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THE FORMATION OF β-GLUCURONIDASE RESISTANT GLUCURONIDES BY THE INTRAMOLECULAR REARRANGEMENT OF GLUCURONIC ACID CONJUGATES AT MILD ALKALINE pH

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Abstract—It is well known to carbohydrate chemists that substituted sugars may undergo facile rearrangement involving the migration of the aglycone from —OH to adjacent —OH. Despite the importance of glycoside conjugates, notably involving glucuronic acid, in the metabolism of xenobiotics, drug metabolism workers have neglected this phenomenon. The potential rearrangement of glucuronides from the biosynthetic C-1 isomers to other positional and stereo-isomers is important, since only 1-*O*-substituted β-D-glucosiduronates are substrates for β-glucuronidase, which is commonly used to identify such conjugates. The intramolecular rearrangement of clofibryl glucuronide has been studied over the pH range 5.2–8.6, by enzymic hydrolysis with β-glucuronidase, and by HPLC. The amount of clofibric acid released from the conjugate by β-glucuronidase falls with increasing pH of preincubation above pH 7.4, and this is accompanied by the appearance of three new peaks, each containing both clofibric and glucuronic acids, in the HPLC traces of the incubation mixtures. Similar experiments with three ether glucuronides, those of *p*-nitrophenol, phenolphthalein and 7-hydroxycoumarin, did not show any conversion to β-glucuronidase resistant forms. The phenomenon of intramolecular rearrangement of ester glucuronides must be considered whenever β-glucuronidase is used in the analysis of conjugates of carboxylic acids.

The conjugation of xenobiotics with glucuronic acid is a very commonly encountered metabolic reaction. Although an enormous variety of xenobiotic aglycones can be linked with glucuronic acid [1] the conjugates so formed always involve attachment of the aglycone to C-1 of glucuronic acid. Glucuronides of xenobiotics are generally identified by their susceptibility to β-glucuronidase, which is commercially available from mammalian and bacterial sources. This enzyme is specific for 1-*O*-substituted β-D-glucosiduronates and other stereo- and geometric isomers are not hydrolysed [2].

It has been known since 1920 [3, 4] that many substituted sugars can undergo intramolecular rearrangement in which aglycones migrate from —OH to adjacent —OH. Most examples involve acyl substituents, but ether migrations have been reported [4]. This phenomenon, although well known to carbohydrate chemists, has been largely ignored by drug metabolism workers. In the 1930s, there was a controversy as to the structure of benzoyl glucuronide: Pryde and Williams [5] showed it to be the 1-*O*-benzoyl isomer, but Quick [6] claimed that it was in fact 2-*O*-benzoyl glucuronide. The unequivocal synthesis of methyl 1-*O*-benzoyl-2,3,4-triacetyl glucuronate by Goebel [7] showed that the biosynthetic form was indeed 1-*O*-benzoyl glucuronide. However, little attention was paid to the possibility that ester glucuronides may undergo intramolecular rearrangement in biological media until the demonstration by Heirwegh, Compernolle and colleagues [8, 9] that bilirubin IXα monoglucuronide readily undergoes rearrangement at mild alkaline pH (*ca.* 8.5), leading to the formation of the 2-, 3- and 4-*O*-acyl glucuronides from the biosynthetic 1-*O*-acyl form.

The widely used hypolipidaemic drug clofibrate (ethyl *p*-chlorophenoxy*iso*butyrate) exerts its activity through its hydrolysis product clofibric acid [10]. Early studies [11] showed that the major metabolite of clofibrate was the ester glucuronide of clofibric acid, but several workers cast doubt upon this, since they were unable to cleave the conjugate completely with β-glucuronidase from a variety of sources [12, 13]. Faed [14] was able to separate by TLC two alkali-labile ester conjugates of clofibric acid from the urine and plasma of patients taking clofibrate, one of which was labile to β-glucuronidase, indicating it to be the 1-*O*-acyl glucuronide. Upon scanty evidence, the other conjugate was claimed to be an isomeric ester glucuronide, produced by an intramolecular rearrangement analogous to that reported for bilirubin IXα monoglucuronide by Heirwegh [8, 9].

This phenomenon of intramolecular rearrangement of ester glucuronides to β-glucuronidase resistant isomers might thus result in the mistaken identification of the conjugates of xenobiotic carboxylic acids. Our interest in the metabolic conjugation of clofibric acid [15, 16] and of carboxylic acids in general [17], together with the availability of an authen-

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tic sample of the ester glucuronide of clofibric acid shown by NMR to be the 1-*O*-acyl β-D-glucopyranosiduronate [16, 18] prompted a study of the possible intramolecular rearrangement at mild alkaline pH of this typical xenobiotic ester glucuronide. For comparison purposes, and to examine the possible aglycone dependence of this phenomenon, three ether glucuronides were also examined.

MATERIALS AND METHODS

Compounds

The following glucuronides were used: (a) ether type: phenolphthalein (Sigma Chemical Co., London, U.K.), *p*-nitrophenol (Koch-Light, Colnbrook, U.K.), 7-hydroxycoumarin (a biosynthetic sample isolated and characterised in this laboratory [19]); (b) ester type: clofibric acid (a sample isolated from rabbit urine and fully characterised by us [16, 18]). All were known to be the 1-*O*-substituted glucuronides. All other materials were the best available commercial grades.

High pressure liquid chromatography (HPLC)

This used a Waters U6K valve loop injector and Model 6000A pump with a Cecil 2012A variable wavelength detector and an LKB Redirac fraction collector. Columns were 100 × 5 mm packed with ODS-Hypersil (Shandon).

Assay of aglycones

Phenolphthalein. Addition of 9 vol. 0.4 M glycine buffer pH 10.4 and O.D. read at 550 nm (Pye SP30).

p-*Nitrophenol.* Addition of 9 vol. 0.4 M glycine buffer pH 10.4 and O.D. read at 400 nm.

7-*Hydroxycoumarin.* Sample adjusted to 40% methanol by the addition of methanol, centrifuged and 10 μl of the clear supernatant injected on to the HPLC column with a mobile phase of 40% methanol, flow rate 2 ml/min and the detector set at 325 nm. 7-Hydroxycoumarin had a retention time of 1.8 min.

Clofibric acid. Sample acidified to pH 1 with 3M HCl and extracted with 3 vol. *n*-hexane containing 1.2% isopropanol with shaking for 15 min. After centrifuging to separate the phases, the O.D. of the hexane layer was read at 226 nm.

The concentrations of the aglycones were determined in each case by reference to previously established calibration curves, which were linear over the appropriate concentration ranges.

pH-Dependent rearrangement of xenobiotic glucuronides

Solutions of the glucuronides of phenolphthalein and *p*-nitrophenol (80 μg/ml), 7-hydroxycoumarin (120 μg/ml), and clofibric acid (500 μg/ml) were incubated in 0.1 M Tris–maleate buffers pH 5.2–8.6 at 37° for 3–24 hr. Aliquots were then assayed as follows:

1. Assay of solution for aglycone liberated during the first incubation.

2. Adjustment to pH 5 with 0.2 M acetate buffer, addition of 5000 units of bovine liver β-glucuronidase (Ketodase, General Diagnostics, Eastleigh, U.K.) and incubation at 37° for a time previously shown to given complete hydrolysis (phenolphthalein 3 hr, *p*-nitrophenol 6 hr, 7-hydroxycoumarin 8 hr, and clofibric acid 4 hr). Following this the incubation mixture was assayed for aglycone liberated. In each case, a control incubation containing 2 mg saccharo-1,4-lactone (Sigma) was performed.

3. For clofibric acid only, an equal volume of 1 M NaOH was added and the whole heated to 37° for 4 hr, followed by assay for liberated aglycone.

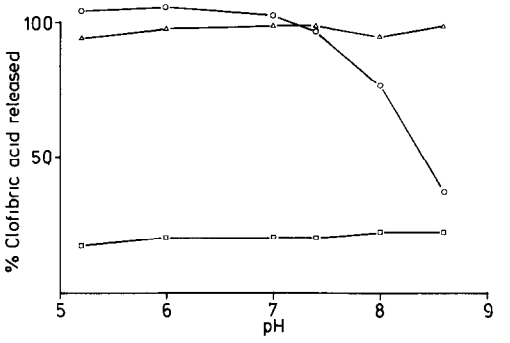

Fig. 1. The conversion of 1-*O*-clofibryl β-D-glucupyranosiduronate to β-glucuronidase resistant compounds by preincubation at various pHs. Details of the experiment are given in the text. The amount of clofibric acid released by the preincubation (□), alkali (△) and β-glucuronidase (○) after preincubation at the pHs indicated on the abscissa are shown by the appropriate curves.

RESULTS

Ether type glucuronides

When the three ether glucuronides were pre-incubated at pHs between 5.2 and 8.6 and subsequently treated with β-glucuronidase, in each case, the preincubation did not result in appreciable liberation of the aglycone, and had no effect on the lability of the β-glycosidic linkage to β-glucuronidase. The role of β-glucuronidase in the hydrolysis of these glucuronides was confirmed by its complete inhibition by saccharo-1,4-lactone (data not shown).

Ester type glucuronides

In contrast to the above, the preincubation of clofibryl glucuronide at various pHs between 5.2 and 8.6 had a profound effect on the subsequent lability of the ester link to β-glucuronidase. The results are presented in Fig. 1. The amount of clofibric acid liberated during the preincubation and subsequent assay, which involves acidification, was some 20% of the total, and this did not vary with the pH of the preincubation. Similarly, treatment of the preincubation mixtures with 1 M NaOH resulted in complete liberation of the clofibric acid, again independent of preincubation pH. However, the lability of clofibryl glucuronide towards β-glucuronidase varied markedly with preincubation pH, being essentially complete at pHs up to 7, but falling precipitously above this value. After a 3 hr preincubation at pH 8.6, only 15% of the total clofibryl glucuronide present was cleaved by β-glucuronidase.

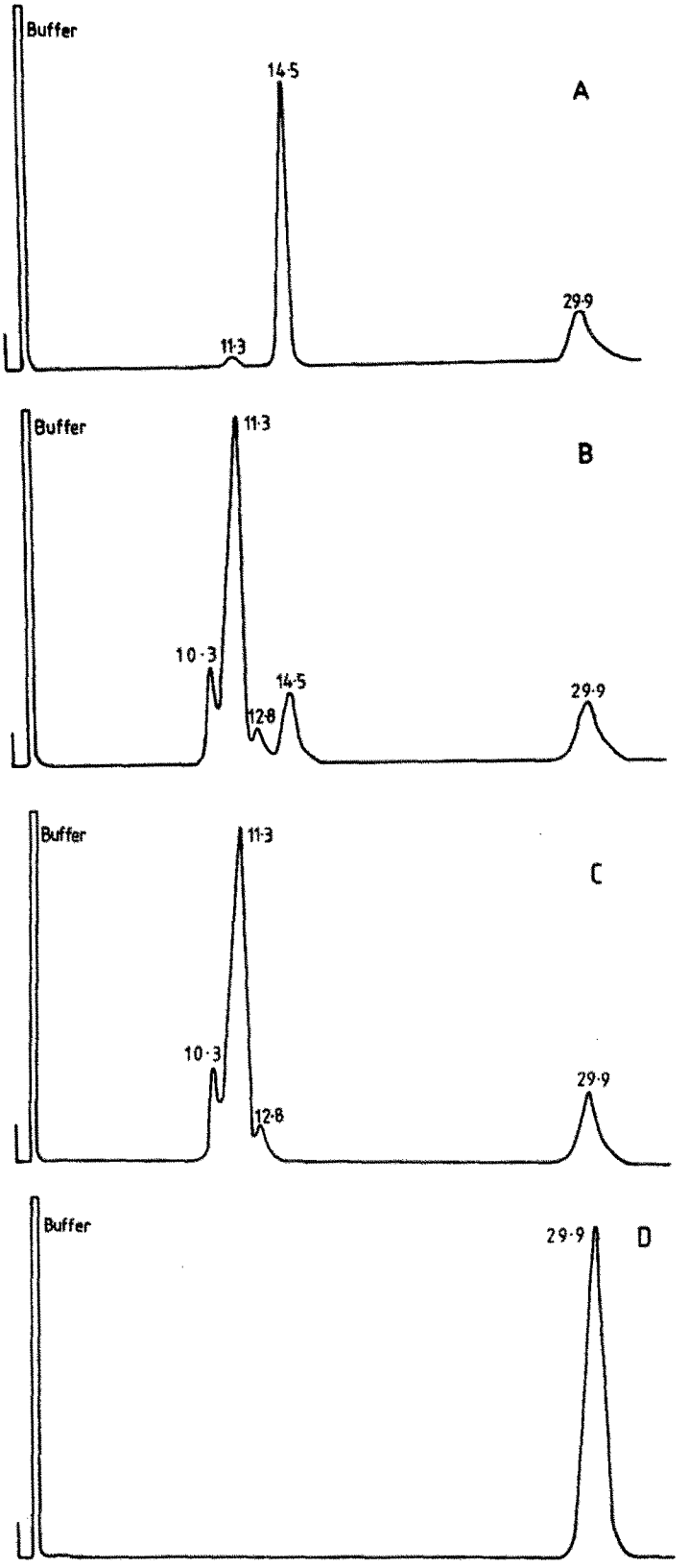

Fig. 2. HPLC separation of isomers of clofibryl glucuronide. Trace A: starting material; trace B: after preincubation at pH 8.6 at 37° for 3 hr; trace C: pH 8.6 preincubation mixture after subsequent treatment with β-glucuronidase; trace D: pH 8.6 preincubation mixture after subsequent treatment with 1 M NaOH. Details of the experiment and HPLC conditions are given in the text. Numbers next to the peaks on the traces are retention times (min).

HPLC of clofibryl glucuronide preincubation mixtures

Clofibryl glucuronide was examined by HPLC as such and after preincubation for 3 hr at pH 8.6. The mobile phase was 40% methanol containing 0.1% trifluoroacetic acid, flow rate 2 ml/min, with u.v. detection at 226 nm. The traces are presented in Fig. 2. The starting material (trace A) gave three u.v. absorbing peaks with retention times (min) 11.3 (very minor), 14.5 (major) and 29.9. The peak at 14.5 min disappeared on incubation with β-glucuronidase and that at 29.9 min, which corresponded to clofibric acid, increased (data not shown). This hydrolysis was blocked by saccharo-1,4-lactone. After preincubation at pH 8.6 as described, peaks appeared at 10.3 (minor) 11.3 (major) 12.5 (very minor) 14.5 and 29.9 min (trace B). Again the peak at 14.5 min disappeared upon treatment with β-glucuronidase (trace C) and this was inhibited by saccharo-1,4-lactone. β-Glucuronidase treatment for 16 hr had no effect on the other peaks in the chromatogram. The peaks at 10.3, 11.3, 12.5 and 14.5 min all gave a blue colour with naphtharesorcinol, indicating that each contained glucuronic acid [20]. Treatment of the pH 8.6 preincubation mixture with 1 M NaOH caused all the peaks to disappear with the exception of the clofibric acid peak at 29.9 min, which was markedly increased in size (trace D).

DISCUSSION

The results presented here show that the lability of the ester glucuronide of clofibric acid to β-glucuronidase is dependent upon the pH at which it has been maintained prior to enzyme treatment. Studies with three ether glucuronides have shown that they are stable in buffers of pH 5.2–8.6 at 37° for 24 hr, and that their subsequent hydrolysis by β-glucuronidase is unaffected by preincubation at any of these pHs.

The question arises as to the nature of the β-glucuronidase-resistant products of clofibryl glucuronide produced by preincubation at alkaline pH. Three new HPLC peaks appear in addition to clofibric acid and 1-*O*-clofibryl-β-D-glucopyranosiduronate, and these are all weak esters labile to mild alkali, giving rise to clofibric acid. They contain glucuronic acid as revealed by the naphtharesorcinol test but are resistant to β-glucuronidase. The evidence thus suggests that these new compounds are isomeric forms of clofibryl glucuronide, in which the aglycone is attached to either C-2, 3 or 4 of the glucuronic acid ring. The assignment of structures to these is not possible at present, as when the aglycone moves away from C-1, mutarotation is possible, as is the interconversion of pyranose, furanose and open chain forms. The formation of 3,6-lactones must also be considered. The number of possible isomers is thus considerable and elucidation of the structures of the 3 new forms of clofibryl glucuronide must await further study.

The phenomenon of intramolecular rearrangement of xenobiotic ester glucuronides to β-glucuronidase resistant forms has considerable implications for the analysis of these conjugates, and must be considered whenever apparently novel conjugates of acids are detected. Although studied here in buffer solutions, we have obtained similar results using urine of animals dosed with clofibric acid, and the rearrangement can occur during the collection of urine even when this is done over solid CO_2 [21]. A re-evaluation of results obtained with a range of carboxylic acids previously studied in this laboratory in the light of this work suggests strongly that the ester glucuronides of hydratropic [22], 1- and 2-naphthylacetic acids [23, 24], diphenylacetic [25], benzoic [26] and salicylic [26] acids all undergo at least partial rearrangement to β-glucuronidase resistant isomers, and that is especially noteworthy in alkaline urine. This was suggested by consistently higher values for the glucuronide obtained by alkaline hydrolysis compared with β-glucuronidase treatment. Since the completion of this work a report has appeared showing that the acyl glucuronide of probenecid excreted in human urine is a mixture of the C-1, -2, -3 and -4 isomers, the C-1 form predominating [27].

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