Malaysian Journal of Pharmaceutical Sciences,
Vol. 5, No. 1, 33–43 (2007)

IN VITRO IRREVERSIBLE BINDING AND DEGRADATION OF (R)- AND (S)-KETOPROFEN GLUCURONIDES TO PLASMA PROTEINS

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This study describes the in vitro degradation studies of the diastereomeric ketoprofen glucuronides, under physiological conditions (pH 7.4, 37°C, (R)-ketoprofen glucuronide $t_{½}$ = 30 min, (S)-ketoprofen glucuronide $t_{½}$ = 70 min) and the irreversible binding of diastereomeric ketoprofen glucuronides (15 μg/ml) to human serum albumin (HSA) (289 μM) and human plasma under physiological conditions (pH 7.4, 37°C). The (R)-ketoprofen glucuronide irreversibly bound to a greater extent in both human plasma and human serum albumin. This is the reverse to that found in previous studies. These findings further support the hypothesis that faster degradation of 1-O-acyl glucuronide (in this case the (R)-diastereomer) is associated with a greater extent of irreversible binding.

Keywords: *Ketoprofen, Ketoprofen glucuronide, Irreversible binding, Human serum albumin, Plasma*

INTRODUCTION

Acylglucuronidation plays a significant role in the elimination of many chiral non-steroidal anti-inflammatory drugs (NSAIDs) including ketoprofen [(R,S)-2-(3-benzoylphenyl) propionic acid, see Fig. 1 for structures] (Upton *et al.* 1980), carprofen (Rubio *et al.* 1980), fenoprofen (Rubin *et al.* 1972), ibuprofen (Castillo and Smith 1995), naproxen (Vree *et al.* 1993) and ximoprofen (Mayo *et al.* 1990). Chiral 2-arylpropionic acid NSAIDs are generally marketed as a racemic mixture although most of their potency is due to the S-enantiomer (Muller *et al.* 1990). Conjugation to the glucuronide produces a strongly acidic compound which, in most cases, is more water-soluble than its precursor and hence more readily eliminated at physiological pH. However, rather than leading to the abolition of biological activity of the parent compound, it appears that the process may have pharmacological and toxicological consequences (Faed 1984; Spahn-Lannguth and Benet 1992; Hayball 1995). Due to these conjugates being susceptible to nucleophilic attack, hydrolysis and intermolecular rearrangement, they are unstable under physiological conditions (Sinclair and Caldwell 1982; Dickinson *et al.* 1984; Smith *et al.* 1985). In addition, they may also bind covalently to biological macromolecules leading to irreversible binding. Such irreversible binding has been reported *in vitro* for a number of drugs including

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fenoprofen (Volland, Sun and Dammeyer 1991), ibuprofen (Castillo and Smith, 1995), and zomepirac (Smith, Mcdonagh and Benet 1986). This irreversible binding, while generally considered to be a minor pathway could account for drug toxicity as it can have a significant effect on the drug disposition such as metabolic clearance, and protein and tissue binding. It has been proposed that the formation of isomeric acyl glucuronides is a prerequisite for covalent binding (Spahn-Lannguth and Benet 1992) and the reaction rate of the degradation of 1-O-acyl glucuronides gives an indication of the covalent binding of 1-O-acyl glucuronides to proteins. Hence, the faster the rate of degradation the greater extent of reversible binding (Ebner *et al.* 1999). We were interested if this rational could be applied to the enantiomers of ketoprofen and the corresponding acyl glucuronides.

Previous research on the irreversible binding of acyl glucuronides, *in vitro*, of racemic and the separate diastereomers of the acyl glucuronides of ketoprofen to HSA showed that the two diastereomers had different reactivity. With the maximum yield of the glucuronide from the (S)-ketoprofen was twice that of its antipode (Dubois *et al.* 1993; Presle *et al.* 1996). This is unexpected as a faster degradation rate of (R)-ketoprofen glucuronide has been previously observed (Hayball, Nation and Bochner 1992) and therefore it is expected, based on the relationship between degradation and irreversible binding (Ebner *et al.* 1999), that (R)-ketoprofen glucuronide would irreversibly bind to a greater extent. To clear up this discrepancy, we investigated the relationship between the degradation of the diastereomers of ketoprofen glucuronide at physiological pH and their extent of irreversible binding to plasma and HSA.

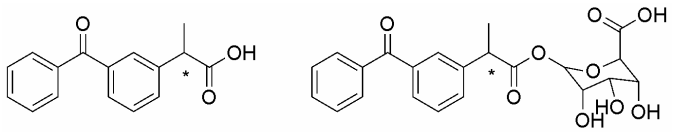


Fig. 1: Structures of ketoprofen and 1-O-acyl ketoprofen glucuronide, *denotes chiral centre in the aglycone.

MATERIALS AND METHOD

Ketoprofen was obtained from the Menari Laboratorios. Tetrabutyl ammonium hydrogen sulfate and HSA were purchased from Sigma. Acetonitrile, ether and methanol were of chromatographic grade (BDH Chemicals).

HPLC analysis and sample collection was performed on a system consisting of a Model M-45 pump, WISP 712 injector (Waters Associates Inc.), SPD-6A UV spectrophotometric detector (Shimadzu) and a Powerchrom version 2.0.7. integrator. The columns were Waters analytical and semi-preparative SymmetryPrep™ C_{18} 7 μm (3.9 × 150 mm and 7.8 × 300 mm). Compounds were detected at 254 nm.

Isolation of (R)- and (S)-Ketoprofen Glucuronides

A single dosage of racemic ketoprofen (Orudis, 200 mg) was given to a human subject. Urine was collected 2.5 h after administration, acidified to pH 3.0 with glacial acetic acid and cooled to 0°C. The glucuronides were extracted from the urine with ethyl acetate (4 × 25 ml). The extracts were concentrated and the (R)- and (S)-ketoprofen glucuronides separated by HPLC [semi-preparative column, mobile phase: acetonitrile/25 mM aqueous phosphate buffer (32:68 v/v), containing tetrabutyl ammonium hydrogen sulfate (20 mM), pH 3.0, 4.2 ml/min (S)-ketoprofen glucuronide t_R = 8.0 min, (R)-ketoprofen glucuronides t_R = 9.0 min]. The glucuronides were separately collected, freeze dried, residues taken up in acetonitrile/water (32:68 v/v, 1 ml) and were separately further purified [semi-preparative column, acetonitrile/water (32:68 v/v, pH 3.0, orthophosphoric acid)]. The glucuronides were separately collected and freeze dried [(R)-ketoprofen glucuronide > 99% diastereomeric excess (d.e.) (and (S)-ketoprofen glucuronides > 99% d.e., (analytical HPLC, mobile phase: acetonitrile/25 mM aqueous phosphate buffer (32:68 v/v), containing tetrabutyl ammonium hydrogen sulfate (20 mM), pH 3.0, 1 ml/min)]. The pure diastereomers were stored at –80°C in a glycine buffer (1 M, 1 ml, pH 3.0), concentrations of 1.125 × 10^{-2} M and 5 × 10^{-3} M for the (R)- and (S)- ketoprofen glucuronide/glycine buffer solutions respectively.

Degradation Studies of (R)- and (S)-Ketoprofen Glucuronides in Aqueous Phosphate Buffer (pH 7.4) at 37°C

(R)-ketoprofen glucuronide (50 μl, 1.125 × 10^{-2} M, glycine buffer 1 M, pH 3) was dissolved in aqueous phosphate buffer (950 μl, pH 7.4). An aliquot (30 μl) of this solution was taken and immediately quenched with mobile phase [60 μl, acetonitrile/aqueous phosphate buffer (32:68 v/v, pH 3.0), total volume of 90 μl]. The remaining sample (920 μl) was incubated at 37°C and aliquots (30 μl) were taken and quenched with mobile phase at timed

intervals of 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, and 8 h and analysed by analytical HPLC (30 µl inject). The remaining solution (60 µl) was hydrolysed with β-glucuronidase enzyme [50 µl enzyme (1000 units/ml) + 50 µl aqueous phosphate buffer] at 37°C for 1 h, this HPLC trace showed the total rearrangment isomers (which are unaffected by the enzyme) plus the quantity of ketoprofen which is formed by enzyme catalysed hydrolysis of the (R)-ketoprofen glucuronide. The resulting solution was analysed by analytical HPLC (30 µl inject). An identical procedure was followed for the (S)-ketoprofen glucuronide.

In vitro Irreversible Binding of the (R)- and (S)-Ketoprofen Glucuronide to HSA

(R)- and (S)-Ketoprofen glucuronides (15 µg/ml, 35 µM) were separately incubated with HSA solutions (290 µM in 0.067 M phosphate buffer pH 7.4) at 37°C. Aliquots (200 µl) were taken at 0 and 10 min, and at 1.5, 3, 6, and 24 h. These aliquots were placed in eppendorf tubes (1.5 ml capacity) and a solution of acetonitrile/phosphoric acid (30:0.01 v/v, 300 µl) was immediately added to precipitate the proteins. After the aliquots were centrifuged the protein pellets were washed with methanol/ether (3:1, 5 × 1 ml) (the aliquots were sonicated for 5 min before removal of the methanol/ether solution, to ensure all the reversibly bound glucuronide was extracted). Analytical HPLC of the last supernatant confirmed that all the reversibly bound ketoprofen glucuronides were removed from the precipitated protein. The protein pellets were completely hydrolyzed (no change in d.e. of the ketoprofen glucuronides was observed) with NaOH (2 M, 1 ml) and heating of the solution at 60°C for 4 h. Analytical HPLC showed that the diastereomeric purity of the glucuronides had been maintained. Acidification of this solution (phosphoric acid, 200 µl) followed by extraction with ether (2 ml) and removal of the solvent by a stream of nitrogen gave the irreversibly bound glucuronides. The remaining residues were reconstituted in 100 µl of acetonitrile/water (32:68 v/v, pH 3, phosphoric acid) and analysed by analytical HPLC.

In vitro Irreversible Binding of the (R)- and (S)-Ketoprofen Glucuronide to Plasma Proteins

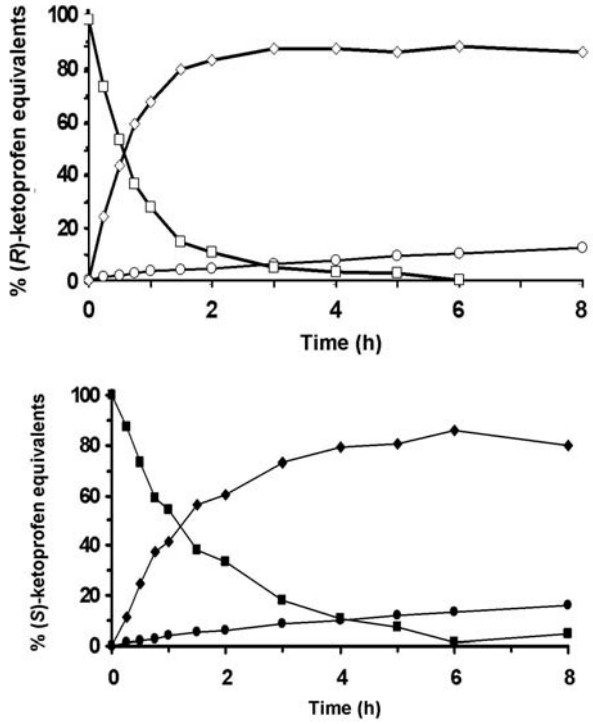
Separate (R)- and (S)-ketoprofen glucuronides (15 µg/ml, 35 µM) were incubated with human plasma (2 ml) at 37°C. Aliquots (200 µl) were taken at times of 0 and 10 min, and at 1.5, 3, 6, and 24 h. These aliquots were placed in

eppendorf tubes (1.5 ml capacity) and a solution of acetonitrile/phosphoric acid (30:0.01, v/v, 300 µl) was immediately added to precipitate the proteins. After the aliquots were centrifuged the protein pellets were washed with methanol/ether (3:1, 5 × 1 ml) (the aliquots were sonicated for 5 min before removal of the methanol/ether solution). Analytical HPLC of the last supernatant confirmed that all the reversibly bound ketoprofen glucuronides were removed from the precipitated protein. The protein pellets were completely hydrolyzed with NaOH (2 M, 1 ml) and heating of the solution at 60°C for 4 h. Acidification of this solution (phosphoric acid, 200 µl) followed by extraction with ether (2 ml) and removal of the solvent by a stream of nitrogen gave the irreversibly bound glucuronides. The remaining residues were reconstituted in 100 µl of acetonitrile/water (32:68 v/v, pH 3, phosphoric acid) and analysed by analytical HPLC.

RESULTS AND DISCUSSION

Ketoprofen glucuronide was isolated from human urine, which is a convenient source for this metabolite as approximately 70% of the administered dose is excreted as the glucuronide metabolite (Hayball, Nation and Bochner 1992; Skordi *et al.* 2004). The diastereomers were separated and purified by HPLC analysis to give the desired diastereomer in greater than a 99% d.e.

Incubation of the separate diastereomers of ketoprofen glucuronide in aqueous phosphate buffer (pH 7.4) resulted in the expected formation of the parent compound and the migration isomers. (R)-ketoprofen glucuronide ($t_{½}$ = 30 min) degraded quicker than (S)-ketoprofen glucuronide ($t_{½}$ = 70 min) which is in agreement with previous degradation studies (Hayball, Nation and Bochner 1992). The results are represented in Figure 2.



(a)

(b)

Fig. 2: (a) Degradation of (R)-ketoprofen or (b) (S)-ketoprofen glucuronide (pH 7.4) at 37°C. Biosynthetic glucuronide (■, ❒), migration isomers (◆, ◊) and ketoprofen (●, ❍).

The *in vitro* irreversible binding of (R)- and (S)-ketoprofen glucuronide to HSA and plasma proteins was examined under conditions described in the materials and methods section. In all experiments it was ensured that all the reversibly bound glucuronide was removed prior to hydrolysis of the protein pellets. The diastereomeric purity of the glucuronides did not change when the protein pellets were hydrolyzed. The time dependency graph of the (R)- and (S)-ketoprofen glucuronides, (n = 5), bound to plasma is shown in Figure 3. For both diastereomers, the acylation kinetics observed display a typical pattern. In each case, the amount of irreversibly bound ketoprofen glucuronide increased in a time dependent manner, reached a maximum value, and then decreased the maximum value was achieved after 3 to 6 h. The (R)-diastereomer irreversibly bound to a greater extent than the (S)-diastereomer with a maximum of 5.1 ± 0.7% of (S)-ketoprofen glucuronide irreversible bound and 5.4 ± 0.9% of (R)-ketoprofen

glucuronide were irreversibly bound after 6 h. The results obtained for each diastereomer were statistically different (two-way ANOVA, $p < 0.05$).

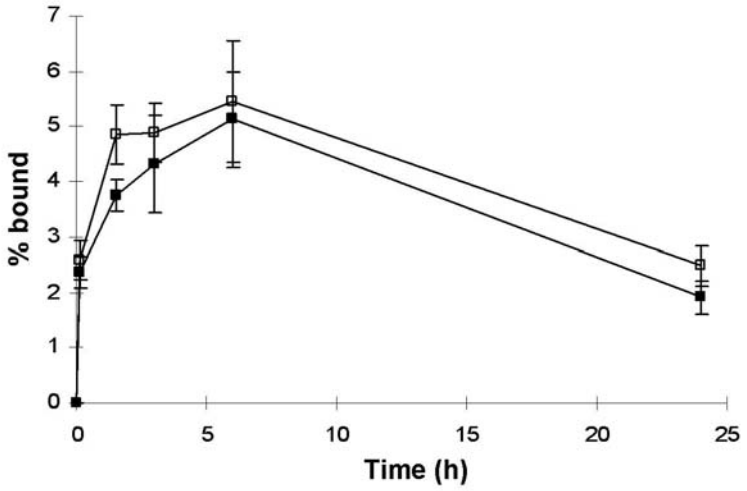


Fig. 3: Time dependent irreversible binding for (R)- and (S)-ketoprofen glucuronide (± SE) in plasma, (n = 5, statistically different, two-way ANOVA, $p < 0.05$). (R)-ketoprofen (❒) and (S)-ketoprofen (■).

In the HSA experiments both diastereomers (at protein concentration of 290 μM, n = 6), exhibited similar kinetics to those observed for the plasma binding (see Figure 4). For each diastereomer the amount of irreversibly bound ketoprofen glucuronide increased in a time dependent manner, reaching a maximum value and then decreasing. The diastereomeric glucuronides had different reactivities with the (R)-diastereomer irreversibly bound to a greater extent than the (S)-diastereomer. A maximum of 2.6 ± 0.4% of the (S)-ketoprofen glucuronide was irreversibly bound after 3 h whereas 3.4 ± 0.6% of (R)-ketoprofen glucuronide was irreversibly bound after 6 h. However, in this case the results were not statistically different (two-way ANOVA, $p < 0.1$).

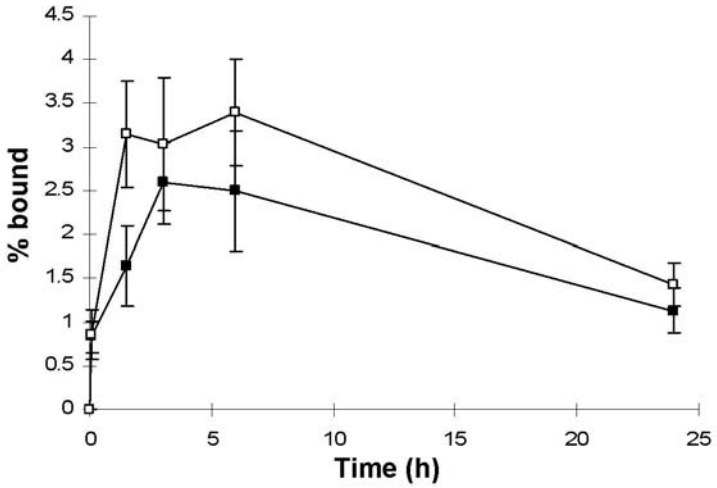


Fig. 4: Time dependent irreversible binding for (R)- and (S)-ketoprofen glucuronide (± SE) in HSA(n = 6, not statistically different, two-way ANOVA, $p < 0.1$). (R)-ketoprofen (❒) and (S)-ketoprofen (■).

The (R)- and (S)-glucuronides of the ketoprofen showed different extents of irreversible binding in HSA and human plasma. The (R)-ketoprofen glucuronide showed preferential irreversible binding, in both HSA and human plasma. The binding of the separate diastereomers of ketoprofen glucuronide to plasma were statistically different ($n = 5$, $p < 0.05$), whereas the binding of the separate diastereomers to HSA were not statistically significant ($n = 6$, $p < 0.1$). This statistical discrepancy could be because of HSA, which is essentially fatty acid free, is purified and therefore influencing the extent of irreversible binding to albumin. In all binding studies the amount of glucuronide bound reached a maximum value and then decreased as time increased which is consistent to that found by Presle *et al*. (1996).

The (S)-ketoprofen glucuronide, in comparison to its diastereomer, has previously exhibited greater reversible binding (Hayball *et al.* 1992) and one would therefore expect it to irreversible bind to a lesser extent than its antipode. This is because reversible and irreversible binding are mutually exclusive processes and hence the greater the extent of reversible binding the lesser the extent of irreversible binding. This is consistent with our experimental results that observed that the (S)-diastereomer irreversibly binds to a lesser extent in both human plasma and HSA than the (R)-diastereomer. However our findings are the reverse to that found by Presle *et al*. (1996), where they observed that it is the (R)-diastereomer irreversibly

bound to a lesser extent in HSA than the (S)-diastereomer. This discrepancy could simply be due to the difference in the concentration of HSA and glucuronide. Therefore at low concentration of ketoprofen glucuronides (15 μg/ml) the (S)-diastereomer irreversibly binds to a lesser extent than the (R)-diastereomer whereas at high concentration of ketoprofen glucuronides (145 μg/ml) the (R)-diastereomer irreversibly binds to a lesser extent than the (S)-diastereomer.

CONCLUSION

In conclusion, we have found that the (R)-ketoprofen glucuronide has a larger degradation rate than the (S)-diastereomer, in aqueous phosphate buffer at pH 7.4 and 37°C. The (R)-ketoprofen glucuronide also shows preferential irreversible binding, in both HSA and human plasma to the (S)-diastereomer. These findings are the reversal to those previously published. However these findings further strengthen the hypothesis by Ebner *et al.* (1999) that the faster the degradation of 1-O-acyl glucuronides, the greater is the extent of irreversible binding.

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