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HEPATIC MICROSOMAL GLUCURONIDATION OF CLOFIBRIC ACID IN THE ADULT AND NEONATE ALBINO RAT

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Abstract—Hepatic microsomal UDP glucuronyltransferase activity towards the acid substrate clofibric acid has been described in the adult and neonate albino rat. The enzyme was maximally activated, approximately 2-fold, in the presence of 0.1–0.4% (w/v) digitonin. Induction of the digitonin activated clofibric acid glucuronyltransferase was observed following phenobarbitone treatment *in vivo* (2.2-fold), and to a lesser extent, following β-naphthoflavone treatment (1.3-fold). Clofibrate treatment *in vivo* (of which clofibric acid is the ester hydrolysis product) had no effect on clofibric acid glucuronidation *in vitro*. The activity of clofibric acid glucuronyltransferase in the liver of rat before and at birth was low (approx. 0.08 nmoles glucuronide formed/min/mg microsomal protein). The activity increased 5-fold during the first three post-natal days. After this time, the activity increased linearly reaching adult levels by four weeks after birth. The data indicated that clofibric acid glucuronyltransferase belongs to the neonatal cluster of enzymes and clofibric acid is a group 2 substrate. Clofibric acid, a common therapeutic agent, is a useful, acid substrate for the estimation of mammalian hepatic microsomal glucuronyltransferase activity.

Glucuronidation is an important reaction in the detoxification of foreign chemicals and also of endogenous substances. The conjugation of a compound with glucuronic acid generally renders it more water soluble and hence readily excretable in urine or bile. The reaction is catalysed by the microsomal enzyme, uridine diphosphoglucuronyltransferase, which couples glucuronic acid (in the high energy form—uridine diphosphoglucuronic acid) with a hydroxyl or carboxylic acid group [1].

Many different model substrates have been used to investigate the activity of glucuronyltransferase in hepatic subcellular preparations [2–4]. Recent studies on the inducibility of the enzyme following phenobarbitone or 3-methylcholanthrene treatment and its activity in the foetal and neonatal period have indicated the existence of two major forms of the enzyme [5, 6]. The existence of at least two forms of glucuronyltransferase has been demonstrated further by the purification of enzymes with specific catalytic activities [7, 8]. There are also tentative suggestions of the existence of other forms [9].

Clofibrate (the ethyl ester of α[*p*-chlorophenoxy]isobutyric acid), a hypolipidaemic drug, is primarily excreted (80–90%) as the glucuronide of the free acid following administration to the rat [10, 11]. In this present study, we have developed an assay for rat hepatic microsomal glucuronyltransferase using clofibric acid as substrate. Peri- and post-natal development and the effect of the liver microsomal monooxygenase inducing agents, phenobarbitone and β-naphthoflavone, on clofibric acid glucuronyltransferase have been investigated and the profile compared with that observed with other substrates of the glucuronyltransferase(s). In addition, the effect of chronic clofibrate treatment *in vivo*, which has recently been shown to induce a unique microsomal cytochrome P-450 [12], on the activity of the transferase *in vitro* has been studied.

MATERIALS AND METHODS

Chemicals

Clofibrate and clofibric acid, sodium salt (unlabelled) and [^{14}C-methyl]clofibric acid (sp. act. 10.2–12.4 μCi/mg) were synthesised by Radiochemical Unit, Imperial Chemical Industries, Pharmaceuticals Division. The radiochemical purity of [^{14}C] clofibric acid was 99.3% determined by silica gel thin layer chromatography using the following solvent systems, toluene: dioxane: acetic acid 90:25:4 and *n*-butanol: acetic acid: water 40:10:5. An authentic sample of clofibryl glucuronide isolated from rabbit urine [13] was kindly supplied by Dr. J. Caldwell, St. Marys Hospital, Medical School, London. Sodium phenobarbitone was purchased from B.D.H. Chemicals plc, Poole, Dorset; β-naphthoflavone from Aldrich Chemical Co., Gillingham, Kent; ketodase from General Diagnostics, Eastleigh, Hants; UDP-glucuronic acid and digitonin from Sigma Chemical Co., Poole, Dorset. Solvents for high pressure liquid chromatography were HPLC grade from Rathburn Chemicals plc, Walkerburn, Peebleshire. All other chemicals were of analytical grade.

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Animal treatments

Sodium phenobarbitone in saline (75 mg/kg/day i.p.) and β-naphthoflavone in corn oil (25 mg/kg/day i.p.) were administered to adult male Alderley Park albino rats (8–10 weeks old; 120–150 g at start) for 3 days and the animals were killed on day 4. Clofibrate (0.4% w/w) was fed in the PCD diet (BP Nutrition, Witham, Essex) for 14 days. (These dose regimens gave maximal induction of hepatic microsomal cytochrome P-450). Control animals received vehicle or drug free diet. Animals were allowed free access to diet and water until they were killed under ether anaesthesia. The livers were then rapidly removed into 10 mM sodium-potassium phosphate buffer pH 7.4 containing 0.15 M potassium chloride at 4°. Liver microsomes were prepared as described by Fouts and Devereux [14].

For the peri- and post-natal studies, virgin female Alderley Park albino rats (225–250 g) were mated with breeder males (250–275 g), four females to one male. The day when sperm were detected in the vaginal smear was regarded as the first day of pregnancy. Neonates were taken from their mothers at various times after birth. Day 17 foetuses (i.e. five days before birth) were removed by caesarian section. The average litter size was 11 pups. Livers were pooled according to litter (regardless of sex) and microsomes prepared as described above.

The protein content of microsomes was determined by the method of Lowry *et al.* [15].

Clofibric acid glucuronidation

Activated glucuronyltransferase activity towards clofibric acid was determined as follows: final reaction mixtures for the standard assay contained [^{14}C] clofibric acid 0.05–2.0 mM; 0.25 μCi added in 10 μl dimethyl sulphoxide, 1–5 mg microsomal protein, 0–0.4% (w/v) digitonin and 75 mM Tris–HCl buffer pH 7.4 containing 10 mM magnesium chloride to a final vol of 1 ml. The reaction was started by the addition of 3 mM uridine diphosphoglucuronic acid (UDPGA). The reaction mixtures were incubated in a shaking water bath at 37° for 10–60 min.

For the peri- and post-natal studies, the low enzyme activity necessitated the use of 0.5 mM; 0.25 μCi [^{14}C] clofibric acid and reaction mixtures were incubated for 20 min. Maximum activation of neonate liver microsomes was achieved with 0.1% (w/v) digitonin.

The reaction was stopped by the addition of 0.2 ml 0.1 M HCl and then extracted with 8 ml ethyl acetate. The recovery of radioactivity into ethyl acetate was > 99% under all reaction conditions. After evaporation of the ethyl acetate, the residue was reconstituted in methanol:water:trifluoroacetic acid (TFA); 55:45:0.1. Separation of the glucuronide was carried out on a Pye Unicam HPLC system with a Lichrosorb RP8 reverse phase column (0.4 × 20 cm). An isocratic eluting solvent system of methanol:water:TFA; 55:45:0.1 at 1.5 ml/min flow rate was used. Radioactivity eluting from the column was detected and quantitated using a Berthold LB 503 radioactivity monitor.

Clofibric acid glucuronide in acid stopped reaction mixtures was hydrolysed by incubation with β-glucuronidase (10,000 units ketodase in 0.2 M acetate buffer pH 5.0 at 37° for 24 hr).

RESULTS

Clofibric acid glucuronide was the only metabolite of clofibric acid produced in the complete *in vitro* system and had the same retention time (7 min) as an authentic sample (detected by absorbance at 229 nm). Clofibric acid had a retention time of 14 min. No glucuronide was formed in the absence of UDPGA. Incubation of extracted reaction mix-

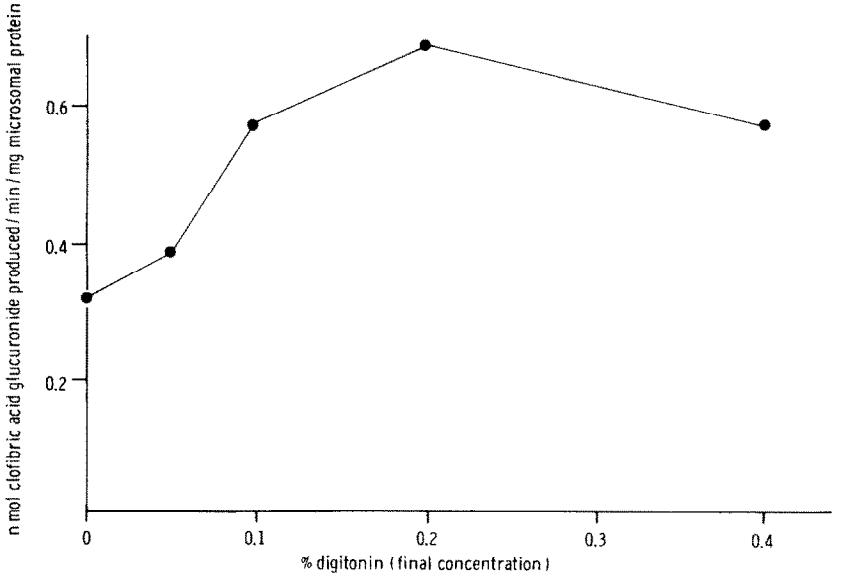

Fig. 1. Activation of microsomal glucuronyltransferase with digitonin. Each point is the mean of two individual reaction mixtures containing [^{14}C] clofibric acid 2 mM; 0.25 μCi, 2 mg microsomal protein, 3 mM UDPGA and incubated for 10 min at 37°.

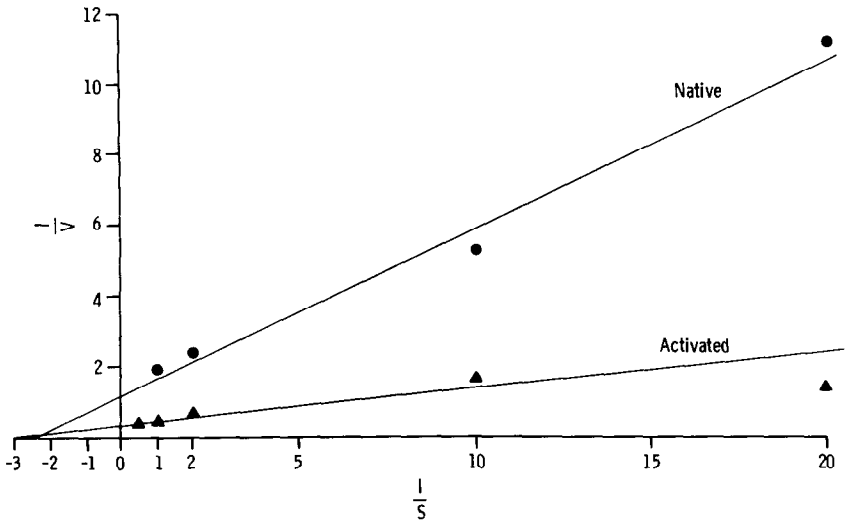

Fig. 2. Double reciprocal plot of the activity of the native and digitonin activated transferase with respect to clofibric acid concn. Each point is the mean of two individual reaction mixtures containing 2 mg microsomal protein, 0.2% (w/v) digitonin (activated enzyme only), 3 mM UDPGA and incubated for 10 min at 37°. Kinetic parameters were determined by linear regression analysis. Correlation coefficients were 0.993 and 0.992 for native and activated plots respectively. V nmol clofibric acid glucuronide produced/min/mg microsomal protein and S mM clofibric acid.

tures with β-glucuronidase abolished the peak corresponding to the clofibric acid glucuronide.

Clofibric acid glucuronidation was linear up to 20 min incubation and 5 mg microsomal protein (data not shown). The activity of clofibric acid glucuronyltransferase was increased in the presence of digitonin (Fig. 1). The concn of digitonin required for maximal activation (2-fold) was 0.1–0.4% (w/v) final concn.

The activity of the native and digitonin activated glucuronyltransferase was dependent on clofibric acid concn. A double reciprocal plot of the data is shown in Fig. 2. The apparent K_m and V_{max} for the native enzyme were 0.40 mM and 0.83 nmol glucuronide formed/min/mg and for the activated enzyme 0.32 mM and 2.96 nmol/min/mg respectively.

The effect of phenobarbitone, β-naphthoflavone and clofibrate treatment on digitonin activated clofibric acid glucuronyltransferase is shown in Table 1. Phenobarbitone treatment increased the enzyme activity significantly (2.2-fold), whilst β-naphthoflavone only caused a small increase (1.3-fold). Clofibrate treatment had no effect on the glucuronidation of clofibric acid.

The peri- and post-natal development of the digitonin activated clofibric acid glucuronyltransferase is shown in Fig. 3. The activity was low at birth (17%

Table 1. Effect of monooxygenase inducers on (digitonin activated) microsomal clofibric acid glucuronyltransferase activity*

Treatment	Clofibric acid glucurononyltransferase† (nmol glucuronide formed/min/mg)
Saline control	1.10 ± 0.13
Phenobarbitone	2.37 ± 0.05‡ (215)
Corn oil control	1.24 ± 0.09
β-Naphthoflavone	1.61 ± 0.20‡ (130)
Control	1.61 ± 0.41
Clofibrate	1.69 ± 0.61 (105)

* Reaction mixtures contained 2 mM; 0.25 μCi [^{14}C] clofibric acid, 2 mg microsomal protein, 3 mM UDPGA, 0.2% (w/v) digitonin and were incubated for 5–10 min at 37°. Treatments with monooxygenase inducers were as described in Materials and Methods.

† Data for phenobarbitone and β-naphthoflavone are from a representative experiment and are the mean ± S.D. of three individual animals with duplicate assays per animal. Data for clofibrate are from three separate experiments and are the overall mean ± S.D. of ten individual animals with duplicate assays per animal.

‡ Statistically significant difference from control ($P < 0.05$; Students t test).

Figures in parentheses are % control.

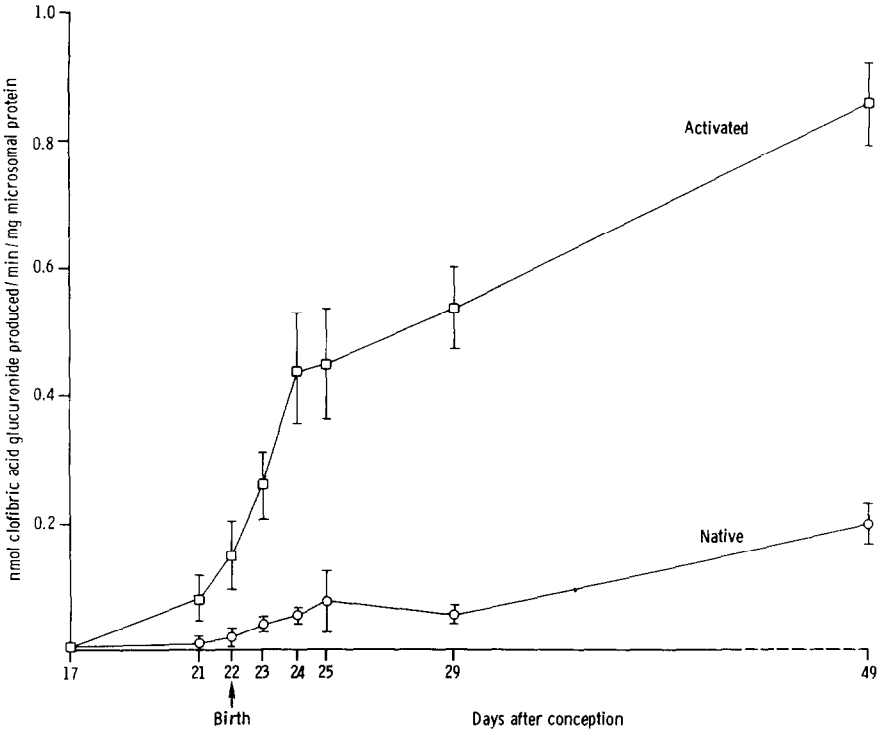

Fig. 3. The peri- and post-natal development of digitonin activated clofibric acid glucuronyltransferase. Each point is mean ± S.D. of values from three or four individual litters. Reaction mixtures contained [^{14}C] clofibric acid 0.5 mM; 0.25 μCi, 2 mg microsomal protein, 0.1% (w/v) digitonin, 3 mM UDPGA and were incubated for 20 min.

of the 49 day juvenile value) and markedly increased approx. 5-fold during the first 3 days (activities approx. 50% of juvenile value). The activity of clofibric acid glucuronyltransferase then increased gradually and at 49 days had reached a level similar to that observed in adult animals (see Table 1).

DISCUSSION

Clofibric acid is a substrate for hepatic microsomal glucuronyltransferase *in vitro*. The formation of clofibric acid glucuronide, identified by coelution with authentic standard, required the presence of UDPGA. The glucuronide was fully cleaved by β-glucuronidase. Clofibric acid glucuronide has been shown to rearrange to β-glucuronidase resistant glucuronides at mild alkaline pH [16]; the acid conditions in the present study obviously precluded this reaction. Digitonin, which has been shown to be a useful activating agent with other substrates [14], gave maximal activation to clofibric acid glucuronyltransferase over a wide concentration range.

The K_m of the digitonin activated enzyme was similar to that for the native enzyme although V_{max} (expressed as nmoles clofibric acid glucuronide produced/min/mg microsomal protein) was higher. This would indicate that the enzyme has the same affinity for the substrate in the activated form. Various workers have suggested that the latency of glucuronyltransferase is due to the enzyme being located within the microsomal membrane, and the effect of digitonin and other membrane perturbants (e.g. phospholipase A) is to facilitate greater access of substrates to the enzyme active site [18].

The low activity of clofibric acid glucuronyltransferase at birth would tend to place it in the 'neonatal' cluster of transferase enzymes, which reach adult levels of activity after birth, rather than the 'late foetal' cluster which develop before birth. The post-natal development of activity is similar to that of glucuronyltransferases for oestradiol and testosterone which belong to the 'neonatal' cluster of enzymes with a marked increase in activity during the initial postnatal days, followed by a gradual increase to adult levels [2].

Clofibric acid glucuronidation was predominantly induced by phenobarbitone although β-naphthoflavone had some effect. This profile would classify clofibric acid as a 'group 2' substrate. The distinguishing feature between the substrate 'groups 1 and 2' appears to be the specific molecular configuration of the compound; small planar molecules belong to 'group 1' and are chiefly 3-methylcholanthrene/β-naphthoflavone inducible and large bulky molecules belong to 'group 2' and are chiefly phenobarbitone inducible [19, 20]. Okulicz-Kozaryn *et al.* [20] suggest a critical molecular 'thickness' of 4 Å divides the two groups. Our calculation gives a thickness of around 5.4 Å for clofibric acid thus placing the compound marginally in 'group 2'. The fact that clofibric acid was induced to a small extent by β-naphthoflavone may indicate some overlapping of catalytic activity within the two groups of transferase.

As clofibrate treatment has been shown to increase

(ester) glucuronidation of bilirubin determined both *in vivo* and *in vitro* [21, 22], this indicated that the clofibric acid glucuronyltransferase is distinct from the bilirubin transferase. The lack of effect of clofibrate treatment *in vivo* on microsomal clofibric acid glucuronyltransferase activity correlates with the constant percentage excretion of clofibric acid glucuronide during chronic clofibrate treatment *in vivo* [10].

The use of a widely prescribed pharmaceutical agent such as clofibric acid as a model substrate may be relevant in studies of the effect of chronic drug treatment on animal and human liver glucuronyltransferase activity.

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