Biochemical Pharmacology, Vol. 46, No. 3, pp. 357–364, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

STUDIES ON THE *IN VITRO* REACTIVITY OF CLOFIBRYL AND FENOFIBRYL GLUCURONIDES

EVIDENCE FOR PROTEIN BINDING VIA A SCHIFF'S BASE MECHANISM

N. GRUBB,* A. WEIL† and J. CALDWELL‡

Department of Pharmacology and Toxicology, St. Mary's Hospital Medical School, Imperial College of Science, Technology and Medicine, London W2 1PG, U.K.

(*Received* 17 *February* 1993; *accepted* 10 *May* 1993)

Abstract—Clofibryl and fenofibryl acyl (ester) glucuronides (CAG and FAG) are major metabolites in humans of the hypolipidaemic drugs clofibrate and fenofibrate, respectively. We have investigated three inter-related aspects of the reactivity of CAG and FAG in human serum albumin (HSA) solution, human plasma and in buffer at pH 7.0: namely (a) rearrangement via acyl migration to glucuronic acid esters of clofibric acid (CA) and fenofibric acid (FA), (b) hydrolysis of the parent glucuronide and rearrangement products to yield CA and FA and (c) the formation of covalent adducts with albumin and plasma protein. CAG was more reactive than FAG in all media, especially the protein solutions. The reactivity of both glucuronides was accelerated in protein solution compared with buffer and this was more marked in plasma than in HSA solution. The predominant reaction during the initial stages of the incubation was formation of isomeric rearrangement products. In the protein solutions, CA and FA were the major reaction products after 24 hr, compared to the rearranged isomers in buffer. Protein binding of ^{14}C to HSA was markedly higher after incubation of CAG and FAG labelled on the glucuronyl moiety compared with the label on the aglycone. This is consistent with the covalent binding of CAG and FAG to protein proceeding via the formation of a Schiff's base rather than by transacylation.

Clofibrate and fenofibrate (Fig. 1) are two structurally related hypolipidaemic drugs which are rapidly and completely hydrolysed to their parent acids clofibric acid (CA§) and fenofibric acid (FA) after oral administration to humans. The use of clofibrate has been decreased due in large part to a short-term multi-centre study by the World Health Organization which showed a higher incidence of non-cardiac related deaths due mainly to diseases of the liver, gall bladder and intestine over the untreated high cholesterol control group [1]. Like many drugs bearing carboxyl groups, the active acid metabolites of these two fibrates are excreted extensively in the urine as their acyl glucuronides [2, 3]. Acyl glucuronides of xenobiotic and endobiotic carboxyl-containing molecules have a reactive potential due to the presence of a relatively electrophilic ester carbonyl carbon atom. Since the early 1980s, acyl glucuronides have received increasing attention. They are no longer perceived as the inert end-products of metabolism, but as potentially reactive intermediates and possible mediators of toxicity [4–7]. This reactivity is manifest in three ways: alkali-catalysed hydrolysis, acyl group migration and covalent protein binding [4, 6]. Intramolecular acyl group rearrangement is a well established reaction in carbohydrate chemistry [8] and is mechanistically related to alkaline hydrolysis. Migration of the acyl moiety occurs from the 1-carbon hydroxyl group to the neighbouring 2-, 3- and 4-hydroxyl groups of the glucuronyl moiety. This results in the formation of β-glucuronidase-resistant glucuronic acid esters. The mechanism is well established and proceeds via nucleophilic attack on the neighbouring hydroxyl group and formation of an ortho-ester intermediate [8, 9]. Migration between the three positional isomers is reversible but reformation of the parent 1-*O*-acyl glucuronide is very unlikely due to the mutarotation at C-1 after movement of the acyl group. Intramolecular acyl migration has been demonstrated for bilirubin glucuronide [10] and a number of xenobiotic acyl glucuronides [11–15] and appears to be a general reaction of acyl glucuronides. Both hydrolysis and rearrangement are accelerated at alkaline pH and with increasing temperature.

To date, *in vitro* irreversible protein binding has been demonstrated for drug glucuronides such as diflunisal [16], oxaprozin [17, 18], tolmetin [19], zomepirac [11], fenofibryl [20], carprofen [21] and bilirubin [22] glucuronides, and *in vivo* for clofibryl

* Present address: Wishard Memorial Hospital, Clinical Pharmacology Section, 1001 West Tenth Street, WOP 320, Indianapolis, IN 46202, U.S.A.

† Present address: Research and Development, Rhone Merieux, 31057 Toulouse, France.

‡ Corresponding author. Tel. (44)-71-723-1252; FAX (44)-71-723-7535.

§ Abbreviations: CAG, clofibryl acyl glucuronide; FAG, fenofibryl acyl glucuronide; CA, clofibric acid; FA, fenofibric acid; HSA, human serum albumin; HPP, human plasma protein; UDPGA, urine diphosphate glucuronic acid; FAB-MS, fast atom bombardment-mass spectrometry; CHAPS, 3-(3-chloramidopropyl)-dimethylammonio-1-propanesulphonate.

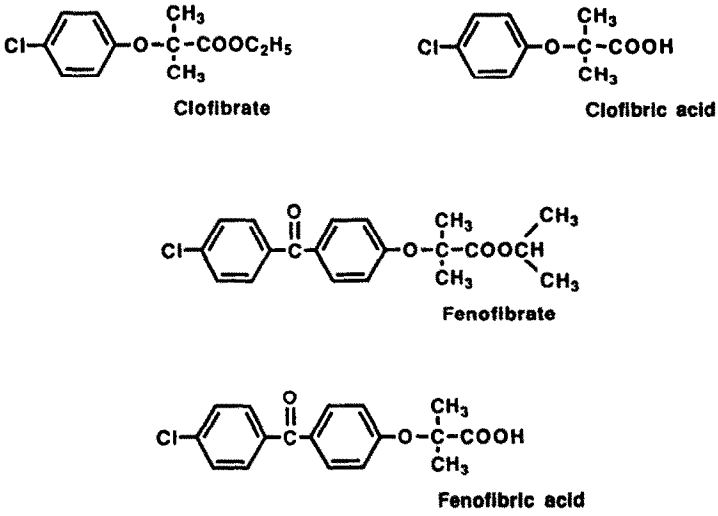

Fig. 1. Structures of clofibrate, CA, fenofibrate and FA.

[23], fenoprofen [24], diflunisal [25] and zomepirac [11] glucuronides. Two distinct mechanisms have been proposed for the covalent binding of xenobiotic acyl glucuronides to proteins and evidence has been produced supporting both. Van Breemen and Fenselau [26] suggested that binding occurs via trans-acylation following nucleophilic attack by a cysteine-sulfhydryl group on the glucuronide ester bond with loss of glucuronic acid. Evidence for the involvement of tyrosine hydroxyl [26] and lysine ε-amino [27] functional groups has subsequently been presented. In contrast Benet and co-workers provided evidence for the binding of zomepirac [28] and tolmetin [19] acyl glucuronides to albumin via the formation of a Schiff's base between a lysine amino group and the aldehyde group resulting from the ring opening of the glucuronyl moiety subsequent to migration of the acyl group from the 1-carbon atom. They suggested that the resulting imine may then undergo the Amadori rearrangement to form the more stable 1′-amino-1-deoxyketose product. Intramolecular acyl migration is a prerequisite for the reaction since it can only occur with rearranged isomers. At the completion of the reaction the glucuronyl moiety is covalently bound to the protein. Irreversible protein binding via Schiff's base formation has been tentatively suggested for carprofen [21], diflunisal [16] and valproic acid [29] acyl glucuronides.

The withdrawal from the market of drugs containing carboxylic acid groups, especially the 2-arylpropionic acid nonsteroidal anti-inflammatory drugs, is disproportionately high. Many adverse side-effects are gastrointestinal. For zomepirac and tolmetin, however, a high incidence of anaphylactic or anaphylactoid reactions was responsible for their withdrawal [30, 31]. Case reports have also described such reactions for diflunisal [32].

Chemical modification of protein via irreversible binding of acyl glucuronides has been suggested as a possible mediator of immunotoxic responses [33]. A current explanation for the different types of hypersensitivity reactions caused by drugs and other small molecules is the "hapten hypothesis" suggesting that formation of a drug–protein adduct can act as an antigen and thereby stimulate an immune response against the drug [34].

The present investigation was undertaken to determine the *in vitro* reactivity of clofibryl and fenofibryl acyl glucuronide (CAG and FAG) in buffer (pH 7.0), human serum albumin (HSA) solution (pH 7.0) and human plasma, and particularly to determine the extent and nature of *in vitro* covalent protein binding.

MATERIALS AND METHODS

[^{14}C]CA [2-(*p*-chlorophenoxy)-2-methylpropionic acid], specific activity 7.33 μCi/mg, radiochemical purity 99% by HPLC was a gift from the Radiochemistry Unit, ICI Pharmaceutical Division (Alderley Park, Cheshire, U.K.). [^{14}C]FA [4-(*p*-chlorobenzoyl)phenoxyisobutyric acid], specific activity 38.4 μCi/mg, radiochemical purity 99% by HPLC was a custom synthesis from Laboratoire de Chimie Pharmaceutique (UER des Sciences Pharmaceutiques et Biologiques, University de Grenoble, France). Uridine diphosphate [^{14}C]-glucuronic acid (UDPGA), specific activity 303.0 μCi/mmol, radiochemical purity 98% by HPLC and paper chromatography was purchased from NEN Research Products (Du Pont Ltd, Stevenage, U.K.). CA and FA for use as reference compounds were supplied by Aldrich Chemical Co. Ltd (Gillingham, U.K.) and Laboratoires Fournier (Dijon, France), respectively. Solvents (HPLC grade) and reagents (analytical grade) were obtained from commercial sources.

Isolation of CAG, [^{14}C]*CAG and* [^{14}C]*FAG.* CAG for use as a standard was isolated from the urine of rabbits dosed with CA and characterized as described previously [2]. Two female Dutch rabbits (weight 2.5 kg, Ranch Rabbits Ltd, Crawley, U.K.) were each dosed orally with a total of 6 g CA (2 g dissolved in 3 mL propylene glycol on 3 successive days). Urine was collected for 7 days in glass vessels containing glacial acetic acid (1 mL) over dry ice.

The bulked urine was centrifuged to remove solid matter and acidified (pH 3.0) with glacial acetic acid. The urine was then lyophilized and the residue extracted with methanol (3 × 200 mL), which was filtered and the solvent removed *in vacuo*. The residue was dissolved in water (80 mL), the pH adjusted to 2.0 with HCl (0.5 M) and the solution extracted with ether (3 × 200 mL). The bulked extracts were dried over anhydrous Na_2SO_4 and the ether evaporated *in vacuo*. The residue was dissolved in dry methanol (80 mL), 20 drops of KOH (1 M) were added and the volume reduced to about 40 mL by rotary evaporation. Cold dry ether was added to the chilled methanolic extract until it became cloudy. Upon standing overnight at −20°, the white precipitate which formed was filtered and recrystallized from ethanol/ether (1:1 by volume) to give *ca.* 1 g of clofibryl glucuronide. This was characterized by fast atom bombardment-mass spectrometry (FAB-MS), ^{1}H-NMR and reaction of the sugar conjugating agent with naphthoresorcinol as described previously [2]. The isolated compound had a retention time of 25 min in HPLC System 4 and upon hydrolysis with β-glucuronidase or mild alkali showed a concomitant appearance of a peak corresponding to CA (retention time 23.7 min).

[^{14}C]CAG was isolated from the 0–24 hr urine of a female Dutch rabbit given orally 1 g [^{14}C]CA (147 μCi) dissolved in propylene glycol (3 mL). Urine was collected in glass vessels containing 1 mL glacial acetic acid, over dry ice, then pooled, centrifuged briefly to remove solid matter and acidified (pH 2.0) with conc. HCl. [^{14}C]CAG was extracted by the method of Emudianughe *et al.* [2] and purified by HPLC. A 50 mL aliquot of pooled urine was washed with hexane (2 × 30 mL) to remove CA and extracted with ethyl acetate (4 × 20 mL). The ethyl acetate extracts were pooled, dried (anhyd. Na_2SO_4) and evaporated to dryness on the rotary evaporator. The residue was taken up in 2 mL of acetonitrile:0.05 M aqueous sodium acetate buffer pH 4.5 (1:3 by volume) and after clarification by centrifugation, subjected to semi preparative HPLC in System 1. Fractions containing CAG (retention time 9.0 min) were collected, combined and the acetonitrile removed on the rotary evaporator. The aqueous residue was then extracted with 3 vol ethyl acetate, the extract dried (anhyd. Na_2SO_4) and evaporated to dryness. The residue was taken up in the minimum volume of dry methanol and kept at −20°. The isolated compound had an identical retention time (25 min) on HPLC System 4 to that of the standard sample of CAG which disappeared upon treatment with mild alkali or β-glucuronidase with the concomitant appearance of CA (retention time 23.7 min).

[^{14}C]FAG was isolated from the 0–24 hr urine of human subjects dosed with [^{14}C]fenofibrate at the Hospital Cantonal, Geneva, Switzerland as described previously [3]. Eight healthy volunteers (four male, four female) took orally 300 mg [^{14}C]fenofibrate (66 μCi/subject). Aliquots of urine were subjected to solid phase extraction on C18 SepPak cartridges (Waters) which were eluted with methanol. The eluates were streaked on TLC plates and developed with $CHCl_3$/methanol/glacial acetic acid (20:80:1, by volume). Scanning for ^{14}C showed the presence of four ^{14}C zones, the major one (R_f 0.23, 75% of ^{14}C) giving a blue colour with naphthoresorcinol. This band was scraped from the plate and eluted with methanol, which was centrifuged (1000 *g*, 10 min) and evaporated to dryness *in vacuo*. The residue was taken up in methanol and aliquots (500 μL) subjected to semi-preparative HPLC in System 2. The fraction corresponding to FAG (retention time 8.1 min) was collected and the mobile phase removed *in vacuo*. Repetition of this procedure gave *ca.* 20 mg [^{14}C]FAG which was stored dry at −20°. The [^{14}C]FAG was characterized using FAB-MS, ^{1}H and ^{13}C-NMR [see Ref. 3], and HPLC before and after hydrolysis in System 3.

Biosynthesis of fenofibryl and clofibryl [^{14}C]*-glucuronides.* These glucuronides were prepared by incubation of CA or FA and UDP[^{14}C]GA with rabbit or human hepatic microsomes, respectively. The incubation mixtures contained the acid (20 mM), $MgCl_2$ (100 mM), UDP[^{14}C]GA (30 mM, 0.1 μCi per incubation), D-saccharic acid 1,4-lactone (0.5 mM) and microsomes (equivalent to 20 mg protein) in 75 mM Tris–HCl buffer pH 7.4, final volume 10 mL. The microsomes were solubilized prior to incubation with 8 mM CHAPS at 4° for 20 min. The mixture was incubated at 37° and the incubation terminated after 3 hr by the addition of 2 mL 0.1 M HCl. Precipitated protein was removed by centrifugation. The mixture was extracted with 3 × 20 mL ethyl acetate and the combined extracts evaporated *in vacuo* and the residue taken up in 50 μL 70% aqueous acetonitrile. HPLC analysis [System 3 (FAG) and 4 (CAG)] showed *ca.* 14% (FA) and 36% (CA) conversion of UDP[^{14}C]GA to the glucuronides, which were isolated as described above for urine.

Incubation of [^{14}C]*CAG,* [^{14}C]*FAG, C*[^{14}C]*AG and F*[^{14}C]*AG in buffer, albumin solution and plasma.* [^{14}C]FAG and [^{14}C]CAG (30 μg/mL, *ca.* 16,000 dpm/mL) were incubated (37°, 30 μg/mL) in duplicate in 0.1 M Tris/maleate buffer pH 7.0 at 37°, freshly collected heparinized human plasma (53.5 mg protein/mL) and a 3% (30 mg/mL, 0.5 mM) solution of HSA (fraction V, essentially fatty acid free, cat. no. A-1887, Sigma Chemical Co., Poole, U.K.) in 0.1 M phosphate buffer, pH 7.0, which was sterilized by ultrafiltration (0.22 μm). Incubations were initiated by the addition of the glucuronide to the prewarmed incubation medium. Two aliquots (1 mL) were removed immediately and then at 2 min, 1, 4, 8 and 24 hr for analysis by HPLC and covalently bound ^{14}C. C[^{14}C]AG and F[^{14}C]AG were incubated only with the albumin solution as described above.

Analysis of CAG and FAG isomers and covalently bound CA and FA. One millilitre aliquots of the buffer incubation were phased through C18 SepPak cartridges (Waters) which were eluted with 1 mL methanol. This was evaporated and the residue taken up in 200 μL HPLC mobile phase for analysis in HPLC System 3 ([^{14}C]FAG) and System 4 ([^{14}C]-CAG). Protein was precipitated from the albumin and plasma incubations by the addition of 3 mL acetonitrile followed by centrifugation (3000 *g* for

15 min). The acetonitrile supernatant was removed, concentrated under a stream of N_2 and the residue taken up in 200 μL HPLC mobile phase for analysis in HPLC System 3 or 4. The protein pellet was washed 10 times with 3 mL methanol/diethyl ether (3:1 by volume) to remove reversibly bound material. Each washing was counted for ^{14}C. The washed protein pellet was digested with 2 mL glacial acetic acid and the ^{14}C content determined after the addition of 15 mL Ecoscint scintillation fluid. Irreversibly bound radioactivity remaining after repeated washing was expressed as pmol CAG (FAG) equivalents bound/mg protein. The irreversible binding of [^{14}C]CA and [^{14}C]FA, *ca.* 30 μg/mL (*ca.* 20,000 dpm), to HSA and human plasma protein (HPP) was determined after incubation of the free acid with HSA solution or HPP for 24 hr as described above.

^{14}C in protein digests and solutions was assayed by liquid scintillation spectrometry using Packard TriCarb instruments Models 4640 or 4450 (Canberra-Packard, Pangbourne, U.K.). Quench correlation was achieved by reference to an external standard using a standard curve stored in the instrument which was established at regular intervals.

HPLC. HPLC employed a Shimadzu LC-4A liquid chromatograph, an SPD 2AS UV detector and C-R3A Chromatopac computing integrator (supplied by Dyson Instruments Ltd, Houghton-le-Spring, Tyne and Wear, U.K.). Samples were introduced on to the column with a Rheodyne 7125 valve loop injector. Radio HPLC used an LKB 2112 RediRac fraction collector (LKB Instruments, Croydon, U.K.) to collect fractions (0.5 mL) into minivials which were then counted for ^{14}C after the addition of 3 mL Ecoscint scintillation fluid. The following systems were employed:

System 1: column, Lichrosorb RP8 7 μ, 250 × 10 mm (E. Merck), UV detector at 254 nm, mobile phase acetonitrile/water 1:1 by volume containing 1% v/v glacial acetic acid, flow rate 3 mL/min, room temperature.

System 2: column, Lichrosorb RP18 7 μ, 250 × 10 mm (E. Merck), UV detector at 226 nm, mobile phase acetonitrile/water 70:30 by volume containing 0.1% v/v trifluoroacetic acid, flow rate 1.5 mL/min, room temperature.

System 3: column, Nucleosil C8 5 μ, 250 × 4.6 mm (SFCC, Gagny, France) cooled to 4°, UV detector at 290 nm, mobile phase acetonitrile/0.01 M phosphate buffer pH 7.5 containing 0.005 M tetrabutyl-ammonium bromide, flow rate 1 mL/min.

System 4: column, Lichrosorb RP18 7 μ, 250 × 4 mm (E. Merck), UV detection at 226 nm, gradient elution with mobile phase A acetonitrile/water 22:78 by volume, mobile phase B acetonitrile/water 40:60 by volume, both containing 0.005 M tetrabutyl ammonium hydroxide. Elution for 16 min with 100% A then a linear gradient over 16–26 min to 100% B, flow rate 2 mL/min, room temperature.

The retention times of compounds of interest are shown in Table 1.

Table 1. HPLC retention times of CA, FA, their glucuronides and rearrangement products

Compound	Retention time (min) in System 1	2	3	4
CA	—	—	—	23.7
Clofibryl glucuronide	9.0	—	—	25.0
FA	—	—	11.8	—
Fenofibryl glucuronide	—	8.1	8.8	—
Rearranged isomer A*	—	—	16.9†	26.6‡
Rearranged isomer B*	—	—	9.5†	15.6‡
Rearranged isomer C*	—	—	6.9†	8.7‡

HPLC systems are as described in the text.
* Assignment in order of appearance during incubation of the glucuronide.
† Rearrangement products of fenofibryl glucuronide.
‡ Rearrangement products of clofibryl glucuronide.

RESULTS

The profiles of rearrangement of CAG and FAG, initial concentration 30 μg/mL, to their acyl-migrated isomers and concomitant liberation of CA and FA by hydrolysis of C(F)AG and/or their isomers in buffer, pH 7.0, albumin and plasma solution (pH 7.0, 30 mM) are shown in Figs 2 and 3, respectively.

FAG was more stable than CAG in all media. The predominant reaction during the initial stages of the incubation in both plasma and HSA solution was formation of rearrangement isomers (73 and 72% after 2 min and 1 hr in plasma and HSA solution for CAG and 75 and 45% after 4 and 24 hr for FAG, respectively) which subsequently declined over the remainder of the incubation (9 and 5% after 24 hr in plasma and HSA solution, respectively for CAG and 29 and 45% for FAG). CA (85 and 92%) and FA (70 and 39%) were the predominant reaction products after 24 hr in plasma and HSA solution, respectively. In buffer, however, rearrangement isomers were the major reaction products after 24 hr (66% for CAG and 65% for FAG).

Binding of ^{14}C to protein after incubation of CAG and FAG with plasma and HSA solution was assumed to occur via a covalent binding mechanism since alkaline digestion of the precipitated protein liberated ^{14}C. Figure 4 shows the irreversible binding of [^{14}C]CAG and [^{14}C]FAG at initial concentrations of *ca.* 30 μg/mL to HPP and HSA expressed as pmol CA (FA) equivalents bound/mg protein. Binding of [^{14}C]CAG was greater than [^{14}C]FAG. Binding of [^{14}C]CAG reached a peak at 8 hr (111 and 123 pmol/mg protein for plasma and albumin, respectively) and subsequently declined (81 and 65 pmol/mg protein at 24 hr, respectively). Irreversible binding of [^{14}C]FAG increased throughout 24 hr (46 and 71 pmol/mg protein after 24 hr in plasma and albumin, respectively).

Irreversible protein binding of ^{14}C was markedly greater ($P < 0.001$, Student's *t*-test) at each time point after incubation of CA[^{14}C]G and FA[^{14}C]G at initial concentrations of *ca.* 30 μg/mL with albumin solution (Fig. 5) compared with [^{14}C]CAG and [^{14}C]FAG. The protein binding of C[^{14}C]AG and F[^{14}C]AG progressively increased during the entire incubation period (934 and 419 pmol/mg protein for plasma and albumin after 24 hr, respectively).

Irreversible binding after incubation of [^{14}C]CA

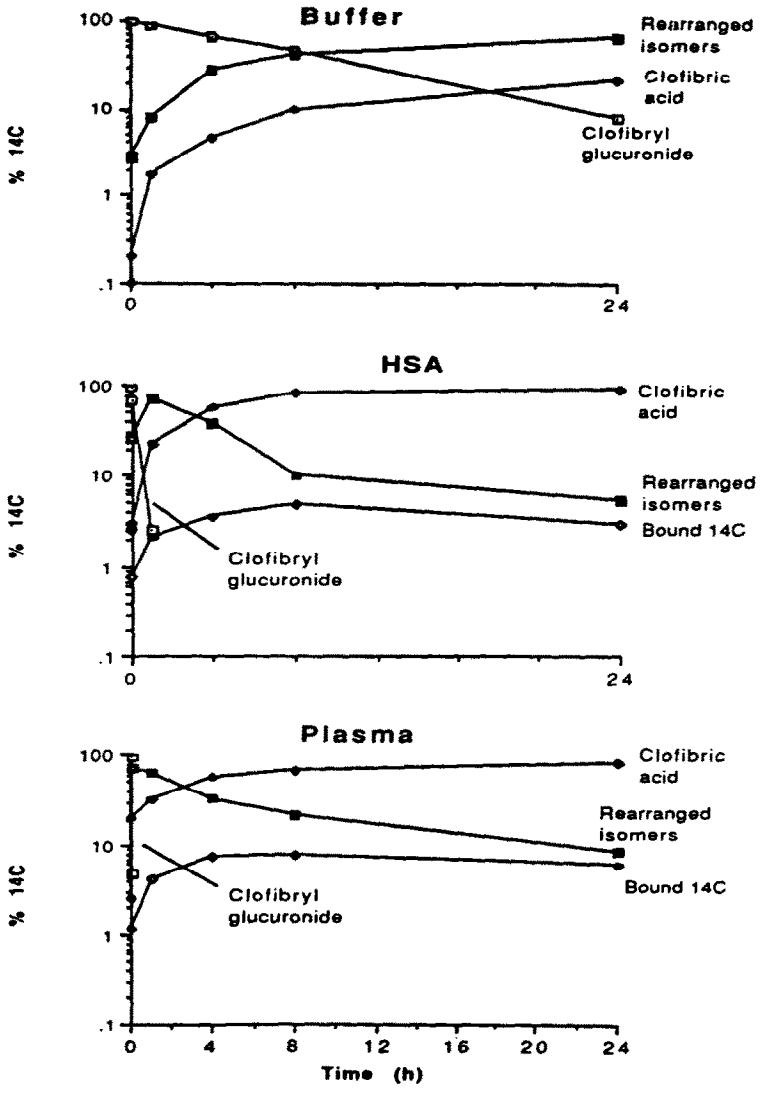

Fig. 2. Time courses of rearrangement, hydrolysis and covalent binding of [^{14}C]clofibryl glucuronide, initial concentration *ca.* 30 μg/mL at 37° in buffer (pH 7.0), HSA (pH 7.0, 0.5 mM) and human plasma.

with human plasma or HSA solution (37°, 24 hr) was 12.9 and 22.7 pmol/mg protein, respectively and for [^{14}C]FA, 8.3 and 9.4 pmol/mg protein, respectively.

DISCUSSION

This report describes the reactivity of CAG and FAG in buffer, pH 7.0, human plasma and HSA solution. The reactivity profiles (hydrolysis and rearrangement) were qualitatively similar to other acyl glucuronides studied (see the introduction) showing a decline in the glucuronide concentration with a concomitant increase in the concentrations of both the rearranged isomers and free acids. The reactivity of CAG and FAG in buffer was essentially identical to previous reports [35, 36].

Although there are several reports in the literature concerning acyl glucuronide reactivity towards protein (see the introduction), none of these concern phenoxyacetic acid glucuronides *in vitro* in protein solutions. CAG and FAG were more reactive in terms of hydrolysis and rearrangement in protein solution than most acyl glucuronides studied. This was markedly greater in protein solutions than in buffer. CAG was more reactive than FAG in all media, although this was much less marked in buffer than in the protein solutions. The marked susceptibility of CAG to nucleophilic attack has been demonstrated by its reactivity towards standard chemical nucleophiles [37]. The greater reactivity of CAG and FAG in plasma compared with HSA solution may in part be due to the presence of esterases.

The disappearance of CAG and FAG in all media followed first-order kinetics over the whole of the measurable reaction. The disappearance of the rearrangement isomers of CAG and FAG, however, was not first-order and was slower than the parent glucuronides. This is consistent with the current perception that, although reformation of the parent

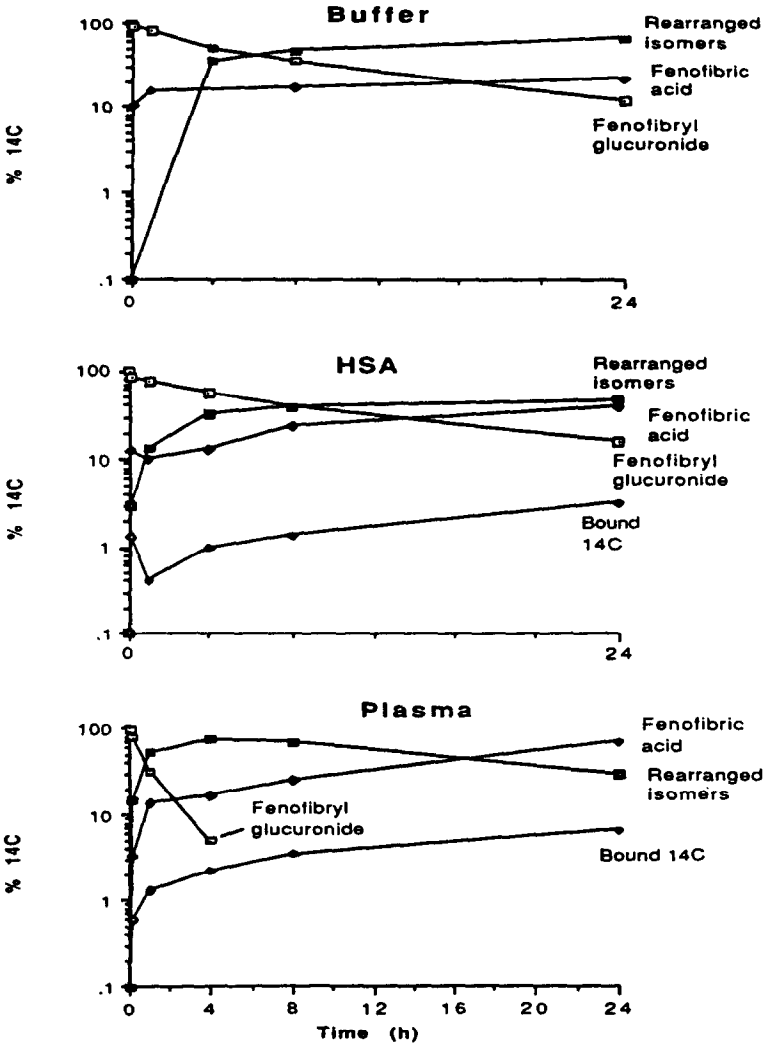

Fig. 3. Time course of rearrangement, hydrolysis and covalent binding of [^{14}C]fenofibryl glucuronide, initial concentration *ca.* 30 μg/mL at 37° in buffer (pH 7.0), HSA (pH 7.0, 0.5 mM) and human plasma.

glucuronide is impossible, the rearranged isomers are interconvertible.

Reports in the literature on the reactivity of acylglucuronides in albumin or plasma vary with the drug studied. The reactivity of the acyl glucuronides of diflunisal [16] and tolmetin [19] was retarded in the presence of HSA, while the reactivity of zomepirac [11] and oxaprozin [18] glucuronides was enhanced in the presence of blood, plasma and HSA, respectively. Dickinson and King [16] proposed that reversible binding of diflunisal occurred at a different site to, and, therefore, inhibited, irreversible binding. The relationship between reversible and irreversible protein binding is, however, unclear since there are few data on the reversible protein binding of acyl glucuronides. Irreversible protein binding of acyl glucuronides is well documented. It was first demonstrated for bilirubin glucuronide in 1966 [22] followed by several xenobiotic acyl glucuronides (see the introduction). The mechanism(s) of binding, site of attachment, and structures of the adducts remain, however, largely unknown.

The extents and profiles of the binding of [^{14}C]-CA and [^{14}C]FA to plasma and HSA, after incubation with CAG or FAG, are similar to that observed with other acyl glucuronides where binding of the aglycone has been measured [11, 16–20, 23]. Maximum binding of CAG was reached after 8 hr. Previous studies with acyl glucuronides [10, 18, 20] have shown that maximum binding of the acyl moiety to HSA peaks at 2–6 hr and subsequently declines slowly. This decline presumably results from hydrolysis of the protein adduct. Dickinson and King [16] have reported a biphasic decline of diflunisal–HSA adducts *in vitro* with a half-life of about 28 days.

The results of this study show higher irreversible protein binding of ^{14}C to protein after incubation with CAG or FAG labelled with ^{14}C on the glucuronyl moiety. Binding of ^{14}C to HSA was between 4.6- (1 hr) and 14.9- (24 hr) fold higher for CAG, and 2.2- (1 hr) and 5.9- (24 hr) fold higher for FAG when labelled on the glucuronyl moiety as compared with the aglycone. This is consistent with

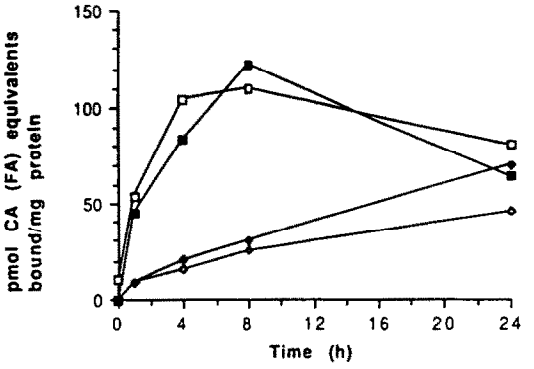

Fig. 4. Time courses of covalent binding of [^{14}C]clofibryl glucuronide (■) and [^{14}C]fenofibryl glucuronide (◆) to HSA (pH 7.0, 0.5 mM) and HPP (□, CAG and ◇, FAG), initial concentration *ca.* 30 μg/mL, 37°.

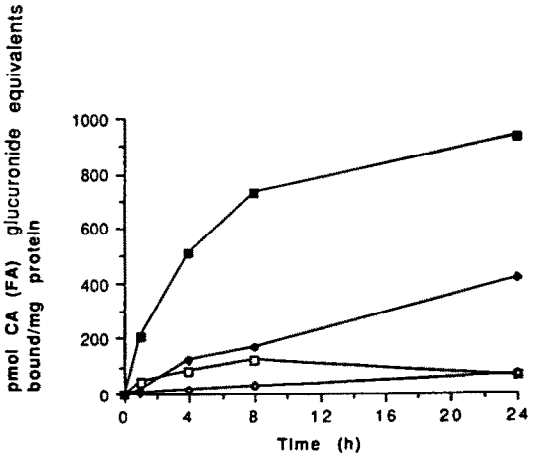

Fig. 5. Time courses of covalent binding of [^{14}C]clofibryl glucuronide (□), [^{14}C]fenofibryl glucuronide (◇), clofibryl [^{14}C]glucuronide (■) and fenofibryl [^{14}C]glucuronide (◆) to HSA (pH 7.0, 0.5 mM), initial concentration *ca.* 30 μg/mL, 37°.

the proposed Schiff's base mechanism in which the glucuronyl moiety becomes covalently bound to the protein [20, 28]. This may arise from hydrolysis of the glucuronic acid ester bond between the acyl group and the migrated glucuronide moiety with subsequent loss of CA or FA. Imine formation, in aqueous solution, is reversible [38, 39]. Hydrolysis may, therefore, occur before or after the Amadori rearrangement has taken place. This is corroborated by experiments using imine trapping reagents with zomepirac acyl glucuronide [28] which show that imine formation exceeds, several fold, the extent of irreversible binding.

It is noteworthy that binding to HSA was consistently higher with the radiolabel on the glucuronyl moiety compared with the aglycone (4.6-, 6.1-, 5.9- and 14.4-fold for CAG; 2.2-, 5.8-, 5.7- and 5.9-fold for FAG, at 1, 4, 8 and 24 hr, respectively). This suggests a similar rate of hydrolysis for the glucuronic acid ester bonds of rearranged CAG and FAG. Figures 2 and 3 suggest that the rate of hydrolysis of the rearrangement isomers appears to be similar when compared with the hydrolysis of the parent glucuronides.

Covalent protein binding of carboxyl containing drugs via their acyl glucuronides is currently attracting interest because of its potential to mediate toxic responses [26, 33]. Irreversible protein binding via acyl glucuronides has been suggested to cause hypersensitivity reactions to tolmetin [19] and zomepirac [11]. Binding is presumed to take place via the acyl glucuronide, however, binding via other reactive metabolites cannot be excluded. Benet, Spahn and co-workers [5, 21] proposed that the potential toxicity of acidic compounds may be predicted on (a) the ability of the corresponding acyl glucuronide to undergo acyl migration, (b) the plasma or tissue time profile of the glucuronide and (c) the stability of the glucuronide in plasma and tissues. The toxicological relevance of these protein adducts in general is uncertain. Protein binding of acyl glucuronides *in vivo* will depend on a number of factors i.e. the half-life of the glucuronide and may well be a general reaction of acyl glucuronides. It is interesting to note that two classes of adduct are formed, those of the glucuronide itself and those from which the aglycone has been lost. These latter will be common to all acyl glucuronides which form adducts. The relative immunogenicities of these remain unclear but it is conceivable that there may be two classes of protein conjugates, one aglycone-specific and one general to all acyl glucuronides. Since the immunological reactions to carboxylic acid drugs are seemingly drug-specific, it would appear that the latter are not antigenic.

In summary, these results support the growing body of evidence demonstrating that the reactions of hydrolysis, acyl group rearrangement and irreversible protein binding are general reactions of acyl glucuronides and furthermore provide evidence for irreversible protein binding of CAG and FAG via imine formation and subsequent Amadori rearrangement.

REFERENCES

1. Oliver MF, Geizeron H, Gyartas I, Heady JA, Morris JN and Strasser T, A cooperative trial in the primary prevention of ischemic heart disease using clofibrate: report from the Committee of Principal Investigators. *Br Heart J* **40**: 1069–1118, 1978.
2. Emudianughe TS, Caldwell J, Sinclair KA and Smith RL, Species differences in the metabolic conjugation of clofibric acid and clofibrate in laboratory animals and man. *Drug Metab Dispos* **11**: 97–102, 1983.
3. Weil A, Caldwell J and Strolin-Benedetti M, The metabolism and disposition of ^{14}C-fenofibrate in human volunteers. *Drug Metab Dispos* **18**: 115–120, 1990.
4. Caldwell J, Sinclair K and Weil A, Acylation of amino acids and other endobiotics by xenobiotic acids. In: *Metabolism of Xenobiotics* (Eds. Gorrod JW, Oelschlager H and Caldwell J), pp. 217–224. Taylor and Francis, London, 1988.
5. Spahn-Langguth H and Benet LZ, Acyl glucuronides revisited: is the glucuronidation process a toxification as well as a detoxification mechanism? *Drug Metab Rev* **24**: 5–48, 1992.
6. Caldwell J, Grugg N, Sinclair KA, Hutt AJ, Weil A

and Fournel-Gigleux S, Structural and stereochemical aspects of acyl glucuronide formation and reactivity. In: *Cellular and Molecular Aspects of Glucuronidation* (Eds. Siest G, Magdalou J and Burchell B), pp. 185–192. John Libbey Eurotext Ltd, 1988.
7. Faed EM, Properties of acyl glucuronides: implications for studies of the pharmacokinetics and metabolism of acidic drugs. *Drug Metab Rev* **15**: 1213–1249, 1984.
8. Haines AH. Relative reactivities of hydroxyl groups in carbohydrates. *Adv Carbohydr Chem Biochem* **33**: 11–109, 1976.
9. Bradlow G, Kan L and Fenselau C, Studies of intramolecular rearrangements of the acyl-linked glucuronides using salicylic acid, and (*S*)- and (*R*)-benoxaprofen and conformation of isomerization in acyl-linked delta 9-11-carboxytetrahydrocannabinol glucuronide. *Chem Res Toxicol* **2**: 316–324, 1989.
10. Compernolle F, Blanckaert N and Heirwegh K, The fate of bilirubin-IXα-glucuronides in cholestatic bile: sequential migration of the 1-acylaglycone to the 2, 3 and 4-positions of glucuronic acid. *Biochem Soc Trans* **5**: 317, 1977.
11. Smith PC, Hesagawa J, Langendijk PNJ and Benet LZ, Stability of acyl glucuronides in blood, plasma and urine: studies with zomepirac. *Drug Metab Dispos* 110–112, 1985.
12. Dickinson RG, Hooper WD and Eadie MJ, pH-dependent rearrangement of the biosynthetic ester glucuronide of valproic acid to β-glucuronidase-resistant forms. *Drug Metab Dispos* **12**: 247–252, 1984.
13. Hynek ML, Munafo A and Benet LZ, Effect of pH on acyl migration and hydrolysis of tolmetin glucuronide. *Drug Metab Dispos* **16**: 322–324, 1988.
14. Hasagawa J, Smith PC and Benet LZ, Apparent intramolecular acyl migration of zomepirac glucuronide. *Drug Metab Dispos* **10**: 469–473, 1982.
15. Sinclair KA and Caldwell J, The formation of β-glucuronidase resistant glucuronides by the intramolecular rearrangement of glucuronic acid conjugates at mild alkaline pH. *Biochem Pharmacol* **31**: 953–957, 1982.
16. Dickinson RG and King AR, Studies on the reactivity of acyl glucuronides—II. Interaction of diflunisal acyl glucuronide and its isomers with human serum albumin *in vitro*. *Biochem Pharmacol* **42**: 2301–2306, 1991.
17. Wells DS, Janssen FW and Ruelius HW, Interactions between oxaprozin glucuronide and human serum albumin. *Xenobiotica* **17**: 1437–1449, 1987.
18. Ruelius HW, Kirkman SK, Young EM and Janssen FW, Reactions of oxaprozin-1-*O*-acyl glucuronide in solutions of human plasma and albumin. In: *Biological Reactive Intermediates, Proceedings of the Third International Symposium* (Eds. Kocsis JJ, Jollow DJ, Witmer CM, Nelson JO and Snyder R), pp. 431–442. Plenum Press, New York, 1986.
19. Munafo A, McDonagh AF, Smith PC and Benet LZ, Irreversible binding of tolmetin glucuronic acid esters to albumin *in vitro*. *Pharm Res* 7: 21–27, 1990.
20. Weil A, Guichard JP and Caldwell J, Interactions between fenofibryl glucuronide and human serum albumin or human plasma. In: *Cellular and Molecular Aspects of Glucuronidation* (Eds. Siest G, Magdalou J and Burchell B), pp. 233–236. John Libbey Eurotext Ltd, 1988.
21. Benet LZ and Spahn H, Acyl migration and covalent binding of drug glucuronides—potential toxicity mediators. In: *Cellular and Molecular Aspects of Glucuronidation* (Eds. G Siest, J Magdalou and B Burchell), pp. 262–269. John Libbey Eurotext Ltd, 1988.
22. Gautam A, Seligson H, Gordon ER, Seligson D and Boyer JL, Irreversible binding of conjugated bilirubin to albumin in cholestatic rats. *J Clin Invest* **73**: 873–877, 1984.
23. Sallustio BC, Knights KM, Roberts BJ and Zacert R, *In vivo* covalent binding of clofibric acid to plasma proteins and rat liver proteins. *Biochem Pharmacol* **42**: 1421–1425, 1991.
24. Volland C, Sun H, Dammeyer J and Benet, Stereoselective degredation of the flurbiprofen acyl glucuronide enantiomers and irreversible binding to plasma protein. *Drug Metab Dispos* **19**: 1080–1086, 1991.
25. McKinnon GE and Dickinson RG, Covalent binding of diflunisal and probenecid to plasma protein in humans: persistence of the adducts in the circulation. *Res Commun Pathol Pharmacol* **66**: 339–354, 1989.
26. Van Breemen RB and Fenselau C, Acylation of albumin by 1-*O*-acyl glucuronides. *Drug Metab Dispos* **13**: 318–320, 1985.
27. Van Breemen RB, Fenselau C, Mogilevsky W and Odell GB, Reaction of bilirubin glucuronides with serum albumin. *J Chromatogr* **383**: 387–392, 1986.
28. Smith PC, Benet LZ and McDonagh AF, Covalent binding of zomepirac glucuronide to proteins: evidence for a Schiff base mechanism. *Drug Metab Dispos* **18**: 639–644, 1990.
29. Williams AM, Worrall S, De Jersey J and Dickinson RG, Studies on the reactivity of acyl glucuronides—III. Glucuronide-derived adducts of valproic acid and plasma protein and anti-adduct antibodies in humans. *Biochem Pharmacol* **43**: 745–755, 1992.
30. Samuel SA, Apparent anaphylactic reaction to zomepirac (Zomax). *N Engl J Med* **304**: 978, 1981.
31. Bretza JA and Novey HS, Anaphylactoid reactions to tolmetin after interrupted dosage. *West J Med* **145**: 55–59, 1985.
32. Cook DJ, Achong MR and Murphy FR, Three cases of diflunisal hypersensitivity. *Can Med Assoc J* **138**: 1020–1029, 1988.
33. Smith PC, McDonagh AF and Benet LZ, Irreversible binding of zomepirac to plasma protein *in vitro* and *in vivo*. *J Clin Invest* **77**: 934–939, 1986.
34. Park BK and Kitteringham NR, Drug-protein conjugation and its immunological consequences. *Drug Metab Rev* **22**: 87–144, 1990.
35. Sinclair KA, Studies on the metabolic conjugation of aryloxycarboxylic acids. PhD Thesis, University of London, 1986.
36. Weil A, Métabolisme du fénofibrate chez l'animal et l'homme. Thèse de Doctorat de l'Université Paris 6, 1987.
37. Van Breemen RB and Fenselau C, Reaction of 1-*O*-acyl glucuronides with 4-(*p*-nitrobenzyl)pyridine. *Drug Metab Dispos* **14**: 197–201, 1986.
38. Flier JS, Underhill LH, Brownslee M, Cerami A and Vlassara H, Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N Engl J Med* **318**: 1315–1321, 1988.
39. Higgins PJ and Bonn HF, Kinetic analysis of the nonenzymatic glycosylation of hemoglobin. *J Biol Chem* **256**: 5204–5208, 1981.