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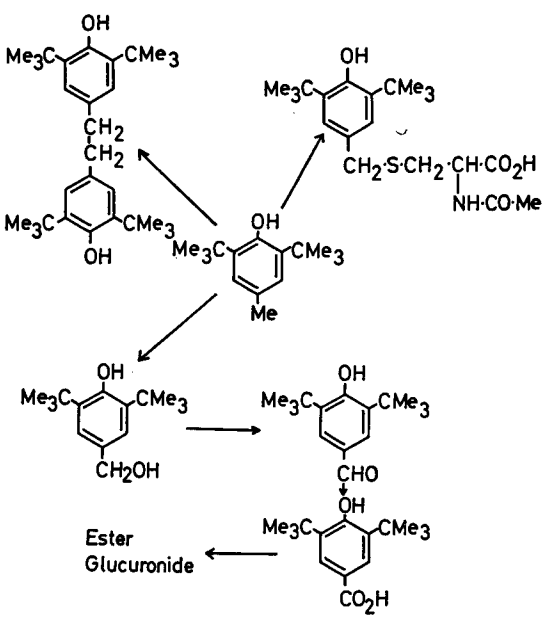
The biliary metabolism of butylated hydroxytoluene (3,5-di-t-butyl-4-hydroxytoluene) and its derivatives in the rat

G. M. HOLDER, A. J. RYAN, T. R. WATSON AND L. I. WIEBE

Department of Pharmacy, University of Sydney, Sydney, Australia

The biliary metabolism of 3,5-di-t-butyl-4-hydroxybenzyl alcohol (BHT–CH_2OH), 3,5-di-t-butyl-4-hydroxybenzaldehyde (BHT–CHO) and 3,5-di-t-butyl-4-hydroxybenzoic acid (BHT–COOH) after parenteral administration has been examined in the rat and compared to that of 3,5-di-t-butyl-4-hydroxytoluene. Quantitative excretion and chemical examination of bile showed that in the enterohepatic circulation BHT–COOH or its ester glucuronide is the recirculating compound from the four compounds studied. Biliary excretion data are also presented for 1,2-bis(3,5-di-*t*-butyl-4-hydroxyphenyl)ethane.

The metabolism of the widely used antioxidant butylated hydroxytoluene (3,5-di-t-butyl-4-hydroxytoluene; BHT) has been reviewed by Hathway (1966). The enterohepatic circulation of its metabolites in the rat was suggested by Daniel & Gage (1965) and demonstrated by Ladomery, Ryan & Wright (1967a), who showed that the metabolites were rapidly excreted into the bile, and relatively slowly into the urine and faeces. The biliary metabolites (Fig. 1) in the rat were later shown to be 3,5-di-t-butyl-4-hydroxybenzaldehyde (BHT–CHO), 3,5-di-t-butyl-4-hydroxybenzyl alcohol (BHT–CH_2OH), 3,5-di-t-butyl-4-hydroxybenzoic acid (BHT–COOH) and 1,2-bis-(3,5-di-t-butyl-4-hydroxyphenyl)ethane (BB) (Ladomery & others, 1967b.)



These findings correlated with urinary metabolites of BHT in the rat and rabbit (Dacre, 1960; Akagi & Aoki, 1962; Wright, Akintonwa & others, 1965). A previously unidentified metabolite in rat urine and bile was shown by Daniel, Gage & Jones (1968) to be *S*-(3,5-di-t-butyl-4-hydroxybenzyl)-*N*-acetylcysteine.

We now report on the biliary metabolism of BHT, BHT-CH_2OH, BHT-CHO, BHT-COOH and B-B in the rat. BHT-COOH was identified as the recirculating compound in the enterohepatic recirculation in the rat. Some additional urinary excretion data are reported.

EXPERIMENTAL

Metabolic experiments

White male Wistar rats (290–350 g), were given doses of [^{14}C]labelled BHT (or derivatives) in aqueous ethanol by intravenous or intraperitoneal injection. Urine and faeces were collected in metabolism cages fitted with all glass separating devices. Bile was collected through a biliary cannula for 6 to 8 h from rats under urethane anaesthesia.

Chromatography

Silica gel G (E. Merck & Co.) was used for both qualitative and preparative thin-layer chromatography (TLC). Various solvent systems used were: A, chloroform–methanol (49 : 1); B, light petroleum b.p. 60–80°; C, light petroleum b.p. 60–80°–ether (2 : 1); D, n-propanol–chloroform (7 : 3); E, methanol–chloroform (2 : 3); F, n-butanol–ethanol–water (2 : 1 : 1). Visualization of BHT and its derivatives was achieved with Gibb's reagent (0·5% 2,6-dichlorobenzoquinone-4-chloroimine in ethanol); glucuronides were detected with naphthoresorcinol reagent (0·3% naphthoresorcinol in ethanol–phosphoric acid (5 : 1). Iodine vapour, and rhodamine (1% in ethanol) viewed under ultraviolet light (254 nm) were used as general reagents.

Methods

Peroxide-free ether was used at all times. All reactions and *work-up* procedures were in a nitrogen atmosphere. Hydrolysis was effected by refluxing with N hydrochloric acid for 0·5 h, or by bacterial β-glucuronidase (Sigma type II) in 0·05M phosphate buffer, pH 7 at 37°.

Reverse isotope dilution analysis involved addition of unlabelled compound to the biological extract before work-up, and purification by preparative TLC. Other radiochemical assays were made on qualitative TLC chromatograms by counting after individual collection of the spots, suspension in scintillation fluid with Cab-O-Sil (Packard Instrument Co.).

"Free-phenols" refer to those compounds extractable from the biological sample by continuous ether partitioning at pH 6.

Materials

The following compounds, used as TLC standards and for reverse isotope dilution, were prepared or obtained from the source indicated: 3,5-di-t-butyl-4-hydroxytoluene (BHT) and bis(3,5-di-t-butyl-4-hydroxybenzyl)ether (Shell Chemical Co., Sydney); 3,5-di-t-butyl-4-hydroxybenzyl alcohol (BHT-CH_2OH), 3,5-di-t-butyl-4-hydroxybenzaldehyde (BHT-CHO), 3,5-di-t-butyl-4-hydroxybenzoic acid (BHT-COOH) were

synthesized according to Ladomery & others (1967b); 1,2-bis(3,5-di-t-butyl-4-hydroxyphenyl)ethane (B-B), 3,3′, 5,5′-tetra-t-butyl-4,4′-dihydroxystilbene (BHT-hydroxystilbene), 3,3′,5,5′-tetra-t-butylstilbene-4,4′-quinone (BHT-stilbenequinone) were prepared according to Cook (1953).

The following [^{14}C] labelled compounds were used: [^{14}C]BHT randomly labelled in the t-butyl groups was obtained from New England Nuclear Corporation, Boston with specific activity $6{\cdot}55 \times 10^6$ d/min mg^{-1} or $2{\cdot}85 \times 10^7$ d/min mg^{-1}.

[^{14}C]BHT-CHO was synthesized by bromine oxidation of [^{14}C]BHT (Coppinger & Campbell, 1953) and purified by preparative TLC (system A). The specific activity after diluent addition was $5{\cdot}58 \times 10^5$ d/min mg^{-1}.

[^{14}C]BHT-CH_2OH was prepared from [^{14}C]BHT-CHO by sodium borohydride reduction and purified by preparative TLC (Solvent A) giving a specific activity after diluent addition of $6{\cdot}19 \times 10^5$ d/min mg^{-1}.

[^{14}C]B-B. Areas corresponding to B-B, BHT-hydroxystilbene and BHT-stilbenequinone were isolated during preparative TLC used to isolate BHT-CHO. Sufficient BHT-stilbenequinone was isolated to reduce with lithium aluminium hydride in tetrahydrofuran (Bohn & Campbell, 1957). Preparative TLC (solvent A) of the product afforded [^{14}C]B-B with a specific activity after diluent addition of $5{\cdot}94 \times 10^5$ d/min mg^{-1}.

The aldehyde, alcohol, and diphenylethane were shown to be radiochemically pure by TLC in solvent systems A and B, A and C, and C respectively.

[^{14}C]BHT-COOH was prepared biosynthetically from 0–8 h bile of rats receiving an intravenous dose of [^{14}C]BHT. Diluent BHT-COOH was added to the bile and isolated by chloroform extraction after refluxing for 0·5 h with an equal volume of 2N hydrochloric acid. The crude acid was purified by preparative TLC using solvents D, E, and C, and the product, specific activity $5{\cdot}47 \times 10^5$ d/min mg^{-1}, was shown to be radiochemically pure (solvent systems A and F).

Radioactivity measurements

Radioactivity measurements were made using a Packard Scintillation Spectrometer, Model 3314, equipped for automatic external standardization. The scintillation fluid consisted of 0·15% PPO and 0·005% POPOP in 10% absolute ethanol in toluene. Aliquots of liquid specimens were counted directly using 10 ml of scintillation fluid; solution of dried finely ground faeces was achieved by digestion of an aliquot with Hyamine hydroxide solution (0·25 ml, Packard Instrument Co. and water 0·1 ml) at 37° overnight. Following addition of fluid, samples were stored in the dark for 24 h before counting.

RESULTS

The hourly excretion of [^{14}C]labelled biliary metabolites of BHT, BHT-CH_2OH, BHT-CHO, BHT-COOH and B-B for 6 h after single intravenous doses is shown in Table 1. Table 2 shows the 2 hrly excretion of the same compounds (excluding B-B) for 8 h after single intraperitoneal doses. Both sets of data show that these compounds are rapidly absorbed, metabolized and excreted in the bile. While the rate of biliary excretion after dosing with the carboxylic acid is greater after intravenous injection, the bulk being excreted in the first hour, the total percentage excreted appears to be independent of the parenteral administration route chosen. By contrast, the total biliary excretion after BHT or BHT-CHO administration is rather less after intra-

Table 1. *Hourly biliary excretion of radioactive metabolites (% dose) during 6 h following single intravenous doses of BHT and related compounds*

Time (h) after dosing	Compound BHT* (100 μg)	BHT–CH_2OH (102 μg)	BHT–CHO (100 μg)	BHT–COOH (112 μg)	B–B (109 μg)
1	46·6 ± 7·5	39·4 ± 8·0	63·1 ± 12·4	78·3 ± 9·8	26·5 ± 7·6
2	25·0 ± 8·0	12·1 ± 3·6	16·1 ± 4·8	1·9 ± 0·1	10·5 ± 3·6
3	12·6 ± 2·6	8·6 ± 5·7	4·2 ± 2·4	0·5 ± 0·1	
4	4·7 ± 1·8	4·2 ± 1·4	2·7 ± 1·9	0·2	
5	2·9 ± 0·6	2·6 ± 0·7	1·0 ± 0·7	0·1	3·3 ± 1·0
6	1·9 ± 0·4	2·1 ± 0·4	0·9 ± 1·2	0·1	
Totals	93·7 ± 11·6	69·2 ± 4·4	88·0 ± 4·5	80·8 ± 9·8	40·3 ± 5·8

* From Ladomery & others (1967a).
Results are the mean values for six rats except for BHT–CHO for which four animals were used. Standard deviations are shown.

peritoneal dosage than after intravenous dosage. The biliary excretion of the alcohol, about 70% of the administered dose, appears to be unaffected by its mode of administration.

The biliary excretion of radiolabelled metabolites of the alcohol, aldehyde and carboxylic acid was also examined for 6 h, 5 days after intraperitoneal administration of these compounds. Table 3 shows the percentage of dose excreted hourly. These results are comparable to those of Ladomery & others (1967a), who found that about 10% of an intraperitoneal dose of BHT was excreted over 6 h, 4 days after dosing.

The total overall excretion pattern of BHT and its related compounds excreted in urine and faeces for 5 days, and bile collected 120–126 h after dosing with a single intraperitoneal dose were examined (Table 4). Although biliary excretion for 120–126 h after intraperitoneal dosage of BHT was not measured, the results of Ladomery & others (1967a) indicate it would not be significantly different from that of its metabolites for this period. There are no significant differences in the total radioactivity excreted over 126 h after dosing, about 70% of the dosed radioactivity for each compound being recovered.

The chemical identity of the metabolites of BHT and related compounds in bile, urine and faeces was examined using reverse isotope dilution analyses, and thin-layer co-chromatography. The Rf values of some of the reference compounds are in Table 5. The predominant metabolite in all biological extracts was the carboxylic acid or its ester glucuronide.

Table 2. *Two hrly biliary excretion of radioactive metabolites (% dose) during 8 h after single i.p. doses of BHT and related compounds*

Time (h) after dosing	Compound BHT* (100 μg)	BHT–CH_2OH (200 μg)	BHT–CHO (300 μg)	BHT–COOH (560 μg)
2	31·5 ± 6·4	57·6 (54·2–61·0)	38·6 (35·4–41·8)	67·1 (62·6–71·5)
4	14·1 ± 1·7	10·1 (9·0–11·1)	12·9 (9·6–16·2)	15·8 (12·9–18·6)
6	6·2 ± 0·9	4·7 (4·3–5·0)	5·6 (4·7–6·4)	3·4 (1·8–5·0)
8	not measured	2·2 (1·9–2·5)	4·2 (3·8–4·6)	1·4 (0·5–2·2)
Totals	51·8 ± 6·6	74·5 (72–77)	61·3 (60–63)	87·6 (86–88)

* From Ladomery (1967a) showing ± standard deviation for six animals.
( ) range of values for two rats.

Table 3. *Hourly biliary excretion of radioactive metabolites (% dose) during 6 h 5 days after single intraperitoneal doses of compounds related to BHT*

Time (h) after dosing	Compound BHT-CH_2OH (102 μg; 8*)	BHT-CHO (100 μg; 5)	BHT-COOH (106 μg; 4)
121	2·3 ± 1·0†	2·0 ± 1·2	2·8 ± 1·5
122	1·5 ± 0·5	1·1 ± 0·7	
123	1·2 ± 0·4	0·7 ± 0·5	2·3 ± 1·2
124	1·0 ± 0·1	0·5 ± 0·3	
125	0·9 ± 0·1	0·2 ± 0·1	2·0 ± 1·5
126	0·9 ± 0·2	0·3 ± 0·1	
Totals	7·9 ± 0·9	4·7 ± 2·2	7·2 ± 4·0

* No. of animals.
† ± Standard deviation.

Little attention was directed to the metabolic products of [^{14}C]BHT except in faeces collected during the 5 day interval after intraperitoneal dosage. Reverse isotope dilution experiments indicated that of the 50% of the dose appearing in the faeces, 40% was free BHT-COOH, and only 0·7% was unchanged BHT. Besides small amounts of nonpolar metabolites a further 40% of the excreted radioactivity was present as conjugated metabolites.

Only the biliary metabolites of [^{14}C]BHT-CH_2OH were examined, the urinary and faecal excretion having been examined by Wright & others (1965). The carboxylic acid, BHT-COOH, was the major metabolite. Of the total radioactivity in 0–6 h bile, 14% was present as free phenols, BHT-COOH predominating, while the ester glucuronide of BHT-COOH was the major component in the remaining metabolites. The only other metabolite detected was a small amount of water-soluble material which was not examined further. Acid hydrolysis, followed by TLC showed that no unchanged BHT-CH_2OH was present. Similar results were obtained after intravenous and intraperitoneal dosage.

In 120–126 h bile from rats receiving intraperitoneal doses of BHT-CH_2OH, traces of the alcohol, aldehyde and BHT-dimers were detected. However the major metabolites were BHT-COOH and its ester glucuronide.

Unchanged BHT-CHO was detected (2% of dose) in the 0–6 h bile of rats given [^{14}C]BHT-CHO (2338 μg) intravenously. Isotope dilution analysis indicated that

Table 4. *Total recovery of radioactive metabolites (% dose) of BHT and related compounds over 126 h after single intraperitoneal doses*

	Compound BHT* (100 μg)	BHT-CH_2OH (102 μg)	BHT-CHO (100 μg)	BHT-COOH (1060 μg)
Urine	32·0 ± 1·2 (10)	14·8 ± 6·9 (4 pairs)	35·2 ± 7·3 (3 pairs)	45·1 ± 7·9 (3 pairs)
Faeces	36·9 ± 1·2 (6)	48·6 ± 4·5 (4 pairs)	28·7 ± 7·6 (3 pairs)	17·4 (2 pairs)
Bile (120–126 h) ..	—	7·9 ± 0·9 (8)	4·7 ± 2·2 (5)	7·2 ± 4·0 (4)
Total.. ..	68·9 ± 1·4	71·3 ± 8·3	68·8 ± 10·8	69·7

± Standard deviation. ( ) No. of animals.
* Ladomery & others (1967a).

Table 5. *Rf values of BHT and its metabolites*

Compound	Rf System A	Rf System B
BHT	0·98	1·0
BHT-CH_2OH ..	0·60	0·50
BHT-CHO	0·85	0·70
BHT-COOH ..	0·15	0·20
BHT-Dimers ..	0·95	0·90–0·95

free BHT-COOH (4% of dose) was present together with conjugated carboxylic acid (57% of dose). These quantities represent 7·8 and 84% of the biliary radioactivity. Acid hydrolysed urine of rats given intraperitoneal [^{14}C]BHT-CHO showed the presence of BHT-COOH (80% of urinary radioactivity) and unchanged aldehyde (1–2%) by TLC analysis. Faeces collected over the 5 days following dosage contained unchanged aldehyde (4% of dose by TLC analysis) while 82% of the radioactivity was present as the free carboxylic acid as demonstrated by reverse isotope dilution analysis.

Bile of rats receiving injections of [^{14}C]BHT-COOH revealed the presence of the free acid and its ester glucuronide as the only metabolites.

Solvent extraction of bile after intravenous injection of [^{14}C]B-B indicated that 3% of the dose (7% of biliary radioactivity) was present as free phenol. Ether extraction at low pH removed an additional 40% of metabolites. TLC investigations of the ether soluble fractions suggested that the metabolites were also dimers. No free BHT-COOH, or its ester glucuronide was detected in these experiments.

DISCUSSION

The 6 h biliary excretion data following injection of low doses of BHT and related compounds indicate that with the exception of the diphenylethane (B-B) these compounds are all rapidly metabolized and excreted in the bile. Examination of the hourly excretion of radioactivity (Table 1) suggests that the rate limiting factor affecting elimination of these compounds or their metabolites into the bile is the rate of oxidation to the carboxylic acid. The total 6 h excretion of the alcohol is significantly ($P < 0{\cdot}05$) less than that of the parent compound, the aldehyde and the acid. The latter compounds are about 80–90% eliminated in the bile in 6 h, compared to the 70% elimination of BHT-CH_2OH.

The small but significant difference in the total label excreted after intravenous injection of BHT and BHT-CH_2OH may arise through pharmacokinetic factors, or through differences in the oxidation pathways followed by these two compounds.

The biliary excretion pattern after intraperitoneal injection of small doses of BHT and related compounds (Table 2) differs from that after intravenous dosage, suggesting that factors such as precipitation and absorption from the peritoneum, and storage of the drug in the body are important in the metabolism of these compounds after intraperitoneal dosage. However, quantitation of metabolites in late bile, 120 h after intraperitoneal dosing with BHT-CH_2OH, BHT-CHO and BHT-COOH (Table 3) indicated no large differences in the amount of radioactivity excreted in a 6 h period. The proportions found are not substantially different to those found by Ladomery & others (1967a) after intraperitoneal administration of BHT.

In total excretion studies over 5 days with all compounds except B-B, substantial agreement was found with the results for BHT of Tye, Engel & Rapien (1965) (Table 4). For low doses of the compounds tested there were no significant differences in the totals of 5 days urinary and faecal excretion and 120–126 h biliary excretion, about 70% of the administered radioactivity being recovered over 5 days. There were individual differences in the ratio of urinary to faecal excretion over this period, faecal excretion appearing more important for doses of BHT-COOH. While these differences appear significant it is unwise to draw any conclusion from them because the rats used were not monitored for the constancy of their nutritional status and urinary pH.

The lack of differences in the overall excretion pattern and the biliary excretion of BHT and related compounds, strongly suggested that the compound or compounds responsible for the enterohepatic circulation of radioactivity following [^{14}C]BHT administration must be common to the metabolic pathways of all these compounds. Only BHT-COOH or its metabolites could reasonably be suspected since BHT-COOH and its ester glucuronide are known to be the main metabolic endproducts of BHT in bile (Ladomery & others, 1967a) and BHT-CH_2OH in urine (Wright & others, 1965).

In verification of this, the major metabolite found in early bile after parenteral administration of BHT-CH_2OH, BHT-CHO and BHT-COOH was the free acid or ester glucuronide, the latter usually predominating. Examination of late bile after acid hydrolysis again showed the carboxylic acid to be the major radioactive component. Similar results were obtained by Ladomery & others (1967b) for late bile after BHT administration. Variations in the ratio of free acid to ester glucuronide in bile, urine and faeces are probably caused by differing degrees of hydrolysis in the large intestine, or during storage and work-up. As would be expected, less free carboxylic acid than the glucuronide ester is present in the bile. This conclusion verifies those of Daniel & others (1968) about the nature of the recirculating compound in BHT recirculation.

The biliary excretion of the diphenylethane (B-B) contrasts with that of the other compounds studied. Amongst the metabolites, BHT-COOH or other fragments were not recognized, the bulk of metabolites appearing as BHT dimers. This contrast with the metabolism of bis-(4-hydroxy-3,5-di-t-butylphenyl)methane(Ionox 220) and bis(3,5-di-t-butyl-4-hydroxybenzyl)ether(Ionox 201) (Hathway, 1966).

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