J. Pharm. Pharmac., 1967, **19**, 383–387 Received January 2, 1967

The excretion of [^{14}C] butylated hydroxytoluene in the rat

L. G. LADOMERY, A. J. RYAN AND the late S. E. WRIGHT

The excretion of small doses of [^{14}C]labelled butylated hydroxytoluene has been examined in the rat. Parenteral doses are excreted slowly in urine and faeces over 4 days. Biliary excretion is rapid. The biliary radioactivity is absorbed readily from the gut and a rapid enterohepatic cycle has been found to operate over at least 96 hr.

BUTYLATED hydroxytoluene (BHT, 3,5-di-t-butyl-4-hydroxytoluene) is an antioxidant used widely in foods. It is therefore important that the toxicology, distribution, metabolism and excretion of this compound should be well understood. Dacre (1961) and Akagi & Aoki (1962) studied its metabolism in rabbits. The quantitative urinary excretion of isotopically labelled BHT has been examined by several workers. Golder, Ryan & Wright (1962) recovered only 33% of the radioactivity in urine from rats given small doses of tritiated BHT. This result was confirmed by Ladomery, Ryan & Wright (1963) using [^{14}C]BHT. Daniel & Gage (1965), using higher doses of [^{14}C]BHT found similar urinary excretion and also noted high faecal excretion. All these authors found a slow rate of excretion, which was not complete even after a week.

The present paper is concerned with the biliary excretion by rats of small doses of [^{14}C]BHT.

Experimental

[^{14}C]3,5-Di-t-butyl-4-hydroxytoluene (2,6-di-t-butyl-*p*-cresol; [^{14}C]BHT) was purchased from New England Nuclear Corp., Boston, Mass., U.S.A. It was used as 0·02% solution in 50% aqueous ethanol.

METABOLIC EXPERIMENTS

White male rats of 300–350 g were given [^{14}C]BHT (100 μg, except where otherwise stated) in aqueous ethanol (0·5 ml) either by intraperitoneal or intravenous injection. Urine and faeces were collected in a metabolic cage fitted with an all-glass separating device. Bile was collected from cannulated rats maintained under urethane anaesthesia. Ringer solution (1 ml) was given every 3 hr to replace lost water and the animals were kept warm. The "linked animal experiment" was carried out by passing the bile cannula from one rat into the duodenum of a second rat. This cannula was cut and joined with a short section of a hypodermic needle in order to check bile flow. The first rat was raised 5 cm above the second to assist the flow of bile into the duodenum. Bile was collected from the second rat.

RADIOACTIVE COUNTING

Urine and bile were counted directly by the addition of 0·1–0·2 ml to 10 ml of scintillation solvent consisting of 2,5-diphenyloxazole (5·0 g), 1,4-bis-2-(5-phenyloxazolyl)benzene (0·05 g), absolute ethanol (300 ml)

From the Department of Pharmacy, University of Sydney, N.S.W., Australia.

and toluene (800 ml). Faeces were dried, weighed and a powdered aliquot extracted continuously with methanol. Aliquots of the methanolic solution were counted as above. Tissues were homogenized in ethanol, centrifuged and the supernatant assayed. The contents from the small intestine were diluted with methanol, centrifuged and the supernatant counted. The contents of the large intestine were treated in the same way as the faeces. The samples were counted in a Packard Scintillation Counter, model 3314. Counting efficiencies were determined with an external source.

Results

The low urinary excretion of [^{14}C]BHT observed earlier (Ladomery & others, 1963) led us to examine the faeces of dosed animals for radioactivity. Table 1 shows that over 4 days 37% of a parenteral dose of

TABLE 1. DAILY URINARY AND FAECAL EXCRETION OF RADIOACTIVITY AFTER A SINGLE INTRAPERITONEAL DOSE OF [^{14}C]BHT

Day	% Excreted in urine*	% Excreted in faeces*
1	5·7 ± 0·5	16·7 ± 1·0
2	10·5 ± 0·8	7·5 ± 0·3
3	10·3 ± 0·6	7·3 ± 0·1
4	5·5 ± 0·4	5·4 ± 0·4
Total	32·0 ± 0·6	36·9 ± 1·2

* ± Standard deviation; 6 rats used.

[^{14}C]BHT was excreted by this route. The urinary excretion agreed with our previous results. Since only about 70% of the dose was accounted for, the general distribution of radioactivity from [^{14}C]BHT in rats was examined. The results are in Table 2. Four groups of six rats were

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN RATS AFTER A SINGLE INTRAPERITONEAL DOSE OF [^{14}C]BHT. Values are the percentage of the administered dose; urine and faeces are cumulative

Day	Urine	Faeces	Contents of small intestine	Contents of large intestine	Liver	Spleen	Kidney	Intestinal wall	Total accounted for
1	4·5	7·6	16·9	6·1	2·1	0·03	0·09	7·5	44·8
2	15·6	20·9	9·8	4·7	1·8	0·02	0·08	4·9	57·8
3	26·3	26·1	9·6	3·9	1·0	0·02	0·02	2·9	69·8
4	31·2	37·9	7·4	2·1	0·9	0·14	0·10	1·6	81·3

dosed. Urine and faeces were pooled at 24-hourly intervals. At the same time a group of rats was killed and some of the tissues examined for radioactivity. The urinary and faecal excretion agree well with those already found. Little activity appeared in the liver, kidney or spleen. However, significant amounts appeared in the intestinal contents and the intestinal wall. This totalled about a third of the radioactivity remaining in the body.

From these results it seemed likely that there was some biliary excretion of radioactivity from [^{14}C]BHT and possibly an enterohepatic cycle. The

biliary excretion of [14C]BHT was examined and found to be surprisingly high. After 6 hr, radioactivity equivalent to 95% of an intravenous dose and 52% of an intraperitoneal dose appeared in the bile (Table 3).

TABLE 3. BILIARY EXCRETION OF RADIOACTIVITY BY RATS AFTER SINGLE DOSES OF [14C]BHT

Time (hr)	% Excreted from 100 μg given intravenously*	% Excreted from 100 μg given intraperitoneally*	% Excreted from 10 mg given intravenously*
1	46·6 ± 7·5	16·0 ± 4·5	28·5 ± 7·8
2	25·0 ± 8·0	15·5 ± 4·6	28·9 ± 6·5
3	12·6 ± 2·6	8·9 ± 1·1	8·2 ± 2·0
4	4·7 ± 1·8	5·2 ± 1·3	3·3 ± 0·5
5	2·9 ± 0·6	3·4 ± 0·7	1·5 ± 0·4
6	1·9 ± 0·4	2·8 ± 0·2	1·0 ± 0·2
Total	93·7 ± 11·6	51·8 ± 6·6	71·4 ± 10·4

* ± Standard deviation; 6 animals used in each experiment.

These high recoveries supported the idea of an enterohepatic cycle of BHT metabolites. This cycling was confirmed using pairs of rats with the bile duct cannulated, in which the cannula from one rat (A) was inserted into the duodenum of the second (B). Bile was collected from rat B after rat A was given an intravenous dose of [14C]BHT. Nearly 30% of the dose given to rat A was excreted by rat B after 10 hr (Table 4).

The efficiency of the biliary excretion of BHT was shown when 10 mg was given intravenously. In this instance over 70% of the dose was recovered in the bile after 6 hr (Table 3).

TABLE 4. BILIARY EXCRETION OF RADIOACTIVITY BY LINKED RATS AFTER A SINGLE DOSE OF [14C]BHT

Time (hr)	% dose excreted*	% dose excreted per hr
2	0·2 ± 0·07	0·10
4	5·1 ± 1·4	2·53
6	7·6 ± 0·6	3·80
8	8·0 ± 1·8	4·01
10	9·0 ± 1·3	4·50
Total	29·9 ± 2·7	

* ± Standard deviation; 5 pairs of rats used.

With this demonstration of the enterohepatic cycling of BHT metabolites it became important to determine for how long the cycle functioned. Sixteen rats were given parenteral doses of [14C]BHT and in four of them the bile duct was cannulated every 24 hr. Radioactivity appeared in significant amounts in the bile even 96 hr after dosing, when nearly 10% of the dose was excreted (Table 5).

The efficiency of the biliary excretion was demonstrated in an additional experiment. Bile obtained from a rat given [14C]BHT was injected intravenously (1 ml) into rats. Bile collected from these animals contained 93·0 ± 2·0% of the injected radioactivity after 6 hr. None was found in the bladder contents. This emphasizes the remarkable affinity of the liver for BHT derivatives. The bile contains mainly conjugates (Ladomery, Ryan & Wright, 1967) and it might be expected

TABLE 5. THE RATE OF BILIARY EXCRETION OF RADIOACTIVITY AT 24-HOURLY INTERVALS BY RATS AFTER A SINGLE INTRAPERITONEAL DOSE OF [^{14}C]BHT

Time after dosing (hr)	% dose excreted in 6 hr*	% dose excreted/hr*	Predicted % dose excreted/hr	Rate of excretion as % dose remaining/hr
24	26·0 ± 4·1	4·3 ± 0·7	3·9	4·8
48	20·5 ± 3·0	3·4 ± 0·5	2·9	5·3
72	13·8 ± 2·0	2·3 ± 0·3	2·2	4·8
96	9·6 ± 1·6	1·6 ± 0·3	1·4	5·1
				mean 5·0 ± 0·2

* ± Standard deviation; 4 groups of 4 rats used.

that intravenous injection would lead to more urinary excretion than was found. However, Williams, Millburn & Smith (1965) have found high biliary excretion of certain glucuronides. It is clear that there is no simple relation between polarity and excretion route.

Discussion

The present work has confirmed the low urinary excretion of tritiated BHT found by Golder & others (1962). The slow urinary excretion of [^{14}C]BHT led us to examine the faecal excretion of activity. After a single parenteral dose urinary excretion is about 32–35% and faecal excretion is 35–37%, accounting for 70% of the radioactivity after 4 days (Table 2).

The result suggested that there might be some accumulation of BHT in tissues. However, under our dosage conditions little radioactivity was found in the organs examined. The liver with 2% contained most. Examination of the intestinal contents, however, revealed that these, together with the gut wall, contained about 30% of the activity remaining in the body after allowing for the urinary and faecal excretion (Table 3). Tye, Engel & Rapien (1965) found only a small amount of BHT in the tissues at much higher dose levels (44 mg/kg) than used in the present study. Daniel & Gage (1965) accounted for about 80% of oral doses of BHT. Faecal excretion in their experiments was much higher than that found in this work. However, this may be due to poor absorption of the BHT since it was given in olive oil, in which it is readily soluble. The laxative properties of olive oil could also be a factor in these results. The slow excretion and the constancy of the radioactive pool in the gut suggested that biliary excretion of BHT might be important. This was shown to be the case when 95% of an intravenous dose of [^{14}C]BHT appeared in the bile of cannulated rats (Table 3). An intraperitoneal dose of [^{14}C]BHT was excreted to the extent of 52% in the bile. This slower rate of excretion of a parenteral dose may be due to the rate of absorption from the peritoneal cavity. However, in both instances the rate of biliary excretion of radioactivity is greater than the daily urinary or faecal excretion. Coupled with the remarkable constancy of the proportion of radioactivity in the gut it is clear that enterohepatic cycling takes place.

Proof of this was obtained from the "linked animal experiment" (Table 4). The 30% of radioactivity recovered in the bile of rat B indicates that the circulation between bile and intestines is rapid. The

steady state attained after about 8 hr (Table 4) could be due to an active intestinal absorption process which becomes saturated. This is supported by the fact that intravenous injections of [14C]BHT at 100 times our usual dose level are excreted to the extent of 71% of the dose in bile (Table 3) showing that the biliary system is freely permeable to the BHT metabolites. From Table 4 the steady state excretion of BHT metabolites in the bile approaches 5% of the residual radioactivity per hr. Using this figure, the biliary excretion of radioactivity was calculated for various time intervals after a parenteral dose. Table 5 shows the results obtained with rats given [14C]BHT and cannulated at the bile duct 24–96 hr after dosing compared with the predicted excretion. A plot of the logarithm of the per cent dose/hr excreted (Wagner, 1961) against mean collection time for the experimental and predicted figures in Table 5 gives two straight and parallel lines. This provides further support that the enterohepatic circulation of BHT metabolites follows first order kinetics at the rate of approximately 5% of the remaining radioactivity per hr.

Acknowledgements. This work forms part of a research programme supported by the National and Medical Research Council of Australia and Grant EF-258 of the U.S. National Institutes of Health.

References

Akagi, M. & Aoki, J. (1962). *Chem. pharm. Bull., Tokyo*, **10**, 200–204.
Dacre, J. C. (1961). *Biochem. J.*, **78**, 758–766.
Daniel, J. W. & Gage, J. C. (1965). *Fd Cosmet. Toxic.*, **3**, 405.
Golder, W. S., Ryan, A. J. & Wright, S. E. (1962). *J. Pharm. Pharmac.*, **14**, 268–271.
Ladomery, L. G., Ryan, A. J. & Wright, S. E. (1963). *Ibid.*, **15**, 771.
Ladomery, L. G., Ryan, A. J. & Wright, S. E. (1967). *Ibid.*, **19**, 388–394.
Tye, R., Engel, J. D. & Rapien, J. (1965). *Fd Cosmet. Toxic.*, **3**, 547.
Wagner, J. G. (1961). *J. pharm. Sci.*, **50**, 359–387.
Williams, R. T., Millburn, P. & Smith, R. L. (1965). *Ann. N.Y. Acad. Sci.*, **123**, 110.