J. Pharm. Pharmac., 1978, 30, 240–243 Received November 21, 1977

Fate of [^{14}C]warfarin in guinea-pigs: effect of a concomitant single dose of salicylate

L. T. WONG*, G. SOLOMONRAJ AND B. H. THOMAS

Drug Research Laboratories, Health Protection Branch, Health and Welfare Canada, Ottawa, Canada, K1A 0L2

When a single dose of sodium salicylate (177·8 mg kg^{-1}, by mouth) was given with [^{14}C] warfarin (1 mg kg^{-1}, i.p.) to guinea-pigs, the salicylate depressed the blood concentrations of ^{14}C for 6 h. At 1 h, salicylate increased the distribution of ^{14}C in the liver and brain, but at 1 and 6 h it was decreased in the blood and kidney. A significant portion of the ^{14}C was excreted into the bile, but was subject to enterohepatic circulation and then excreted by the kidney. There was an enhancement of the biliary elimination of ^{14}C in the first 5 h after salicylate and a decrease in ^{14}C concentration in blood; the proportion of warfarin to its metabolites excreted in the urine and bile was unchanged. Salicylate displaced serum protein bound [^{14}C]warfarin *in vitro*. Salicylate increases the initial biliary elimination of warfarin by displacing some of that bound to plasma protein. This facilitated uptake of warfarin by the liver where it was metabolized. This effect of salicylate did not modify the hypoprothrombinaemia produced by warfarin.

Therapeutic doses of salicylate usually have no effect on the anticoagulant action of warfarin in man (Koch-Weser & Sellers, 1971; MacLeod & Sellers, 1976). In the rat, however, a single dose of sodium salicylate causes antagonism of the hypoprothrombinaemia (Coldwell, Buttar & others, 1974). Unlike man who excretes warfarin and its metabolites predominantly in the urine, the rat excretes a significant amount by the faecal route (Coldwell & others, 1974) and this difference prompted an investigation of the interaction in an animal closer to man in its response to the drug. The metabolism of [^{14}C]warfarin and its interaction with a single dose of salicylate has therefore been examined in guinea-pigs.

MATERIALS AND METHODS

Treatment. Male guinea-pigs, Hartley strain, 275–350 g, acclimatized for at least 7 days, had free access to water but no food for 18 h before being used after which they were divided randomly into two groups of 5. One Group was given sodium salicylate (177·8 mg kg^{-1}, by mouth: ≡200 mg kg^{-1} aspirin) followed by [^{14}C]warfarin (1 mg, 15 μCi kg^{-1}, i.p.) 30 min later. The second group received saline by mouth and [^{14}C]warfarin. The animals were then housed individually in metabolism cages, and blood, urine and faeces were collected.

For biliary excretion experiments, the animals were anaesthetized with halothane and a polyethylene tube (PX-011, Beckton, Dickinson and Co.) was inserted into the common bile duct, the cystic duct being ligated. They were then transferred to restraining cages (Bollman, 1948) and allowed to recover for 1 h and then dosed as described above. Rectal temperature was monitored and body temperature maintained at 36–38°.

Collection and determination of radioactivity. Blood (20 μl) was taken in duplicate from the toe at 0·5, 1, 2, 4, 6, 8, 12, 24, 35 and 48 h after warfarin. Urine was collected at 4, 8, 12, 24 and 48 h and faeces at 24 and 48 h after dosing. In a separate experiment, animals were dosed and killed at 1 and 6 h. Blood, bile, brain, liver, kidney and muscle were frozen at −20°. Bile samples were collected at 1, 2, 4, 6, 8 and 12 h. All samples were analysed for total radioactivity (Thomas, Coldwell & others, 1972).

Prothrombin time. Blood, from the dorsal aorta of ether-anaesthetized animals, was mixed with 3·1% sodium citrate (0·1 ml ml^{-1} blood) and centrifuged at 2000 *g*. The one-stage prothrombin time was then measured using commercial thromboplastin.

Warfarin and metabolites in urine and bile. Aliquots (1 ml) of bile and urine were freeze-dried, redissolved in 0·1 ml of water and 25 μl applied as a band to Whatman No. 1 paper and chromatographed with descending solvent (t-butanol–benzene–conc. NH_4OH–water (45:90:9:3) (Barker, Hermodson & Link, 1970). The various spots were located by radiochromatographic scanning and 1-cm wide strips were counted in a liquid scintillation counter. Glucuronide conjugates and metabolites of warfarin

* Correspondence.

were estimated by incubating equal volumes of bile or urine with Ketodase (Warner-Chilcott) at 37° for 16 h, before processing as described above.

Warfarin and 4′-hydroxywarfarin in bile and urine were identified by mass spectrometry after isolation from the paper chromatogram and purification by t.l.c. (Lewis & Trager, 1970). Unchanged warfarin was further identified by the inverse isotope dilution techniques (Barker & others, 1970).

Serum protein binding. Guinea-pig serum containing 5 μg ml^{-1} of [^{14}C]warfarin was incubated at room temperature for 1 h with salicylate 0–500 μg ml^{-1}. The incubates (2 ml), transferred to washed Visking tube bags mounted in centrifuge tubes, were centrifuged (12 000 *g*; 20 min). The dialysate (20 μl) and undialysed plasma were counted.

Statistics. Significance of difference between the control and treated groups was determined by Student's *t*-test with values of $P < 0.05$ being considered significant. Where data are expressed as a percentage, the arcsin transformation was performed before determining the *t*-values.

RESULTS

Blood profile. Fig. 1 shows that the maximum blood concentration of ^{14}C occurred within 1 h of warfarin injection and the concentration in salicylate-treated animals was significantly depressed over the initial 6 h. Calculation of the elimination half-lives ($T\frac{1}{2}$) using a computer best-fit plot, however, showed no significant difference between the two groups. The $T\frac{1}{2}$ values were 9·62 ± 0·52 h and 10·01 ± 0·29 h for the control and treated group respectively.

Tissue distribution. Table 1 gives the concentrations of radioactivity in tissues at 1 and 6 h after dosing.

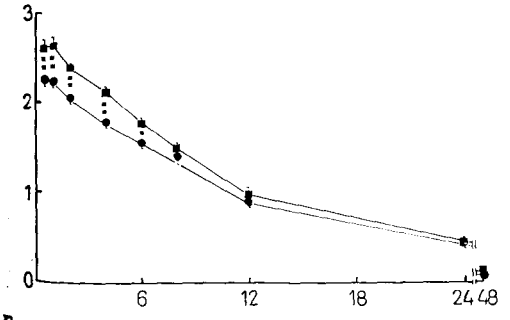

FIG. 1. Blood concentration of radioactivity expressed as μg of warfarin ml^{-1} of blood (ordinate) in guinea-pigs dosed with [^{14}C]warfarin (1 mg kg^{-1}, i.p.) alone (■——■) and in combination with sodium salicylate (177·8 mg kg^{-1}, orally) (●——●). Values are mean ±s.e. from 5 animals (*$P < 0.05$; **$P < 0.005$). Abscissa: Time (h).

At 1 h, the blood and kidney concentrations were depressed while those in the liver and brain were increased by salicylate. The liver/blood ratio (1·15 in the control) was significantly increased (1·92; $P < 0.02$) by salicylate. In both groups ^{14}C concentrations in bile were higher than those in blood. The bile/blood ratio was increased significantly from 1·49 in the control to 2·54 by salicylate ($P < 0.005$). Blood and kidney concentrations were still depressed by salicylate at 6 h.

Table 1. *Total radioactivity† in tissues of guinea-pigs at 1 and 6 h after administration of [^{14}C]warfarin (W1 mg kg^{-1}, i.p.) alone and with sodium salicylate (SAL, 177·8 mg kg^{-1}, orally).*

	1 h		6 h	
Tissue	W	W + SAL	W	W + SAL
Blood	3·08 ±0·01	2·42 ±0·04**	2·07 ±0·05	1·88 ±0·05*
Bile	4·52 ±0·60	6·21 ±0·75	5·40 ±0·61	6·77 ±0·98
Brain	0·23 ±0·01	0·32 ±0·02**	0·14 ±0·01	0·13 ±0·01
Muscle	0·45 ±0·02	0·43 ±0·02	0·27 ±0·02	0·28 ±0·02
Kidney	6·24 ±0·28	2·72 ±0·08**	7·48 ±0·80	3·40 ±0·33**
Liver	3·51 ±0·20	4·56 ±0·46*	1·90 ±0·08	2·08 ±0·23

† Radioactivity expressed as mean ±s.e., μg g^{-1} or ml^{-1} tissue; n = 5.
* $P < 0.05$; ** $P < 0.005$.

Excretion of warfarin and metabolites. Fig. 2 shows the cumulative amounts of radioactivity excreted in the urine and faeces by the control and treatment groups. Over 48 h, ~70% of the dose was excreted in the urine and 6% in faeces. Salicylate did not significantly alter the pattern.

Paper chromatography resolved the radioactivity in urine into six peaks. According to the reported R_F, Peak 1 (R_F = 0·86) was 2,3-dihydro-2-methyl-4-phenyl-5-oxo-γ-pyrano(3,2-c)(1)benzopyran (Dihydrogimmel, DHG). Peak 2 (R_F = 0·72) was unchanged warfarin (confirmed by the inverse isotope dilution technique). Peak 3 (R_F = 0·53) on mass spectrometric analysis showed a molecular ion at *m/e* 324 and a fragmentation peak at *m/e* 187 characteristic of 4′-hydroxywarfarin (Trager, Lewis & Garland, 1970). Peaks 4 (R_F = 0·32) and 5 (R_F = 0·20), most likely 8- and 7-hydroxywarfarin respectively (Barker & others, 1970), were not further confirmed. A significant amount of radioactivity remained near

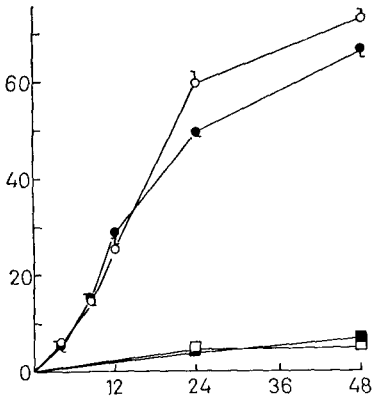

FIG. 2. Cumulative excretion of radioactivity (ordinate) in urine (circles) and faeces (squares) expressed as percentage of the dose of [^{14}C]warfarin administered in guinea-pigs dosed with [^{14}C]warfarin (1 mg kg^{-1}, i.p.) alone (○——○; □——□) and in combination with sodium salicylate (177·8 mg kg^{-1}, orally) (●——●; ■——■). Values are mean ± s.e. from 5 animals. Abscissa: Time (h).

the origin (Peak 6). Ketodase caused a reduction in this peak and corresponding increases in Peaks 2 and 3. Therefore some of Peak 6 appeared to contain the glucuronide conjugates of warfarin and 4′-hydroxywarfarin.

Table 2 shows the urinary excretion of warfarin and its metabolites by the control and salicylate groups at 4, 8 and 12 h. 4′-Hydroxywarfarin comprised 30–70% of the total ^{14}C excreted while unchanged warfarin accounted for less than 6%. Salicylate had little, if any, effect on metabolism but appeared to reduce the urinary excretion of glucuronides at 4–8 h.

The higher concentration of ^{14}C in the bile compared with blood (Table 1) was confirmed by biliary excretion results. After 4 h, the bile concentration in the salicylate group was about twice that of the controls, the % increase was from 17·5% (controls) to 35·3% (treated) (Fig. 3). Cumulative amounts over 12 h were 43·2% and 56·7% for the respective groups.

The amount of warfarin and metabolites excreted into the bile was twice as much at 4 h in the salicylate group as in the controls, in proportion to the increased total excretion of radioactivity (Table 2). The proportion of warfarin and metabolites was unchanged at any time.

Serum protein binding. About 2% of warfarin was unbound. This increased proportionally when salicylate 0–500 μg ml^{-1} was present. At 500 μg ml^{-1}, the % warfarin unbound rose to 13·6%. Since in *in vivo* experiments salicylate concentrations of 100–300 μg ml^{-1} were reached in 6 h, the proportion of free warfarin over this time was increased from 100–300% by salicylate.

Prothrombin time. Warfarin (1 mg kg^{-1}) caused a maximum elevation in 24–36 h (Deckert, 1973). The

Table 2. *Effect of sodium salicylate (SAL, 177·8 mg kg^{-1}, orally) on the urinary and biliary excretion of warfarin (1 mg kg^{-1}, i.p.) and its metabolites at 4, 8 and 12 h after dosing*†.

Time (h)	Treatment	Warfarin	DHG	4′Hydroxy-warfarin	Warfarin + 4′-hydroxy-warfarin glucuronides	Other warfarin metabolites‡	Total
Urinary							
0–4	Warfarin	0·27 ± 0·05	0·14 ± 0·03	2·44 ± 0·74	0·84 ± 0·10	1·83 ± 0·20	5·51 ± 1·05
	Warfarin + SAL	0·25 ± 0·07	0·11 ± 0·02	1·81 ± 0·70	0·78 ± 0·20	2·22 ± 0·40	5·20 ± 1·36
4–8	Warfarin	0·32 ± 0·05	0·25 ± 0·02	5·02 ± 0·93	1·40 ± 0·11	3·49 ± 0·38	10·50 ± 1·25
	Warfarin + SAL	0·38 ± 0·05	0·18 ± 0·01	5·32 ± 0·95	0·81 ± 0·11**	3·17 ± 0·40	9·86 ± 1·37
8–12	Warfarin	0·53 ± 0·10	0·20 ± 0·03	7·36 ± 2·40	0·94 ± 0·06	2·70 ± 0·30	11·77 ± 2·89
	Warfarin + SAL	0·84 ± 0·20	0·18 ± 0·04	10·70 ± 1·40	0·56 ± 0·30	3·42 ± 0·60	15·70 ± 2·05
Biliary							
0–4	Warfarin	2·61 ± 0·13	0·04 ± 0·01	4·32 ± 0·44	1·58 ± 0·26	8·97 ± 1·36	17·50 ± 1·95
	Warfarin + SAL	4·69 ± 0·69*	0·11 ± 0·03	11·69 ± 0·80**	2·89 ± 0·21**	15·94 ± 0·97**	35·30 ± 1·52**
4–8	Warfarin	2·00 ± 0·38	0·02 ± 0·01	5·17 ± 0·60	0·76 ± 0·16	7·40 ± 0·83	15·35 ± 1·65
	Warfarin + SAL	1·71 ± 0·21	0·03 ± 0·01	6·70 ± 0·49	0·82 ± 0·12	6·76 ± 0·53	16·01 ± 0·78
8–12	Warfarin	1·01 ± 0·20	0·02 ± 0·01	3·18 ± 0·53	0·60 ± 0·09	3·81 ± 0·76	8·62 ± 1·36
	Warfarin + SAL	0·69 ± 0·14	0·02 ± 0·01	1·93 ± 0·29	0·33 ± 0·06	2·42 ± 0·32	5·40 ± 0·73

* $P < 0·05$; ** $P < 0·005$.
† Results (mean ± s.e.) are expressed as % dose excreted; n = 5.
‡ Consists of 7- and 8-hydroxywarfarin and unidentified polar warfarin metabolites.
DHG-Dihydrogimmel.

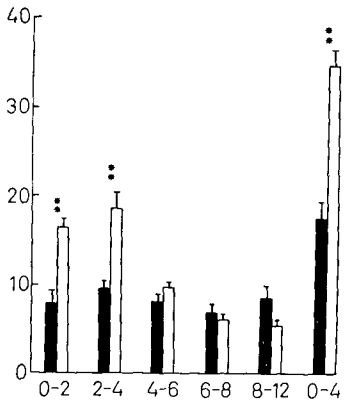

FIG. 3. Biliary excretion of radioactivity (ordinate) expressed as percentage of the dose of [^{14}C]warfarin administered in guinea-pigs dosed with [^{14}C]warfarin (1 mg kg^{-1}, i.p.) alone (closed columns) and in combination with sodium salicylate (177·8 mg kg^{-1}, orally) (open columns). Values are mean ± s.e. from 5 animals (** $P < 0.005$). Abscissa: Time (h).

same dose (i.p.) increased the prothrombin time from 25·9 (normal, to 40·5 s and 34·7 s at 18 and 42 h. Salicylate (177·8 mg kg^{-1}) given with the anticoagulant had no effect on the values.

DISCUSSION

The guinea-pig, like man, excretes warfarin and its metabolites predominantly in urine—70% ^{14}C in 48 h, a result comparable with that of Deckert (1973).

About 43% of the warfarin dose was excreted into the bile in 12 h after injection, a significant portion of this consisted of unconjugated warfarin and its hydroxy-metabolites. Since Hirom, Millburn & others (1972) reported the molecular weight threshold for biliary excretion in the guinea-pig to be 400 ± 50 it is surprising that significant amounts of unchanged warfarin (mol. wt = 308) and hydroxy-warfarin metabolites (mol. wt = 324) were excreted into the bile. Our results suggest that factors other than molecular weight may affect the biliary elimination of drugs (Smith, 1971).

Salicylate depressed the initial (0–6 h) blood concentration of radioactivity following administration of [^{14}C]warfarin. This is likely to be a result of salicylate enhancing biliary elimination of the drug. The increase is probably a result of the rapid uptake of warfarin into the liver by the salicylate-treated animals. At 1 h the liver concentration of ^{14}C was increased and the blood concentration decreased, which resulted in the liver/blood ratio changing from 1·1 in the control to 1·92 ($P < 0.02$) in the salicylate-treated animals.

The more rapid uptake of warfarin from the blood to liver is probably due to displacement by salicylate of bound warfarin from plasma protein. This is supported by the *in vitro* finding that salicylate competes with warfarin for protein binding sites thereby increasing the pool of unbound drug.

Salicylate enhanced the biliary elimination of warfarin only in high concentrations. The overall effect of a single dose on the pharmacokinetics and anticoagulant action of warfarin is small. This is in contrast to the rat in which salicylate increased the rate of elimination and decreased the hypoprothrombinaemia of warfarin (Zawidzka, Coldwell & Grice, 1972). The difference in effect of salicylate in these two species could be due, at least partly, to the route of disposition of warfarin which in the rat is excreted with its metabolites in approximately the same proportion in the urine and faeces whereas in the guinea-pig, excretion is predominantly in urine suggesting that warfarin and its metabolites are excreted in bile and undergo enterohepatic circulation. Because biliary excretion of warfarin and its metabolites is not a true route of elimination in the guinea-pig but rather a storage depot, the overall effect of enhancement of biliary excretion on the elimination and anticoagulant effect of warfarin is probably small in this species compared with the rat.

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