolites in the above tissues as well as in plasma and urine were determined by paper electrophoresis.

Experimental

Chemicals——BHA (2-*tert*-butyl-4-methoxyphenol) was from Wako Pure Chemical Industries, Ltd. BHA-G was prepared from collected urine of rats given BHA orally, according to the method of Astill *et al.*[6] BHA-S was synthesized from BHA and chlorosulfonic acid in a mixture of carbon disulfide and diethylaniline by the method of Feigenbaum and Neuberg.[7] BHA-G and BHA-S preparations each showed a single peak on DEAE-Sephadex column chromatography (Fig. 2-A).

Preparation of Labeled Compound——^{3}H-labeled BHA was prepared by the platinum-catalyzed $^3H_2$ gas exposure technique of Shinroihi Co., Ltd. The crude sample was recrystallized from *n*-hexane. About 30 mg of ^{3}H-labeled BHA was dissolved in 1 ml of 1 N NaOH and acidified immediately with 2 ml of 4 N H_2SO_4 in an ice bath. The sample was extracted 3 times with 3 ml of *n*-hexane. The extracts were combined and evaporated to dryness *in vacuo*. The residue was dissolved in methanol, passed through a quartz column

(1.5×15 cm) packed with Kiesel gel 60 (Merck) containing 10% mixed fluorescent agent (B5-FM, Wako Pure Chemical Industries Ltd.), and eluted with a mixture of *n*-hexane–isopropyl ether (20: 1). The fluorescent band visible under UV light (at 254 nm) was collected and evaporated to dryness *in vacuo*. The specific radioactivity of ^{3}H-BHA was 487 μCi/mg and the radiochemical purity was found to be greater than 98% by thin-layer chromatography (polyamido FM plate (Wako), methanol–acetone–water (3: 1: 1, by volume)). ^{3}H-BHA-S was synthesized from ^{3}H-BHA by the method used for the synthesis of nonlabeled BHA-S. The specific activity of ^{3}H-BHA-S was 12.8 μCi/mg and the radiochemical purity was greater than 96% as determined by paper electrophoresis.

Animals and Treatment——Male Wistar rats weighing between 105 and 160 g were used. The animals received a single oral administration of 420 μCi (862 μg in 0.5 ml of olive oil) of ^{3}H-labeled BHA per rat. At 1 or 3 hr after administration, animals were anesthetized with pentobarbital (40 mg/kg, *i.p.*) and the blood was withdrawn by heart puncture. The liver, kidney and pancreas were removed and homogenized in 10 vol. of methanol. After centrifugation, the methanol sample solutions were stored at −20° until use.

In the excretion study of BHA-S, 6.5 μCi (508 μg) of ^{3}H-labeled BHA-S was given intravenously or intraperitoneally to rats and then each animal was placed in a separate metabolic cage to collect urine for 24 hr.

Macroautoradiography——After oral administration of ^{3}H-labeled BHA (420 μCi, 862 μg), rats were frozen in acetone-dry-ice at 1, 3, 6, and 24 hr, and 25-μm thick slices were cut with an Autochriotome (Nakagawa Seisaku Co., Ltd.) according to the method of Ullberg.[5] Autoradiograms were obtained by contact with MK-A macroautoradiography films (Konishiroku Co., Ltd.).

DEAE-Sephadex Column Chromatography——DEAE-Sephadex was washed with 1% $(NH_4)_2CO_3$ and changed to CO_3 form. One milliliter of the mixture of BHA-G and BHA-S was applied to a column of the DEAE-Sephadex (1×25 cm). The column was eluted with 1% $(NH_4)_2CO_3$ and fractions of 5 ml were collected. After the 40th fraction had been collected, the eluting solvent was changed to 5% $(NH_4)_2CO_3$ and elution was continued until the 80th fraction. The absorbance of each fraction at 284 nm for BHA-G and at 276.5 nm for BHA-S was estimated. The glucuronyl group was also estimated at 565 nm by the naphthoresorcinol method.[8] Inorganic sulfate was measured at 375 nm by the method of Egami and Takahashi.[9]

Paper Electrophoresis——The methanol sample solutions were concentrated, applied to paper strips (2×40 cm) of Toyo Roshi No. 51A and run with authentic BHA, BHA-G and BHA-S at 300 V for 4 hr in the following buffer system: 0.2 M sodium acetate–acetic acid buffer, pH 3.8. The bands of authentic samples on the strip were visualized with Lumicolor plate SB (Wako) under UV light (PAN UV lamp, Toshiba). The distances of migration of BHA, BHA-G and BHA-S to the anodic side were 0, 7 and 11 cm, respectively.

Radioactivity Measurement——The paper strips were cut in 1.0-cm strips from the origin and the radioactivity of each piece was determined in a toluene scintillator consisting of 0.4% 2,5-diphenyloxazole (DPO) and 0.01% 1,4-bis(2-(5-phenyloxazolyl)) benzene (POPOP). To determine the total radioactivity in tissues and plasma, aliquots of the sample (0.1 ml of 10% tissue homogenate or 0.1 ml of plasma) were solubilized by the addition of 0.5 ml of Soluene 350 (Packard) with gentle shaking at room temperature overnight. To the solubilized sample was added 10 ml of the toluene scintillator. In the case of urine, the sample was added directly to a scintillator consisting of 2 vol. of toluene, 2 vol. of dioxane, 1 vol. of methanol, 8% naphthalene, 0.4% DPO and 0.01% POPOP.

Results and Discussion

Whole-body autoradiography was carried out at 1, 3, 6, and 24 hr after administration of ^{3}H-labeled BHA. The autoradiograms are shown in Fig. 1.

The radioactivity of the gastrointestinal tract was mainly located in the stomach and duodenum at 1 and 3 hr, and in the cecum and lower part of the large intestine at 6 hr. At 24 hr, most of the radioactivity had disappeared from the stomach and small intestine and remained mainly in the large intestine. On the other hand, at 1 and 3 hr ,the radioactivity was markedly accumulated in the liver, kidney and pancreas, but considerable amounts of ^{3}H had disappeared from those organs at 6 hr. At 24 hr slight radioactivity remained in the kidney. As the high radioactivities were mainly distributed in the liver, kidney and pancreas at 1 and 3 hr, the chemical forms present in those organs were investigated.

Fig. 2 shows the separation of authentic BHA–G and BHA–S, and the profile of radioactivity of a pooled plasma sample obtained from rats given ^{3}H-labeled BHA orally. The results showed that BHA–G and BHA–S were separated well ,and that the radioactive peaks of the plasma sample coincided with those of authentic BHA–G and BHA–S. However this procedure is too tedious for the analysis of many samples. BHA and its conjugates could be separated

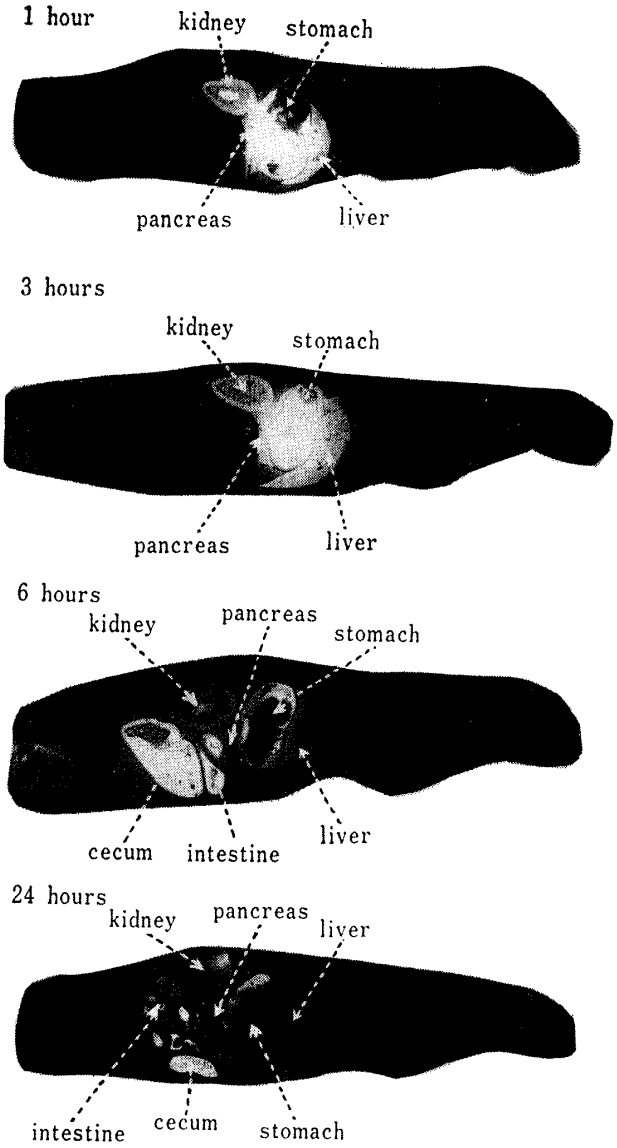

Fig. 1. Autoradiograms of Rats after Oral Administration of ^{3}H-BHA

Dose: 420 μCi (862 μg) ^{3}H-BHA/rat.

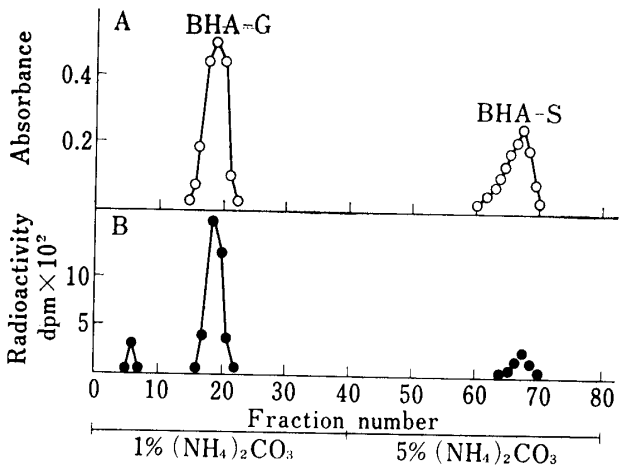

Fig. 2. Separation of BHA-G and BHA-S on a DEAE-Sephadex Column

Column, DEAE-Sephadex CO_3 (1×25 cm), volume of each fraction, 5 ml.
A: elution profile of authentic samples of BHA-G (284 nm) and BHA-S (276.5 nm).
B: elution profile of the radioactivity of plasma sample.

of the different metabolites in various samples. The total radioactivities in the tissues were in the order kidney>liver>plasma>pancreas at each time. The proportions (%) of BHA and its metabolites in the samples were in the following order: for BHA, liver and pancreas (24—36%)>plasma and kidney (3—5%)≫urine (0.5%); for BHA-G, urine (98%) >kidney (81—89%)>plasma (62—72%)>liver and pancreas (45—66%); for BHA-S, plasma (23—28%), kidney (6—11%), liver and pancreas (4—8%)≫urine (1%). The proportion of BHA-S was the highest in plasma, but extremely low in urine. We then investigated whether BHA-S could be converted into the corresponding glucuronide *in vivo* and excreted in urine. After intravenous and intraperitoneal administration of ^{3}H-labeled BHA-S to rats, 24 hr urine was analyzed by paper electrophoresis.

As shown in Fig. 4, there was a considerable individual variation in the urinary excretion ratio of BHA; it was 40—50% in two of five rats and 2—3% in the other animals. Contrary to our expectation, BHA-G was not detected by paper electrophoresis. The tailing regions of BHA-S towards BHA-G in Fig. 4 were collected and subjected to re-electrophoresis. A single band corresponding to authentic BHA-S was detected.

conveniently by paper electrophoresis under the conditions described in "Experimental."

Fig. 3 shows some examples of the electrophoretic patterns of samples from the liver, kidney, pancreas, plasma and urine of rats at 3 hr after administration of ^{3}H-labeled BHA. In the urine sample, the distances of migration of BHA-G and BHA-S were slightly different from those of other samples. This might be due to the effect of salts present in urine. Other samples obtained at 1 or 3 hr showed similar profiles.

Table I presents the total amount of radioactivity (as BHA) and the proportions

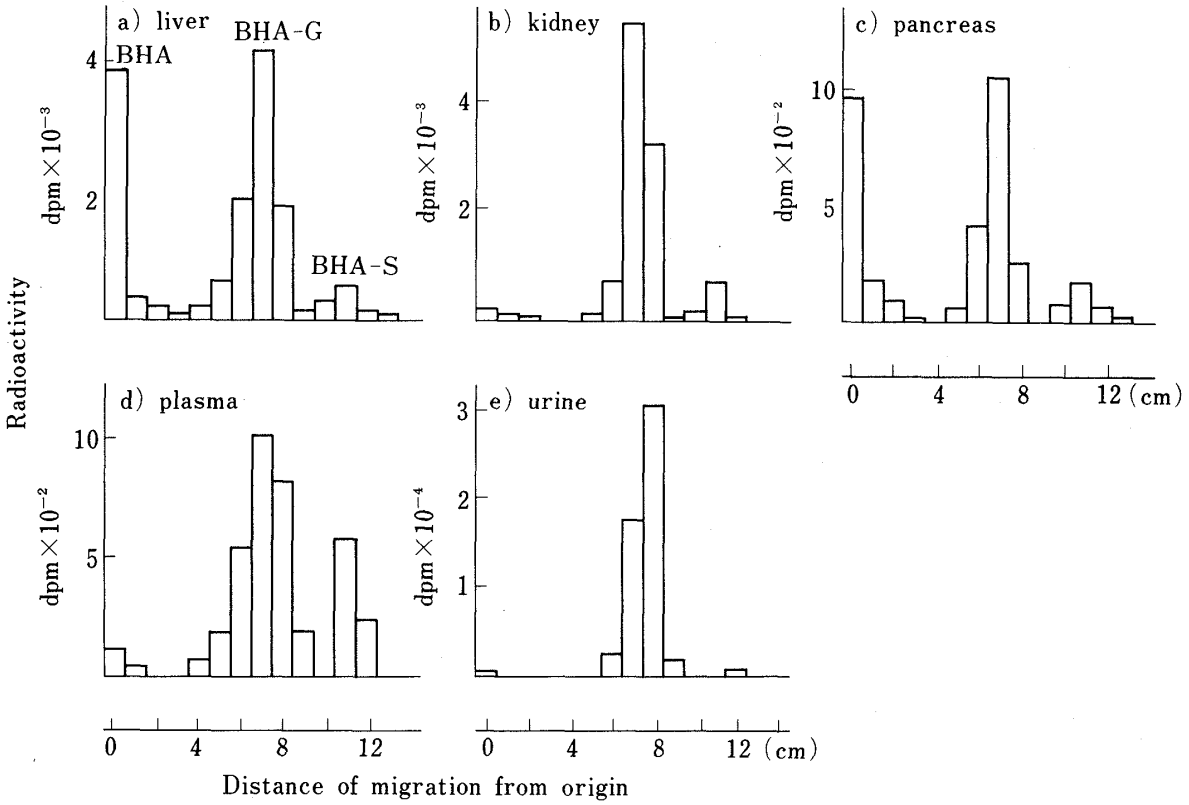

Fig. 3. Paper Electrophoresis of Tissue Extracts of Rats after Oral Administration of ^{3}H-BHA
0.2 M acetate buffer (pH 3.8); 300V; 4 hr. Dose: 420 μCi (862 μg) ^{3}H-BHA/rat.

TABLE I. Distribution of BHA, BHA-G and BHA-S

Tissue		Total amount (as BHA)		Per cent		
		mμCi/g or ml	ppm	BHA	BHA-G	BHA-S
Liver	1 hr	1098± 98.1	2.3	26.9± 6.5	65.8± 5.9	3.5±0.5
	3 hr	1075±245.1	2.2	31.7±13.3	52.2±13.6	4.2±1.2
Kidney	1 hr	2396±665.3	4.9	2.9± 0.6	88.8± 2.1	5.8±1.6
	3 hr	1524±392.8	3.1	3.0± 1.0	81.4± 3.4	10.8±3.6
Pancreas	1 hr	246± 54.3	0.5	23.5± 2.8	61.7± 4.0	7.0±2.4
	3 hr	258± 22.7	0.5	36.3±14.2	45.4±14.6	7.7±2.8
Plasma	1 hr	408± 94.2	0.8	3.0± 0.5	71.9± 5.8	22.6±6.5
	3 hr	426± 96.3	0.9	5.3± 1.2	61.7± 4.9	27.5±3.7
Urine	1 hr	34± 7.1*		0.4± 0.2	97.8± 0.6	0.6±0.2
	3 hr	118± 15.5*		0.6± 0.2	97.8± 0.6	0.9±0.2

Animals: male Wistar rats (110—160 g). Dose: 420 μCi (862 μg) ^{3}H-BHA/rat.
* μCi/total urine. Each value is a mean of five rats (with ± S.E.).

Astill *et al.*[6] reported that a considerable amount of ethereal sulfate was present in 5-day urine after oral administration of BHA to rats. In our experiments, 23—28% BHA-S was found in the 1 and 3 hr plasma but only a little (>1%) in the 1 and 3 hr urine. When ^{3}H-labeled BHA-S was injected intravenously or intraperitoneally, BHA-G was not detected in 24 hr urine, suggesting that the conversion of BHA-S to BHA-G did not occur under these conditions. It is possible that the excretion rate of BHA-S is slower than that of BHA-G, although further studies are clearly necessary.

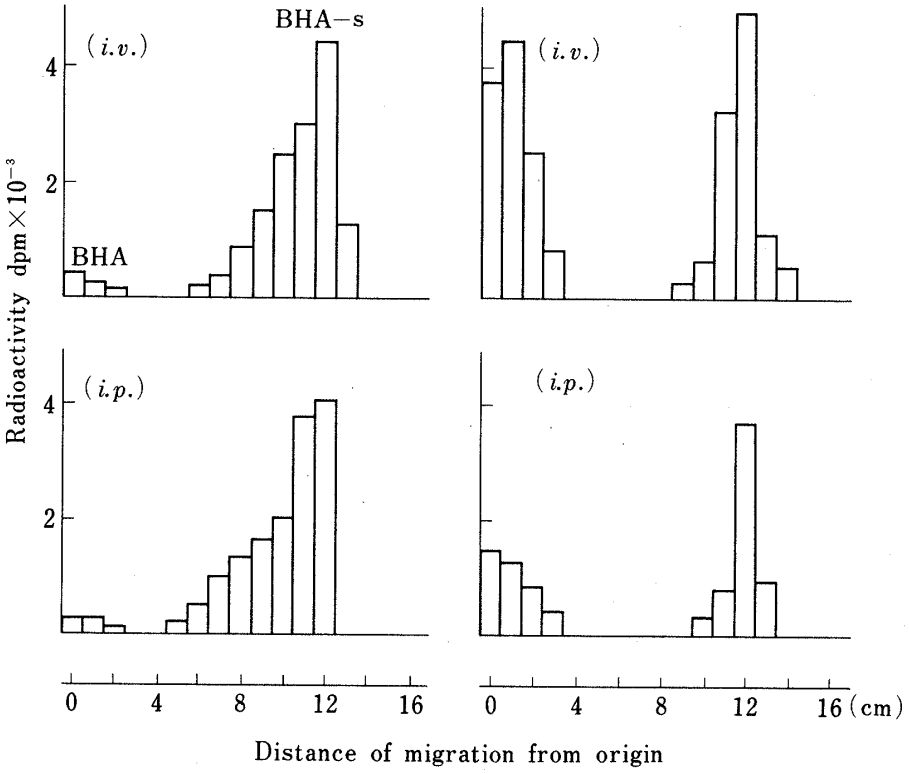

Fig. 4. Paper Electrophoretic Patterns of the Urine of Rats after Intravenous or Intraperitoneal Administration of ^{3}H–BHA–S

0.2 M acetate buffer (pH 3.8); 300 V; 4 hr. Dose: 6.5 μCi (508 μg) ^{3}H-BHA-S/rat.

References and Notes

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