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COMPARATIVE STUDY OF CLOFIBRIC ACID AND BILIRUBIN GLUCURONIDATION IN HUMAN LIVER MICROSOMES

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Abstract—Hepatic microsomal glucuronoconjugation of the hypolipidemic drug clofibric acid was characterized in human liver and compared to the acylglucuronide formation of an endogenous substrate, bilirubin. The affinity of UDP-glucuronosyltransferase for bilirubin was 15-fold higher than for clofibric acid; the V_{max} for the transformation of the two substrates were similar. The analysis of the specific activity in 32 liver biopsies showed that glucuronidation of clofibric acid or bilirubin were comparable in man and in rat. However, UDP-glucuronosyltransferase activity towards clofibric acid exhibited a large interindividual variation in man. Sex or age did not influence the glucuronidation of bilirubin and clofibric acid. Among the drugs given to the patients only clofibrate was able to increase the bilirubin conjugation. No effect of alcohol or smoking on the conjugation of the two substrates was observed. The absence of correlation between UDP-glucuronosyltransferase activities towards clofibric acid and bilirubin together with the specific induction of bilirubin glucuronidation by clofibrate suggested that these arylcarboxylic substrates were conjugated by separate forms of UDP-glucuronosyltransferase in human.

Clofibrate has been successfully used as a hypolipidemic drug for many years and its pharmacological and toxicological implications are extensively documented [1–3]. In man as well as in rodents, clofibrate is readily hydrolyzed into the pharmacologically active parent acid [2-(4′-chlorophenoxy)-2-methylpropionic acid], which is then quickly eliminated in urine as clofibryl ester glucuronide (60–90% of the oral dose) [4, 5]. In this regard the crucial role of UDP-glucuronosyltransferases (UDPGTs, EC 2.4.1.17) in the biotransformation and disposition of arylacetic acids becomes obvious. If the properties of this family of membrane-bound enzymes are well defined in laboratory animals [6, 7] and some of their corresponding genes recently isolated [8, 9], by contrast little is known concerning the characteristics of the human enzymes and particularly their ability to form ester glucuronides.

Glucuronidation, the predominant mammalian phase II reaction is generally considered to be a metabolic pathway for detoxification. Nevetherless, it has been suggested that drugs like clofibrate, containing a carboxylic acid function group, could be responsible for long-term toxicity through their electrophilic acylglucuronides, which can covalently react with biomolecules [10]. In fact, the safety of this compound in patients on long term therapy has been questioned [11] and the relevance to humans of its effect on peroxisome proliferation and carcinogenicity in rodents remains to be established [2].

On the other hand, like other arylacetic and aryloxyacetic acids, clofibrate specifically induces in rats the conjugation of bilirubin, leading to the excretion of bilirubin acylglucuronides [12–14]. Even if, to our knowledge, this inductive effect has not been directly demonstrated in humans, it has been administered by the clinicians to lower plasma bilirubin level in Gilbert's syndrome [15] and neonatal icterus when resistant to phototherapy [16].

In this paper, we compare the formation of ester glucuronides from clofibric acid and bilirubin in human liver. For this purpose a sensitive liquid chromatographic assay for the analysis and quantification of arylcarboxylic acid glucuronides was recently developed [17]. This method was adapted to the determination of the kinetic constants of UDPGT towards clofibric acid. Attempt is made to evaluate the influence of sex, cigarette smoking, alcohol and drug intake on the glucuronidation of these two compounds.

MATERIALS AND METHODS

Liver donors. One liver fragment (removed 3 hr after death) was obtained post mortem, from a 45-year-old man presenting no noticeable hepatic damage. Thirty-two liver biopsies were collected from donors having undergone a laparotomy for surgical reasons (Service de Chirurgie Digestive, Pr. Grosdidier, Centre Hospitalier Universitaire de Brabois, Vandoeuvre-lès-Nancy, France). They were kept in liquid nitrogen until used. In this population (20 females, 12 males), the age ranged from 20 to

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77, 7 were smokers (more than 10 cigarettes/day), 9 were alcohol drinkers (more than 80 g/day). Diazepam was administered to 5 patients and cimetidine to 5 patients (usual posology), one received prednisolone and another nandrolone (usual posology). Finally one patient was treated by clofibrate (500 mg/day) for more than one year.

The microsomes were prepared as follows: liver samples were individually homogenized in 0.25 M sucrose, 1 mM Tris–HCl buffer (pH 8.0) with a Potter Elvejhem apparatus (3000 rpm, 3 strokes) to give a 10% (of the original liver weight) homogenate. The fraction was centrifuged at 1300 *g* for 10 min and the supernatant centrifuged again at 10,000 *g* for 20 min in a L5-75 Beckman ultracentrifuge. The microsomal pellets were finally obtained by a centrifugation at 100,000 *g* for 60 min. The microsomal pellets were suspended in 100 mM Tris–HCl buffer (pH 8.0) containing 1 mM EDTA, 1 mM dithioerythritol (Sigma, St Louis, MO) and 20% (v/v) glycerol. They were kept at −80°, being stable for at least 2 years under these conditions (personal data and ref. 18).

The microsomal protein content was determined according to Lowry *et al.* [19] after precipitation with 10% (w/v) trichloroacetic acid.

Glucuronidation assays. The microsomes were fully activated by addition of digitonin (Merck, Darmstadt, F.R.G.) [digitonin/protein (w/w) optimal ratio of 1.0]. Conjugation of bilirubin was performed according to the Heirwegh method [20] with this modification: bilirubin (Merck, Darmstadt, F.R.G.) was dissolved in a minimal volume of sodium hydroxide (0.1 N) instead of bound to bovine serum albumin. Final concentrations of the substrates in the incubation mixture were respectively 2.50 mM and 0.13 mM for UDP-glucuronic acid and bilirubin respectively. The activity towards clofibric acid (Ega-Chemie, Steinheim, F.R.G.) was assayed using (^{14}C) UDP-glucuronic acid (Amersham, Les Ulis, France). The reaction was performed in a total volume of 300 μl at 37° for 20 min. The labelled glucuronide formed was separated from (^{14}C) UDP-glucuronic acid by HPLC on a reverse-phase column (Lichrosorb RP-18, Hibar RT 250-4, 7 μM, Merck) as previously described [17]. Incubation conditions were the following: UDP-glucuronic acid (3.5 mM) and [^{14}C] UDP-glucuronic acid (0.5 μCi per assay), clofibric acid (1 mM) and Tris–HCl buffer (100 mM) (pH 7.0). Control reaction without clofibric acid was simultaneoulsy run. Under these conditions the production of clofibric acid glucuronides was linear up to 20 min and to 1.5 mg of microsomal protein.

The assays for determination of kinetic parameters were done in duplicate. But for determination of specific activities, due to the restricted amount of human biopsies only single assays were run.

Correlation coefficients were calculated by linear regression analysis. Otherwise, the test of Wilcoxon was used.

RESULTS

The optimal activation of bilirubin and clofibric acid UDPGT activities was obtained with a digitonin/protein weight ratio of 1.0 in humans. As latent activities were of the same order, glucuronidation of bilirubin was more enhanced (4.3-fold) by the detergent than clofibric acid conjugation (2.0-fold) (Table 1).

Table 1. Activation of human liver UDPGT activities towards clofibric acid and bilirubin by digitonin

Fractions	Substrate	
	Clofibric acid	Bilirubin
Latent activity	0.38	0.25
Digitonin activated activity	0.77	1.08
Activation factor	2.0	4.3

UDPGT activity is maximally activated by preincubation of microsomes with digitonin (digitonin/protein weight ratio : 1.0) at 4° for 30 min. Results are expressed as nmol . min^{-1} mg^{-1} protein.

The values of the apparent kinetic constants for glucuronidation of clofibric acid and bilirubin in a post-mortem liver were determined from the double reciprocal representation of Michaelis–Menten shown in Fig. 1 (inserts A and B respectively).

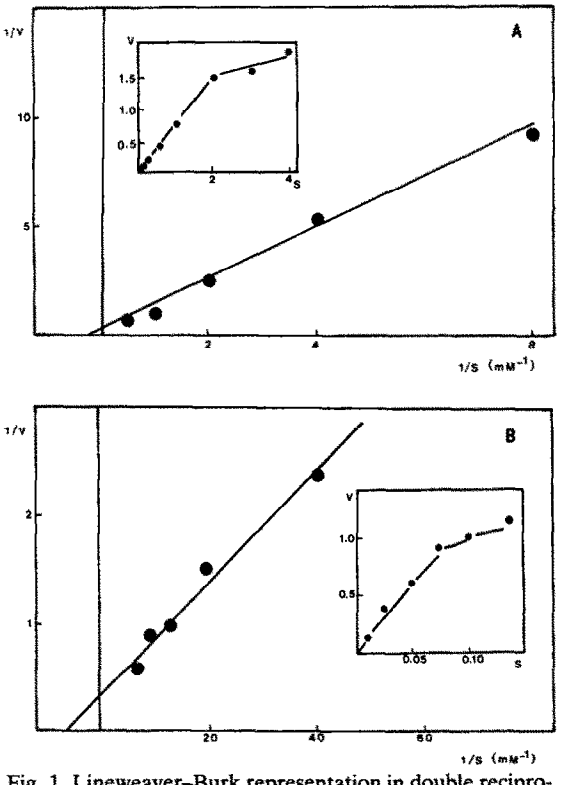

Fig. 1. Lineweaver–Burk representation in double reciprocal plots of human microsomal conjugation of (A) clofibric acid, (B) bilirubin. Inserts represent the specific activity expressed in nmol . min^{-1} mg^{-1} protein as a function of the substrate concentration expressed in mM. Final concentration of UDPGA was 3.5 and 2.5 mM for clofibric acid and bilirubin UDPGT activity measurements respectively. Concentrations of clofibric acid and bilirubin varied from 0.125 mM to 4 mM and from 0.026 mM to 0.130 mM respectively.

Table 2. Apparent kinetic constants of bilirubin and clofibric acid UDPGT in human compared to rat

	Bilirubin UDPGT		Clofibric acid UDPGT	
	K_m	V_{max}	K_m	V_{max}
Human	0.160	3.10	2.50	5.00
Rat	0.064[1]	1.54[1]	0.095[1]	1.17[1]
			0.32[2]	3.0[2]

Apparent K_m and V_{max} are expressed in mM and nmol . $min^{-1} mg^{-1}$ protein respectively.
[1] Fournel *et al* [22].
[2] Odum and Orton [23].

Although the V_{max} of the reaction was quite similar, 3.1 and 5.0 nmol/min/mg protein for bilirubin and clofibric acid conjugation respectively, the UDPGT affinity towards clofibric acid was 15 times lower (K_m 2.50 mM) than towards the endogenous substrate, bilirubin (K_m 0.16 mM).

In human, the V_{max} for both conjugations were twice those measured in the rat. But as the affinity of UDPGT for clofibric acid was in the same range as that for bilirubin in rat, it was very weak in human (K_m being 25 times higher) (Table 2).

Specific UDPGT activities towards bilirubin and clofibric acid measured in human liver biopsies were reported in Fig. 2. With bilirubin as substrate they ranged from 0.05 to 2.30 nmol/min/mg protein and in two cases, the activity was undetectable. For clofibric acid, the activities were from 0.1 to 6.0 nmol/min/mg protein, meaning that the interindividual variation was around 60 (i.e. about twice that for bilirubin conjugation). The mean value was 1.2 nmol/min/mg protein (N = 31). In one sample no activity was detectable even though the conjugation of other substrates such as 4-nitrophenol, nopol (results not shown) and bilirubin was in a normal range.

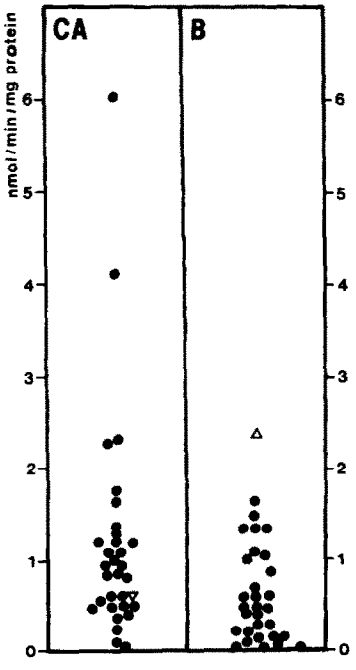

Fig. 2. Specific activities of UDPGT towards clofibric acid (CA) (N = 32) and bilirubin (B) (N = 31) in human liver biopsies. The △ represents the clofibrate-treated patient.

We were not able to find any influence of drinking or smoking on the conjugation of bilirubin or clofibric acid. No effect of drugs was observed except in one patient which had been treated with clofibrate (500 mg/day) for at least one year. In this case, UDPGT activity towards bilirubin was enhanced almost three times when the activity towards clofibric acid remained close to the mean value of the population.

Finally no correlation was found between bilirubin and clofibric acid UDPGT activities in the tested population (data not shown).

DISCUSSION

In agreement with previous studies [18, 21] our results clearly show that both in human post-mortem liver or liver biopsies, the microsomal specific activity of bilirubin UDPGT is of the same order of magnitude as in rat [12, 20, 22]. Likewise the rate of clofibric acid glucuronidation was similar in both species [17, 23] in contrast to some activities previously tested in man, namely towards other exogenous substrates as phenols, coumarines, monoterpenoid alcohols, which were generally three times lower than in rat [18, 24, 25]. The apparent K_m and V_{max} values of UDPGT for both substrates were higher in man than in rat. The affinity of the human enzyme was 15-fold higher for bilirubin than for clofibric acid but the maximal velocities were comparable for both substrates. It should be noted that arylcarboxylic acids, such 1- and 2-naphthylacetic [17, 26] or 2-phenylpropionic acids (unpublished data) are poorly glucuronidated in rodents and man liver microsomes although they undergo *in vivo* efficient and exclusive glucuronidation [27, 28].

In the population tested, there was no significant variation of microsomal UDPGT activities depending on the sex or age. This could suggest that higher serum bilirubin levels in post pubertal males when compared with females are unlikely to be the result of a lower conjugating capacity, in contrast to the hypothesis of Muraca and Fevery based on experiments done in rat [29]. Nevertheless, the confirmation of this point would need further studies on larger populations.

We did not observe any clear connection between the rate of bilirubin or clofibric acid conjugation and

drug-intakes or habits of the patients except for the long-term clofibrate-treated patient. In this sample the bilirubin conjugation was increased without any modification of that of clofibric acid. This finding represents the first direct evidence that the specific inductive effect of clofibrate on bilirubin UDPGT activity observed in rat could also be relevant to man.

The interindividual variations may be discussed in term of genetic variability. Indeed, in a population of 85 patients, Faed and McQueen reported that the amount of urinary clofibrate conjugates varied from 25 to 75% [30].

Accumulating evidence obtained in rat liver suggests the existence of at least five enzyme forms of UDPGT. This proposal is mainly based on physical separation of the different enzymes forms [31–33], on functional heterogeneity of enzyme activities during perinatal development [34], on substrate specificities and regulation by inducers [12, 35, 36] and recently on the characterization of some of their corresponding genes [8, 9]. According to these criteria, two groups of activities have been described: group 1 substrates including planar phenolic aglycones conjugated by at least one form of UDPGT and group 2 substrates including bulkier molecules glucuronidated by other forms of UDPGT.

In addition distinct forms for endogenous compounds like bilirubin and steroids have been reported [7]. In contrast to phenols, the substrate specificity of the conjugation of carboxylic acids has been poorly defined until now, even in animals. Based on the neonatal development and the pattern of induction of this activity in the rat, clofibric acid should be classified as a group 2 substrate [23]. Similarly valproic acid behaves as a group 2 substrate [37]. By contrast, the UDPGT activity towards *p*-phenylbenzoic acid appears before birth suggesting that it belongs to the late foetal cluster of substrates, i.e. group 1 [38]. In man, our knowledge about UDPGTs is scarce and no attempt has ever been made to characterize this enzyme in microsomal preparations until recently [18, 21, 24, 25]. Some of these data show that the same clusters of UDPGT activities found in mammals are present in human [25] even if some discrepancies appeared in the regulation processes, ontogenic development [39] and level of inducibility [18]. The lack of correlation between glucuronidation of bilirubin and clofibric acid in the human population tested here and their differential induction by clofibrate suggested that the formation of these ester glucuronides was catalyzed by separate forms of enzymes.* In rat, convincing evidence suggests that clofibric acid and bilirubin are conjugated by two different forms of enzyme. Gunn rats which are defective in bilirubin glucuronidation are able to conjugate clofibric acid [9]; clofibric acid was found to specifically induce bilirubin glucuronidation and not that of the hypolipidemic drug [40]. Finally we have previously reported [12] the absence of inhibition of bilirubin conjugation by 1 mM clofibric acid added in the incubation medium. Certainly, more studies are needed to characterize the formation of ester glucuronides in man.

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