Biochemical Pharmacology, Vol. 36, No. 22, pp. 3951–3955, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

EVIDENCE FOR A DIGITOXIN CONJUGATING UDP-GLUCURONOSYLTRANSFERASE IN THE DOG*

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(*Received* 4 *December* 1986; *accepted* 9 *June* 1987)

Abstract—Liver microsomes of male Beagle dogs contain a form of UDP-glucuronyltransferase which is capable of conjugating digitoxin and its cleavage products digitoxigenin-bisdigitoxoside and digitoxigenin-monodigitoxoside. The highest reaction rates (V_{max} 236 pmoles/mg microsomal protein min) were found for digitoxin and digitoxigenin-monodigitoxoside whereas the lowest K_m was obtained for digitoxigenin-bisdigitoxoside (29 μM). Digoxin cannot be glucuronidated and digitoxigenin is glucuronidated only in traces. The result may explain the fast digitoxin elimination in dogs. Mutual induction experiments utilizing cardenolides and model substrates of UDP-glucuronyltransferase result in the conclusion that a specific form of UDP-glucuronyltransferase is responsible for glucuronidating digitoxigenin glycosides.

Investigations on rat livers have shown various forms of UDP-glucuronyltransferase with different substrate specificity [1–10]. Utilizing guidelines for an interim terminology [11] these enzymatic forms are distinguishable into substrate specific and acceptor unspecific forms as well as forms for additional substrates. Specific UDP-glucuronyltransferase enzymes were purified for 4-nitrophenol [2, 3], bilirubin [4], testosterone [3, 5, 6], androsterone [3] and morphine [2, 9]. Recently an additional form could be isolated for the substrate digitoxigenin-mono digitoxoside [12–14]. The latter plays an important role for the elimination of digitoxin (dt-3) in the rat. Elimination of dt-3 in the rat occurs as a metabolic pathway: glucuronidation of dt-3 is a consecutive reaction following oxidative cytochrome P-450 catalyzed cleavage of dt-3 to digitoxigenin-bisdigitoxoside (dt-2) and digitoxigenin-monodigitoxoside (dt-1), whereas the initial dt-3 proved to be no substrate for purified dt-1 UDP-glucuronyltransferase [14]. In rats the elimination rate of dt-3 depends on the monooxygenase system as well as the glucuronidation reaction [16, 17]. Recently Schuetz *et al.* [15] showed that dt-1-UDP-glucuronyltransferase is inducible by PCN and other glucocorticoids in cell culture, as shown by Schmoldt and Promies [13] for rat liver dt-1-UDP-glucuronyltransferase.

Preliminary studies showed that in contrast to rats dogs show a considerably higher elimination rate of dt-3 (Benthe, unpublished), which, moreover, exceeds severalfold that of digoxin (dg-3). Therefore, in dogs the LD_{50} for dt-3 is twice as high as for dg-3. In spite of the fast dt-3 elimination it was not possible to detect significant oxidative or hydrolytic cleavage of dt-3 in dog livers.

It may be hypothesized that dogs are able to conjugate unmetabolized dt-3. However, glucuronide formation of dt-3 in dogs could not be proven yet, because following glucuronidase incubation of water soluble dt-3 metabolites no dt-3 was detectable (Benthe, unpublished).

The aim of the present study is to prove this hypothesis utilizing *in vitro* investigation with dog liver microsomes. For further characterization of this possible enzymatic reaction cleavage products of dt-3 (dt-2, dt-1, dt-0) and dg-3 were tested. In addition, the substrate specificity for glucuronidation in dogs should be further characterized with model substrates like 4-nitrophenol, 4-hydroxybiphenyl, pregnanediol, and testosterone with enzyme kinetic experiments.

MATERIALS AND METHODS

Animals. Male Beagle dogs were obtained from inbreed facilities of the hospital and had free access to chow and water *ad libitum* until sacrifice.

Chemicals. 20,22-^{3}H-dt-3 was a kind gift of Beiersdorf (Hamburg, F.R.G.). Cleavage products of this compound were prepared according to Satoh and Aoyama [18]. 1,2-^{3}H-pregnanediol, 4-^{14}C-testosterone and the liquid scintillation cocktail Biofluor were from New England Nuclear (Dreieich, F.R.G.). The unlabelled substrates dt-3, dt-2, dt-1 and dt-0 as well as UDP-glucuronic acid were from Boehringer Mannheim (Mannheim, F.R.G.), Triton X-100 and Brij 58 were from Serva (Heidelberg, F.R.G.). Bovine serum albumin was obtained from Behring Werke (Marburg, F.R.G.). All other chemicals were of analytical grade from Merck (Darmstadt, F.R.G.).

Incubation reactions. Livers were obtained from male Beagle dogs directly after death (sacrifice with

* Some of the results were presented at the European Drug Metabolism Workshop, Nancy, 1984.

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Abbreviations used: dt-3, digitoxin; dt-2, digitoxigenin-bisdigitoxoside; dt-1, digitoxigenin-monodigitoxoside; dt-0, digitoxigenin; dg-3, digoxin; UDP-GT, UDP-glucuronosyltransferase EC 2.4.1.17; UDPGA, UDP-glucuronic acid.

Table 1. Glucuronidation rates for digitoxigenin-digitoxosides (dt-3, dt-2. dt-1), digitoxigenin (dt-0), and digoxin (dg-3)

Substrate (50 μM)	Glucuronidation rate (pmoles/mg protein min)
dt-3	33.7 ± 1.33
dt-2	45.1 ± 0.84
dt-1	69.2 ± 5.24
dt-0	10.1 ± 0.22
dg-3	not detectable

Values are means ± SD from 3 individual experiments with dog liver microsomes (2.24 mg/ml microsomal protein, incubation time 10 min).

Table 3. Inhibition of digitoxin (dt-3) glucuronidation

Substrate	Inhibitor	Inhibition type	app. K_i (μM)
dt-3	dt-2	Comp.*	2.8
dt-3	dt-1	Comp.*	4.7
dt-3	dt-0	Non-comp.*	42
dt-3	4-Nitrophenol	Uncomp.*	382
dt-3	4-Hydroxybiphenyl	Uncomp.*	200
dt-3	Pregnanediol	Non-comp.*	390
dt-3	Testosterone	Non-comp.*	78

Kinetic data were obtained from Lineweaver–Burk plots. Comp.* = competitive.

pentobarbital), cooled with chilled Tris–KCl-buffer (50 mM Tris, 150 mM KCl, pH 7.4) and microsomes were prepared according to Kutt and Fouts [19]. Incubations were run in volumes of 250 μl except for 4-hydroxybiphenyl for which 500 μl were used. The standard incubation mixture contained 50 mM Tris–HCl, pH 7.45, 150 mM KCl, 10 mM $MgCl_2$, 0.05% Brij 58 (w/v), 3.2 mM UDPGA, and one of the following substrates (μM) dt-3 (2–100), dt-2 (2–100), dt-1 (10–100), dt-0 (10–100), 4-nitrophenol (100–3000), 4-hydroxybiphenyl (100–300), pregnanediol (2–100), or testosterone (10–300).

Radioactivities (in μCi ^3H) in the incubation assays were as follows (in μCi): dt-3: 0.11, dt-2: 0.14, dt-1: 0.14, dt-0: 0.13, pregnanediol: 0.15, and testosterone: 0.16. Concentration of microsomal protein was 0.64–4 mg/ml, in general 2.24 mg/ml. Incubation reactions were run after a 3 min preincubation period by starting with UDPGA at 37° in a shaking water bath. Incubation time was 5 or 10 min, depending on linearity of the reaction rates. Reactions were terminated by adding 10% trichloroacetic acid (w/v) or in the case of 4-nitrophenol 10% Triton X-100 in triethanolamine (w/v). Blanks were run by omitting UDPGA. The amount of radioactive glucuronide formed was determined following centrifugation and extensive sample extraction with water saturated chloroform [13]. Determination of radioactivity was performed in a Beckman LS 3800 model using Bioflour as scintillation cocktail. Radio-TLC controls were run from incubation samples according to Rao *et al.* [20].

Determination of 4-nitrophenol was performed photometrically at 405 nm according to Erickson *et al.* [21], determination of 4-hydroxybiphenyl according to Bock *et al.* [22].

Reaction rates were linear to protein concentration and incubation time. All assays were run in triplicate at least, standard deviations were lower than 4%. Enzyme constants were estimated from Lineweaver–Burk plots.

RESULTS

Glucuronidation rates for dt-3 and other cardiac glycosides were determined in Beagle dog liver microsomes. In contrast to all other species tested so far [23] Table 1 shows that Beagle dog liver microsomes are capable of glucuronidating dt-3. All other glucuronidation rates were similar as for the rat [13]. Glucuronidation of dg-3 was not detectable.

These high glucuronidation rates for cardiac glycosides were further characterized by establishing the kinetic data for the different glycosides.

Table 2 summarizes kinetic data of cardiac glycosides glucuronidating UDP-glucuronyltransferase activity. K_m and V_{max} values are similar for dt-3 and dt-1 whereas dt-2 as substrate shows three times higher affinity. The lowest glucuronidation rate was

Table 2. Kinetic constants for the glucuronidation of various substrates with dog liver microsomes

Substrate	app. K_m (μM)	app V_{max} (pmoles/mg protein min)
dt-3	113 ± 3	171 ± 141
dt-2	29	69
dt-1	111	222
dt-0	63	22
4-Nitrophenol	1540	71,400
4-Hydroxybiphenyl	182	57,100
Pregnanediol	80	50
Testosterone	133	1110

Data were obtained from Lineweaver–Burk plots utilizing microsomes from three individual animals (for dt-3 with standard deviation) or from pooled microsomes from different animals.

Table 4. Inhibition of glucuronidation of various substrates with digitoxin (dt-3) as inhibitor

Substrate	Inhibitor	Inhibition type	app. K_i (μM)
dt-2	dt-3	Comp.*	63.7
dt-1	dt-3	Comp.*	241
dt-0	dt-3	Non-comp.*	3.2
4-Nitrophenol	dt-3	No inhibition	
4-Hydroxybiphenyl	dt-3	No inhibition	
Pregnanediol	dt-3	Activation	
Testosterone	dt-3	No inhibition	

Kinetic data were obtained from Lineweaver–Burk plots. Comp.* = competitive.

observed for dt-0. In this case it was not investigated whether dt-0 glucuronide was converted to 3-epi-dt-0 during the incubation.

Inhibition experiments were utilized for further characterization of the dt-3 glucuronidating enzyme. In the case of competitive inhibition the enzyme should glucuronidate both substrates.

To prevent an overlapping substrate specificity if very high substrate concentrations were present substrates and inhibitors were used at concentrations around K_m. Because K_m-values of dog liver UDP-glucuronyltransferase were unknown they were determined before.

Table 2 shows that the K_m for 4-nitrophenol with 1.54 mM is twice as high as in the rat [13] whereas the K_m for 4-hydroxybiphenyl is significantly lower in dogs than in rats (0.3 mM according to Schmoldt and Promies [13]). In comparison to dt-3 4-nitrophenol was glucuronidated 400-times higher and 4-hydroxybiphenyl was conjugated 250-times higher, whereas the endogenous substrate testosterone was conjugated only 4-times higher.

Inhibition experiments were performed utilizing other substrates for UDP-glucuronyltransferase to determine inhibition types and to further evaluate the question which enzyme form is involved in the glucuronidation of cardiac glycosides in dogs (Tables 3 and 4).

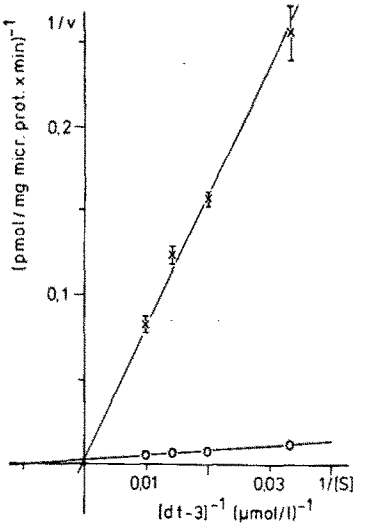

Fig. 2. Competitive inhibition of microsomal digitoxin (dt-3) glucuronidation by 75 μM digitoxigenin-mono-digitoxoside (dt-1). Values are means of 3 experiments ± SD. O——O without, ×——× with inhibitor.

Figures 1 and 2 show competitive inhibition of dt-3 glucuronidation by dt-2 and dt-1.

In contrast to that, dt-0 provided to be a non-competitive inhibitor (Fig. 3).

p-Hydroxymercuribenzoate acted as an inhibitor at very low concentrations (10, 20, 30 μM). However, the maximum inhibition reached 40% only.

Pregnanediol and testosterone acted as non-competitive inhibitors (Fig. 4) whereas 4-hydroxybiphenyl and 4-nitrophenol inhibited the dt-3 conjugation uncompetitively (Fig. 5).

In a reverse assay procedure with dt-3 as the inhibitor dt-2 and dt-1 were competitively inhibited and dt-0 was noncompetitively inhibited (Table 4). For conjugation of all other substrates dt-3 proved to be no inhibitor. The activation of pregnanediol glucuronidation by dt-3 (Fig. 5) needs further investigation.

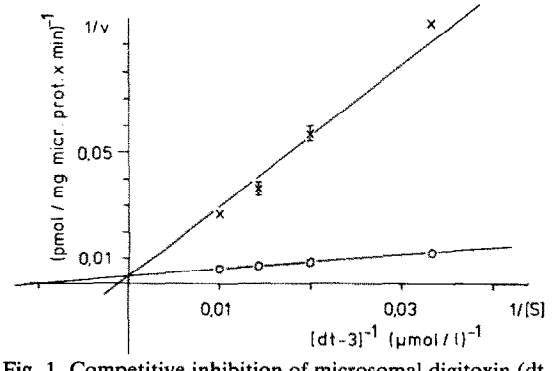

Fig. 1. Competitive inhibition of microsomal digitoxin (dt-3) glucuronidation by 16 μM digitoxigenin-bisdigitoxoside (dt-2). Values are means of 3 experiments ± SD. O——O without, ×——× with inhibitor.

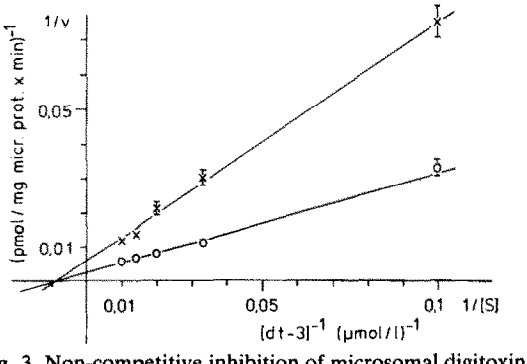

Fig. 3. Non-competitive inhibition of microsomal digitoxin (dt-3) glucuronidation by 50 μM digitoxigenin (dt-0). Values are means of 3 experiments ± SD. O——O without, ×——× with inhibitor.

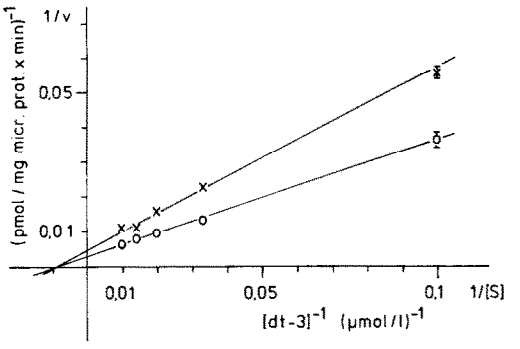

Fig. 4. Non-competitive inhibition of microsomal digitoxin (dt-3) glucuronidation by 50 µM testosterone. Values are means of 3 experiments ± SD. O——O without, ×——× with inhibitor.

DISCUSSION

The results of the present study show that in contrast to other species the dog is capable of glucuronidating unmetabolized dt-3. The yields in respect to glucuronidation rates are at least 30 times higher compared to all tested species [23]. For the first time the fast elimination rate of dt-3 in dogs [23] may be explained with high glucuronidation rates *in vitro*. Until now it was unproven that a dt-3 glucuronide is existent in dogs due to its non-cleavage with β-glucuronidase *in vitro* by β-glucuronidase of *Helix pomatia* and *E. coli*. The conjugate produced in these experiments should be glucuronides because of their formation only in the presence of UDPGA. In addition, the TLC R_f-values of the isolated dt-3 conjugate corresponded to the digitoxin-16′-glucuronide and the urinary excreted water soluble metabolite.

Dt-2 is glucuronidated very slowly in rodents [23, 24], whereas the dog glucuronidates dt-2 with high rates. Similar results were obtained in man [25]. In accordance with results published previously, dt-0 is much slower glucuronidated than dt-1 [13].

It is still under discussion which hydroxyl group of the digitoxose chain is the target of glucuronidation. Our basic assumption is the axial hydroxyl group of the terminal sugar (3′-, 9′-, and 15′-position). Due to the steric hindrance the 16′-position should be the worse target.

As mentioned earlier the widely discussed heterogeneity of UDP-glucuronyltransferase was restricted to inhibition experiments in this investigation. The kinetic constants of some model substrates were determined to elucidate this problem because data for the dog are not yet available. The results are in good accordance with data obtained with liver microsomes from other species [13].

Inhibition experiments support the conclusion that all cardiac glycosides are glucuronidated by the same enzyme form due to their character as competitive inhibitors. Dt-0 and all other substrates tested so far show no significant competitive inhibition and therefore are no substrates for this enzyme within the concentration range of their K_m-value.

The thiol-group blocking agent p-hydroxymercuribenzoate proved to be only a partial inhibitor for the dt-3 conjugation in dog liver microsomes. This may lead to the conclusion that in dog liver microsomes the digitoxin UDP-glucuronyltransferase contains no thiol groups in the active site [26, 27] or, theoretically, there are more than one enzyme with and without thiol-groups in the active site.

Neither *in vivo* nor *in vitro* experiments with liver homogenates or microsomes cleavage products of dt-3 could be detected. Therefore, the activity of the dt-3 conjugating UDP-glucuronyltransferase seems to be the rate limiting step for the elimination of cardiac glycosides. On the other hand, dg-3 elimination should depend, like that of other species, on the renal function only.

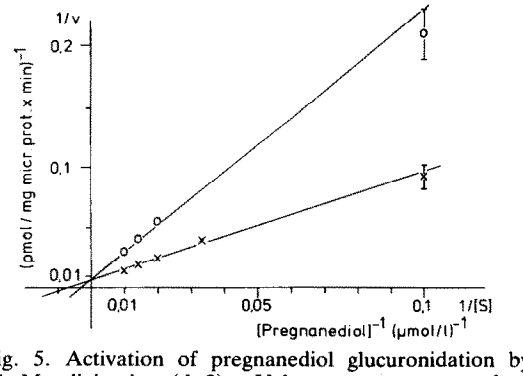

Fig. 5. Activation of pregnanediol glucuronidation by 74 µM digitoxin (dt-3). Values are means of 3 experiments ± SD. O——O without, ×——× with inhibitor.

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