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The Journal of Pharmacology and Experimental Therapeutics

Supplemental Data

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Supplemental Methods.

Supplemental Figures 1-3.

Supplemental Tables 1-5.

Supplemental Methods

Isomerisation and Hydrolysis of Diclofenac 1-β Acyl Glucuronide in Phosphate Buffer, HSA Solution and Human Plasma. Diclofenac 1-β acyl glucuronide (AG; final concentration, 400 μM or 2 mM) was incubated in triplicate independent experiments at 37^{o}C in either 0.1 M potassium phosphate buffer (pH 7.4), a solution of human serum albumin (HSA; 40 μM; 2.66 mg/ml) in the phosphate buffer or pooled human plasma (protein concentration, 48.63 mg/ml). Because tautomeric hydroxyimine/ketoamine glycation adducts of lysyl ε-amino groups can to labile to organic nucleophiles (Acharya and Sussman, 1984), the buffer composition is a potentially important consideration. Hence a phosphate buffer rather than a Tris buffer was used. The buffer, HSA solutions and plasma were equilibrated at 37°C for 20 min before addition of the AG (3 mM in 0.1 M phosphate buffer, pH 7.4; solution prepared immediately before use). Equal volumes of buffer were added to the 400-μM and 2-mM incubations of AG with plasma. Protein concentrations were determined throughout by the Bradford assay (Bradford, 1976). Aliquots of the incubations were removed at zero time and at intervals between 1 min and 16 h. For the incubations in buffer alone, further degradation of AG in the aliquots (99 μl) was stopped by rapid addition of 1 μl of orthophosphoric acid (Kenny et al., 2004). The acidified solutions were analysed immediately by liquid chromatography with UV detection (LC-UV). For the incubations in HSA solution and human plasma, two volumes of ice-cold acetonitrile containing 2% acetic acid (v/v) and one volume of ice-cold ethanol were added immediately and sequentially to the aliquots (100 μl). The final mixture was vortexed briefly. Precipitated protein was pelleted by centrifugation at 14,000 rpm and 4°C. The supernatant was removed, evaporated to dryness under nitrogen at 37^{o}C and reconstituted in 100 μl of 0.1 M potassium phosphate buffer, pH 7.4, containing 1% orthophosphoric acid (v/v) for immediate LC-UV analysis.

LC-UV Analysis of Diclofenac 1-β AG Isomerisation and Hydrolysis. Diclofenac 1-β AG, its α-anomer, the positional isomers and the free drug were resolved on a Zorbax Eclipse XDB-C8 column (150 × 4.6 mm, 5μm; Agilent Technologies, Santa Clara, CA) fitted with a C8 SecurityGuard guard column (Phenomenex, Macclesfield, Cheshire, U.K.). The Summit LC-UV system comprised an ASI-100 automated sample injector, a P580 pump and a UVD170S UV detector (Dionex Ltd., Macclesfield, Cheshire, U.K.). Single aliquots of the samples (20 μl of those derived from phosphate buffer incubations; 40 μl of those derived from HSA and plasma incubations) were eluted at room temperature, at 0.9 ml/min, with a linear gradient of methanol-acetonitrile (9:1, v/v) in 10 mM ammonium acetate, pH 4.5: 15-67.5% over 75 min. The column was purged with 90% methanol-acetonitrile mixture (9:1, v/v) for 10 min and re-equilibrated for 10 min. The absorbance of eluted compounds was monitored at 254 nm. It was assumed diclofenac, the 1-β AG and its anomeric and positional isomers have the same molar extinction coefficient at 254 nm; the isomolar absorbency of other non-steroidal anti-inflammatory drugs (NSAIDs) and their AG at the relevant analytical wavelengths having been reported previously (Dickinson and King, 1991; Terrier et al., 1999). The isomolar absorbency of diclofenac and its AG conjugates (1-β AG and isomers) was confirmed by showing that the total chromatographic peak areas of analyte(s) in an acid-stabilised (1% orthophosphoric acid) 2-mM solution of 1-β AG and a 2-mM solution after it had been incubated at pH 7.4 and 37^{o}C for 90 min and then acidified (Fig. 2A) were equal (100-μl aliquots were spiked with 10 μl of 10-mM aqueous zomepirac as internal standard (IS) before the LC-UV analysis). Analyte peak areas were integrated using Chromeleon software (Dionex Ltd.), and represented individually as a percentage of the combined peak areas of the 1-β AG, isomers and aglycone. No peaks were detected on the UV chromatograms of samples derived from either blank buffered HSA solution or pooled human plasma that interfered with the analyte peaks. A peak at the retention time of an analyte that had a signal-to-noise ratio of less than three was considered to be below the limit of detection and was omitted from the analysis. The 1-β AG and its positional isomers in analytical samples were stable throughout their storage in the autosampler at room temperature: when zero-time and 120-min samples were re-analysed at the end of a batch, the

analyte profiles were unchanged (<5% variance of analyte:IS peak-area ratio for 1-β diclofenac AG, its regioisomers and aglycone). The identities of the chromatographic peaks corresponding to diclofenac and the 1-β AG from their retention times were confirmed by liquid chromatography-mass spectrometer (LC-MS). The resolved AG isomers (1α-, 2-, 3- and 4-*O*-acyl) yielded qualitatively identical positive-ion electrospray mass spectra – dominated by neutral loss of dehydroglucuronic acid – that were indistinguishable from the spectrum of the 1-β AG. The positional isomers (2-, 3- and 4-*O*-acyl) were assigned conventionally from their chronological order of appearance in phosphate buffer at pH 7.4 (Fig. 2B). The minor peak immediately in front of diclofenac 1-β AG was assigned to the 1α-anomer by chromatographic comparisons with the fully stereochemically characterised 1-*O*-acyl anomers of other AG (Corcoran et al., 2001; Akira et al., 2002). Furthermore, the order of the isomers' elution (4-, 1α-, 1β-, 3-, 2-*O*-acyl) is the most frequently observed elution order for AG isomers chromatographed on reversed-phase columns (Iwaki et al., 1999; Corcoran et al., 2001; Akira et al., 2002; Stachulski et al., 2006; Ebner et al., 2010). Although complete or near complete separation of the positional isomers was achieved, anomeric separation, as is generally reported, was accomplished only in the case of the 1-*O*-acyl isomer. Even chromatographic resolution of the α- and β-anomers of 1-*O*-acyl glucuronides is not attained invariably (Ebner et al., 1999). No attempt was made to obtain additional resolution of the AG anomers, which is often incomplete and associated with distorted peak shapes due to rapid inter-conversion of the anomers (Stachulski et al., 2006).

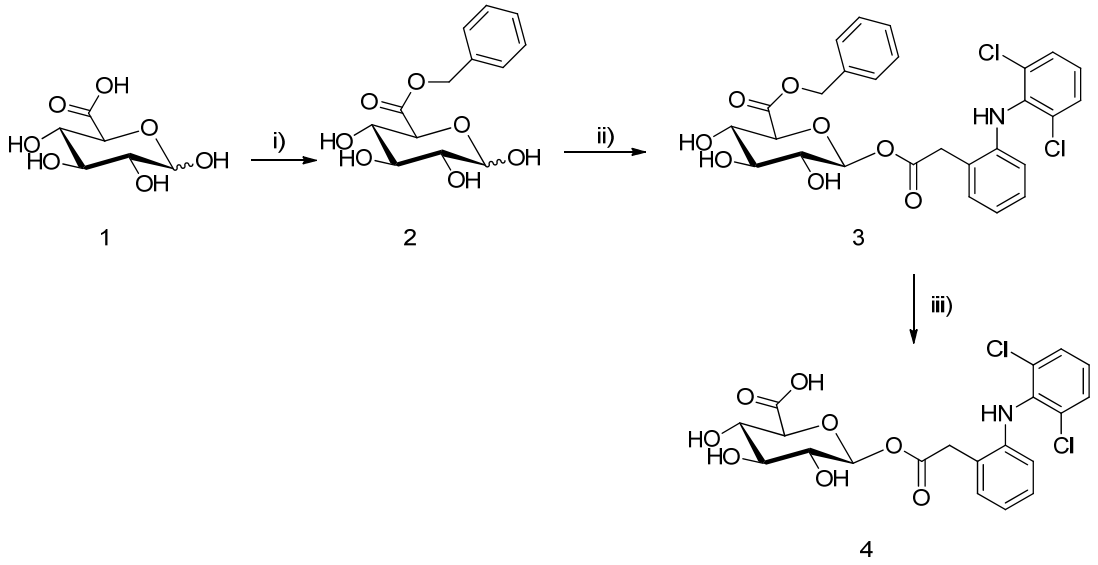
Covalent Binding of Diclofenac Residues to HSA Incubated with 1-β AG In Vitro. Diclofenac residues bound covalently to HSA that had been incubated with synthetic diclofenac 1-β AG were assayed following non-specific hydrolytic deconjugation. The liberated drug was assumed to derive from glycation structures and acyl residues on protein side chains (Fig.1). The adduct hydrolysis and diclofenac extraction methods were developed from those used to measure the covalent protein binding of tolmetin AG (Hyneck et al., 1988; Ding et al., 1995); another unstable NSAID conjugate (Stachulski et al., 2006) that forms a combination of glycation and acyl adducts on HSA in vitro (Ding et al., 1995). The AG (150 μM and 750 μM) was incubated in triplicate with HSA (15 μM) in 0.1 M potassium phosphate buffer, pH 7.4, at 37°C. Aliquots (500 μl) were removed at intervals between 5 min and 16 h. The protein was precipitated immediately by sequential addition of ice-cold acetonitrile (1.5 ml) and ice-cold isopropanol (0.5 ml), and separated by centrifugation at 2500 rpm. Non-covalently bound drug was washed from the protein pellet through vortex mixing with seven 5-ml volumes of methanol-diethyl ether (3:1, v/v). The 6^{th} and 7^{th} extracts were retained, evaporated to dryness under nitrogen at 37°C and reconstituted in 250 μl of ammonium acetate (500 mM, pH 4.5) – acetonitrile (3:1, v/v) for LC-MS/MS analysis. No diclofenac was detected in these extracts. The fully extracted protein pellet was dried under a stream of nitrogen at 37°C, dissolved in KOH (0.25 M, 1 ml) at room temperature and incubated at 80°C for 90 min. An aliquot of the solution (20 μL) was removed for protein assay (Bradford, 1976) after cooling. Protein recovery was 64.71±10.91% (mean±SD, n=204). The remaining hydrolysate was acidified with orthophosphoric acid (43%, v/v; 45 μl). Zomepirac (100 μM in acetonitrile-water (1:1, v/v); 55 μl) was added as IS, and the mixture was vortexed. Ethyl acetate (10 ml) was added. The combination was mixed thoroughly and centrifuged (2500 rpm, 5 min). An aliquot of the upper layer (8 ml) was removed, evaporated to dryness under nitrogen at 37°C and reconstituted in 250 μl of ammonium acetate (500 mM, pH 4.5) – acetonitrile (3:1, v/v) for LC-MS/MS quantification of liberated diclofenac. These values were normalised to the 10-ml volume of ethyl acetate. Single aliquots (25 μl) of the analytical samples, calibration standards and quality control standards were eluted at room temperature, at 1.0 ml/min, from a Zorbax Eclipse XDB-C18 column (150 × 2.1

mm, 5 μm) connected to a PerkinElmer series 200 HPLC system (PerkinElmer, Norwalk, CT) and an API 2000 triple quadrupole mass spectrometer (AB Sciex, Warrington, U.K.). The eluent was a linear gradient of methanol in 8.5 mM ammonium acetate containing 0.0075% formic acid (v/v): 65-85% over 7 min. The column was purged with 90% methanol for 1 min and re-equilibrated for 3 min. MS operating parameters for the multiple reaction monitoring (MRM) assay of diclofenac are shown in Supplemental Table 1. Calibration (0.05-10 μM; 6 calibration points) and quality control (0.05, 0.5 and 5.0 μM; triplicates) standards were prepared by spiking diclofenac in 0.25 M KOH into HSA solution (15 μM, 0.5 ml, in 0.1 M potassium phosphate, pH 7.4). The solutions were evaporated to dryness under a stream of nitrogen. The residues were treated with 0.25 M KOH, the resulting digests acidified, zomepirac IS added and the solutions extracted with ethyl acetate as described above. Analyte (R_t 6 min) and IS (R_t 3.8 min) peaks in MRM chromatograms were integrated by Analyst software (AB Sciex). Drug concentrations in analytical samples were estimated from batch-specific linear regression lines (r^2=0. 0.9982 ± 0.0011; mean ± SD, n=3) on plots of area ratios (diclofenac/IS) against diclofenac concentrations. All the analytical concentrations were within the range 0.104-1.59 μM. The lower limit of detection of diclofenac (peak signal-to-noise ratio > 3) spiked into the buffered HSA solution before alkaline incubation was 0.01 μM. The corresponding lower limit of quantification, defined by accuracy between 80-120% and precision (coefficient of variation) < 20%, was 0.1 μM (accuracy, 90.7%; precision, 17.3%). An assay was accepted if more than six of the nine quality control standards (high, medium and low concentrations) were within 85-115% of value and at least one standard of each concentration was within the acceptable variation. The buffered HSA solutions did not contain any materials that interfered with the analyte and IS peaks in the MRM channels selected for this assay. Covalent binding of diclofenac was normalized to the protein content for each analytical sample, and was expressed as nmol of drug bound per μmol of HSA (molecular weight used, 66 kDa).

LC-MS/MS Analysis of Diclofenac and Diclofenac AG in Clinical Plasma Samples. The stored acidified plasma samples were thawed at room temperature and processed immediately. To 50-µl aliquots of plasma, zomepirac IS (3 µM, 10 µl) was added in acetonitrile-water (1:1, v/v) containing 0.1% formic acid. Protein was precipitated with four volumes of ice-cold acetonitrile. Precipitated protein was pelleted by centrifugation at 14,000*g* for 5 min at 4°C. The supernatant was passed through a 0.45-µm Multiscreen Solvinert PTFE filter plate according to the manufacturer's instructions (Millipore Ltd, Cork, Republic of Ireland). The filtrate was evaporated to dryness at 37^{o}C under nitrogen, and reconstituted in 60 µl of acetonitrile-water (1:1, v/v) containing 0.1% formic acid. Single aliquots (10 µl) were eluted at room temperature, at 0.21 ml/min, from a Zorbax Eclipse XDB-C18 column (150 × 2.1 mm, 5 µm) connected to a Dionex Ultimate 3000 HPLC system and a 4000 QTRAP hybrid quadrupole mass spectrometer (AB Sciex). Samples were maintained at 4°C in the autosampler. The eluent was a linear gradient of acetonitrile containing 0.1% formic acid in water containing 0.1% formic acid: 50-95% over 10 min. Under these conditions diclofenac 1-β AG and its isomers eluted as a single peak (R_t 2.7 min); the R_t of diclofenac and zomepirac were 7 min and 5.2 min, respectively. MS operating parameters for the MRM analyses of diclofenac and diclofenac AG are shown in Supplemental Table 2. Calibration (duplicate) and quality control (quintuplicate) standards were prepared by spiking aqueous solutions of diclofenac (final concentrations: calibrators, 50-1000 nM, 10 calibration points; quality controls, 50, 500 and 1000 nM), 1-β AG (final concentrations: calibrators, 30-1000 nM, 11 calibration points; quality controls, 30, 500 and 1000 nM) and zomepirac (final concentration, 500 nM) into pooled blank plasma. The samples were processed and analysed as described above. Analyte and IS peaks in MRM chromatograms were integrated by Analyst software. Drug and AG concentrations in analytical samples were estimated from batch-specific linear regression lines (diclofenac: r^2=0.9906 ± 0.0069, mean ± SD, n=5; AG: r^2=0.9867 ± 0.0194, mean ± SD, n=5) on plots of area ratios (analyte/IS) against analyte concentrations. The lower limit of detection of diclofenac and the 1-β AG (signal-to-noise ratio >3) spiked into pooled human plasma was 10 nM. The lower limit of quantification (accuracy, 80-120%; precision, <20% coefficient of variation) was 50 nM (accuracy,

93.2%; precision, 15.9%) for diclofenac and 30 nM (accuracy, 108.7%; precision, 3.9%) for the AG. The assay was accepted if the mean accuracy, and precision as measured by coefficient of variance, of all quality control samples was ≤ ± 15% of the actual value, excepting the lower limit of quantification which was ≤ ± 20% the actual value. No endogenous or artefactual materials interfering with analyte and IS signals in the selected MRM channels were found in control plasma.

Synthesis of diclofenac 1-β acyl glucuronide. The glucuronide was synthesized by S.N.L.B. using a modified form of the method of Bowkett et al. (2007) as shown in Supplemental Scheme 1.



i) 1M TBAF in THF, benzyl bromide, DMF, 0°C, 18h; ii) diclofenac, NMM, HATU, ACN, 0°C; iii) 10% Pd/C, cyclohexa 1,4-diene, IPA/THF(v/v,1:1), 60°C, 1h.

Supplemental Scheme 1.

Benzyl bromide (5.15 mL, 43.27 mmol) was added dropwise over 5 min, with stirring, to a solution of D-glucuronic acid **1** (8 g, 41.21 mmol) in DMF (50 mL) and 1 M TBAF in THF (45.3 mL, 45.33 mmol) cooled to 0°C under nitrogen. The resulting solution was left for 18 h, during which time it was allowed to warm to ambient temperature.

The solvents were firstly evaporated and then co-evaporated with toluene (100 mL × 4) to give an orange oil containing residual DMF. The oil was triturated with toluene (200 mL), the solvent decanted, and the crude product was purified by flash-column silica chromatography by loading it onto the column with DCM and performing gradient elution with 0-10% MeOH in EtOAc. Pure fractions were evaporated to dryness to afford benzyl α, β-D-glucuronate **2** (8.5 g, 72.6%) as a colourless gum.

N-methylmorpholine (1.431 mL, 13.02 mmol) was added dropwise to diclofenac (1.927 g, 6.51 mmol), benzyl glucuronate **2** (1.85 g, 6.51 mmol) and HATU (2.475 g, 6.51 mmol) in acetonitrile (25 mL) under nitrogen (mildly exothermic reaction, 20-30°C). The resulting pale yellow solution was stirred at ambient temperature for 18 h. The reaction was monitored by TLC (silica plates/EtOAc): all of the starting acid was consumed. The solution was evaporated to a dark orange oil. The crude product was purified by flash-column silica chromatography (loaded in DCM to aid solubility), using gradient elution with 60-100% EtOAc in isohexane. Fractions containing the product (seen as a blue spot on the TLC plate) were evaporated to a yellow oil. TLC revealed a non-UV active impurity; the ^{1}H NMR spectrum of the material appeared to contain an impurity peak at 2.75 ppm. Flash-column chromatography was repeated, using elution with 80% EtOAc in isohexane. Pure fractions were evaporated to dryness to afford diclofenac acyl glucuronide benzyl ester **3** (400 mg, 11%) as a pale yellow oil. The material's ^{1}H NMR spectrum was consistent with a pure 1-β anomer.

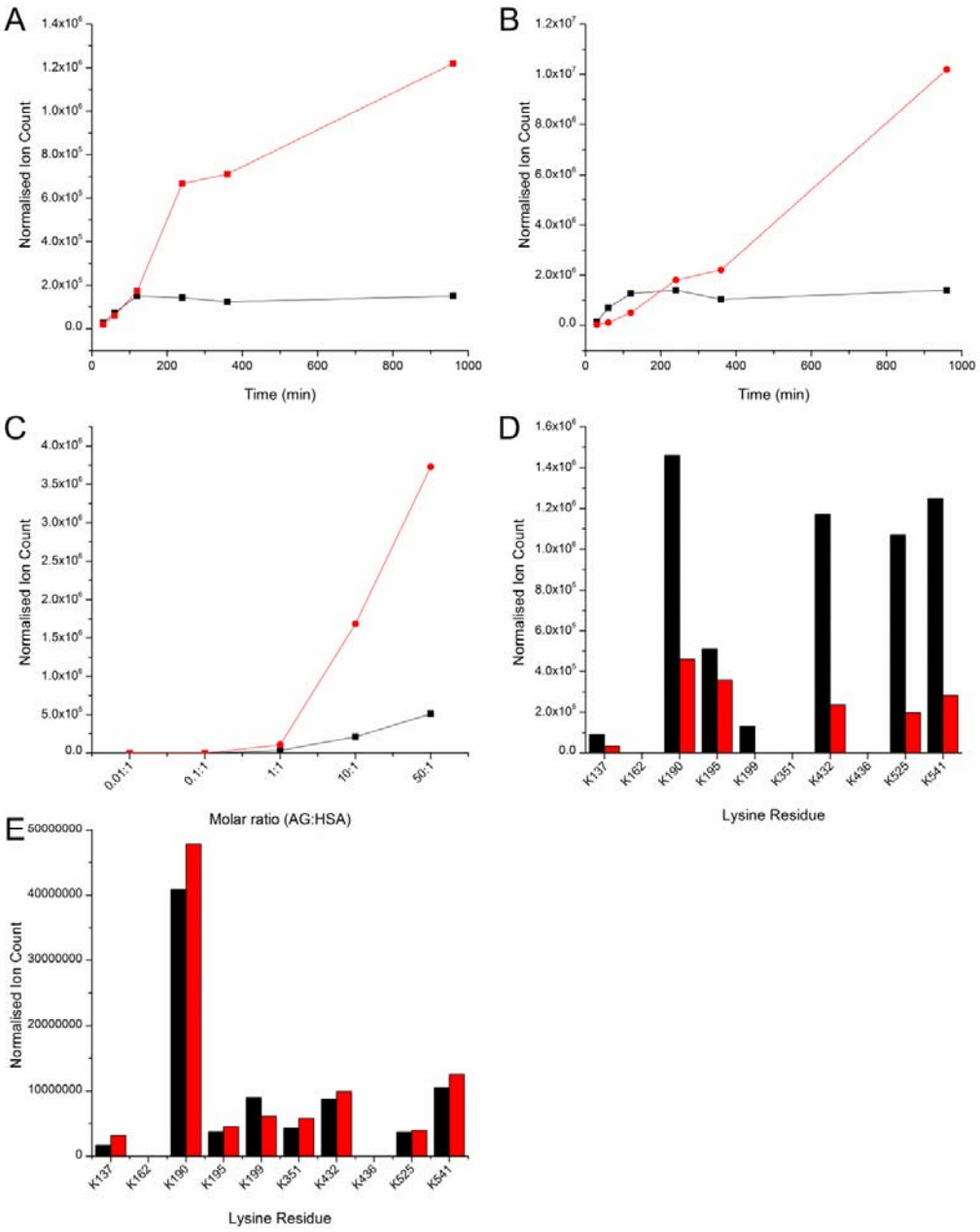
^{1}H NMR (400 MHz, D_6-acetone): δ 3.50 - 3.55 (1H, m), 3.58 - 3.63 (1H, m), 3.69 - 3.75 (1H, m), 3.90 - 3.99 (2H, m, ArC$\underline{H}_2$CO), 4.12 (1H, d, 5′-H), 5.21 (2H, s, ArC$\underline{H}_2$CO), 5.70 (1H, d, J=8 Hz,1′-Hβ), 6.48 - 6.53 (1H, m, ArH), 6.78 (1H, s, broad, NH), 6.93 - 6.98 (1H, m, ArH), 7.12 - 7.20 (2H, m, ArH), 7.30 - 7.38 (4H, m, ArH), 7.40 - 7.43 (2H, m, ArH), 7.48 - 7.51 (2H, m, ArH).

Palladium on carbon (10%; 322 mg, 3.02 mmol) was added in one portion to **3** (1700 mg, 3.02 mmol) and cyclohexa-1,4-diene (16.98 mL, 181.37 mmol) in IPA (30 mL) and THF (30 mL). The resulting suspension was stirred at 60 °C for 1 h (exothermic reaction after about 5 min heating that caused vigorous reflux for about 2 min). The reaction was monitored by TLC: all the starting material was consumed. The solution was cooled and filtered, the catalyst was washed with THF, and the filtrates were evaporated. The residue was triturated with ether. Most of the material appeared to dissolve in the ether. The ether liquors were evaporated to give a pale yellow foam. ^{1}H NMR analysis detected residual IPA. The foam was stirred with isohexane (5 mL) for 1 h. The solvent was decanted, and the residual solid dried under vacuum to yield a pale yellow powder (1.05 g). ^{1}H NMR revealed a persistent trace of IPA plus minor unidentified impurities (possibly

dechlorinated side-products of the hydrogenolysis). A batch of the powder (100 mg) was submitted to preparative reversed-phase HPLC (formic acid eluent modifier). Fractions were evaporated to dry residue at about 25°C. Following confirmation by ^{1}H NMR analysis that the recovered material was pure, the remaining impure glucuronide was purified by reversed-phase HPLC. The diclofenac acyl glucuronide **4** was obtained as a white amorphous solid (550 mg, 38.5%). ^{1}H NMR gave no evidence for the alpha anomer.

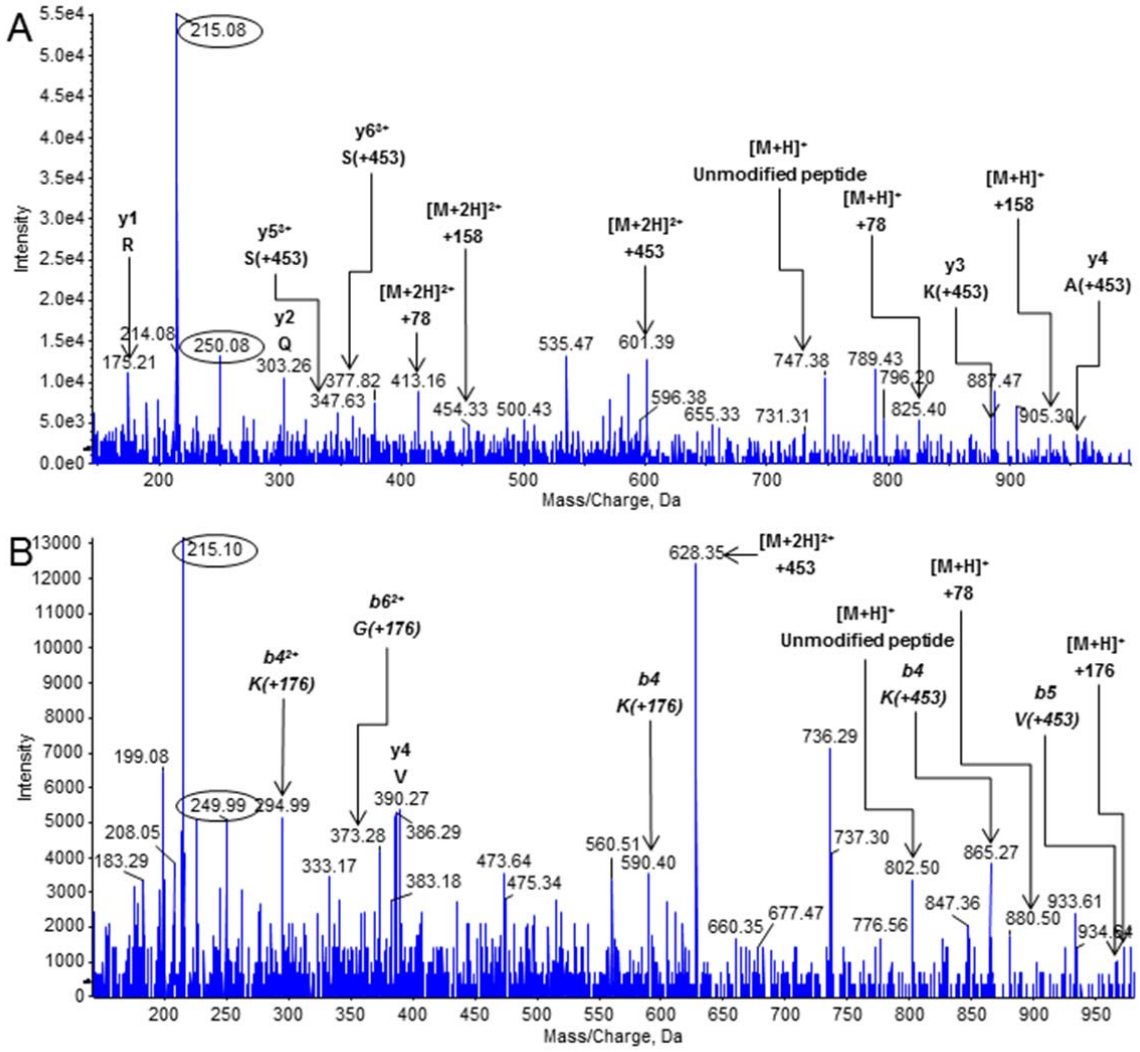
^{1}H NMR (400 MHz, D_6-acetone) : δ 3.53 (1H, t, J=8.01 Hz), 3.61 (1H, t, J=8.88 Hz), 3.70 (1H, t, J=9.19 Hz), 3.93 (1H, d, J=18 Hz), 3.97 (1H, d, J=18 Hz), 4.04 (1H, d, J=9.51 Hz), 5.68 (1H, d, J=7.96 Hz), 6.49 (1H, d, J=8.0 Hz), 6.80 (1H, s, broad), 6.96 (1H, td, J=0.98, 7.45 Hz), 7.13 (1H, td, J=1.56, 7.78 Hz), 7.17 (1H, t, J=7.98 Hz), 7.32 (1H, dd, J=1.39, 7.54 Hz), 7.48 (2H, d, J=8.08 Hz).

C-8 column LC-MS (ES^+): single peak at *m/z* 472/474 ($[M+H]^+$).



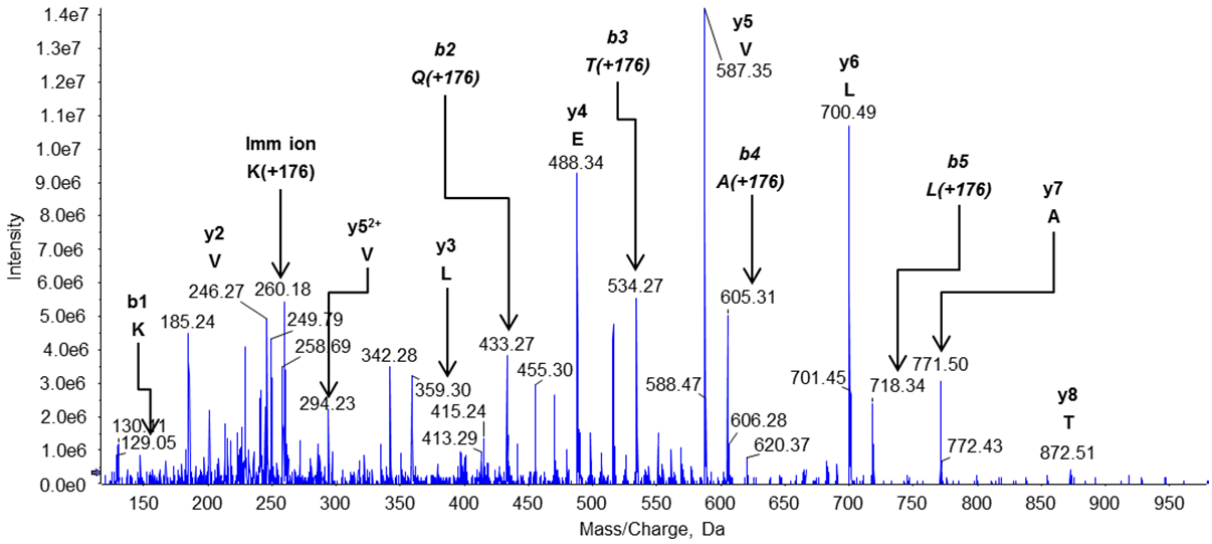
Supplemental Figure 1. Time-, concentration- and regioisomer-dependent adduction of individual HSA lysine residues by 1β-AG and a pre-formed mixture of AG positional isomers in vitro. (A) Time-dependent acylation (black line) and glycation (red line) of the Lys195 peptide. (B) Time-dependent acylation (black line) and glycation (red line) of the Lys525 peptide. (C) Concentration-dependent acylation (black line) and glycation (red line) of the Lys195 peptide. (D) Acylated peptides of HSA that had been incubated with 1β-AG (black) or pre-formed positional isomers (red). (E) Glycated peptides of HSA that had been incubated with 1β-AG (black) or pre-formed positional isomers (red). For time-dependent analyses, HSA (40 μM) was incubated with 1β-AG (2

mM). For concentration-dependent analyses, HSA (40 μM) was incubated with AG (final AG:HSA molar ratio, 0.01:1-50:1) for 16 h. For the comparison of adductions by 1β-AG and pre-formed positional isomers, HSA (40 μM) was incubated for 16 h (final AG:HSA molar ratio, 50:1). These comparisons demonstrated the transacylation reactions were much diminished by prior depletion of the 1-β AG isomer. Glycated Lys162 was detected in other analyses but only inconsistently. Acylated Lys351 was not detected consistently and only at the 50:1 molar ratio. Modified Lys436 was not detected consistently (Table 2). All incubations were performed in 0.1 M potassium phosphate, pH 7.4, at 37^{O}C. The mixture of positional isomers was produced by incubating 1β-AG in phosphate buffer, pH 7.4, at 37 ^{O}C for 3 h (see Fig. 2B). The extent of covalent modification was represented by the normalised ion counts of a peptide detected during LC-MS/MS analysis of a tryptic digest. However, because the proportionalities of ion counts and peptide abundances are unknown it cannot be assumed a peptide's abundance differs from that of any other peptide.



Supplemental Figure 2. Product-ion spectra of glycated HSA peptides (peptide + 453 amu; incorporating diclofenac carboxyl and glucuronyl residues) acquired during LC-MS/MS analysis of a tryptic digest of the protein isolated from diclofenac patient N08. (A) Peptide ^{191}ASSAKQR^{197} (parent ion, $[M+2H]^{2+}$ at *m/z* 601.39) modified at Lys195. (B) Peptide ^{429}NLGKVGSK^{436} (parent ion, $[M+2H]^{2+}$ at *m/z* 628.35) modified at Lys432. The MRM survey scans were set up to search for acylated and glycated HSA peptides with missed trypsin cleavages, i.e. covalent modifications, at lysine residues. The *m/z* values of the modified peptides and fragments (b and y ions) correspond to the $^{35}Cl_2$ isobars. The y_3 ion (KQR) and the y_4 ion (AKQR) but not the y_2 ion (QR; *m/z* 303.26) of ^{191}ASSAKQR^{197} were adducted. Therefore it was confirmed the mis-cleaved peptide was modified at Lys195; the position of the missed tryptic cleavage. The b_4 ion (NLGK; *m/z* 865.27) and the b_5

ion (NLGKV) but not the y_4 ion (VGSK; *m/z* 390.27) of ^{429}NLGKVGSK^{436} were adducted. These assignments conformed with the mis-cleaved peptide being modified at Lys432; the position of the missed tryptic cleavage. The peptide+158 amu ion was assigned to a whole-peptide species that retained the dehydrated residue of the dehydroglucuronic acid moiety. The peptide+176 amu and peptide fragment+176 amu ions retained the complete dehydroglucuronic acid moiety. Ions derived from fragmentation of the adduct are circled.



Supplemental Figure 3. Product-ion spectrum of a glucuronylated/hexuronylated HSA peptide (^{525}KQTALVELVK^{534}; parent ion, $[M+3H]^{3+}$ at *m/z* 435.6) acquired during LC-MS/MS analysis of a tryptic digest of the protein isolated from diclofenac patient N08. The peptide was adducted at Lys525. The b_2 ion (KQ; *m/z* 433.27), and the b_3, b_4 and b_5 ions, but not the y_7 ion (ALVELVK; *m/z* 872.51), were adducted. These assignments conformed with the mis-cleaved peptide being modified at Lys525. Note, the LC-MS/MS analysis was not able to differentiate between isomeric hexuronyl residues. This unexpected modification was found through manual searching of spectra.

Supplemental Table 1. Operating parameters of AB Sciex API 2000 for the multiple reaction monitoring assay of diclofenac liberated from covalent HSA adducts formed in vitro.[a]

Parameters	Diclofenac	Zomepirac[b]
Transition (*m/z*)[c]	296.1→214.9	292.0→139.0
Declustering potential (V)	11	16
Focussing potential (V)	360	360
Entrance potential (V)	6	10.5
Collision energy (eV)	25	27
Collision cell entrance potential (V)	12	12
Collision cell exit potential (V)	10	6
Dwell time (ms)	150	150

[a]Parameters were optimised by separate direct infusion of diclofenac and zomepirac into the source of the mass spectrometer.

[b]Internal standard.

[c]Parent ions are $[M+H]^+$

Supplemental Table 2. Operating parameters of AB Sciex 4000 Qtrap for the multiple reaction monitoring assays of diclofenac and diclofenac AG in clinical plasma samples.[a]

Parameter	Diclofenac AG	Diclofenac	Zomepirac[b]
Transition (m/z)[c]	472.0→296.1	296.1→215.1	292.0→139.0
Declustering potential (V)	37	31	40
Entrance potential (V)	10	10	10
Collision energy (eV)	14	41	27
Collision exit potential (V)	15	15	15
Dwell time (ms)	200	200	200

[a]Parameters were optimised by separate direct infusion of diclofenac and zomepirac into the source of the mass spectrometer.

[b]Internal standard.

[c]Parent ions are $[M+H]^+$

Supplemental Table 3. Diagnoses for diclofenac prescriptions , co-morbidities and complete lists of co-medications

Patient	Diagnosis for Diclofenac Prescription and Comorbidities[a]	Co-medications
N01 (male, 42 yrs)	RA, 1 yr sciatica	folic acid, leflunomide, methotrexate
N02 (female, 65 yrs)	RA, 13 yrs osteoporosis, erosive antral gastritis	alendronate, calcichew D3 forte, citalopram, dermovate, etanercept, folic acid, methotrexate, omeprazole, prednisolone
N03 (female, 52 yrs)	RA, 21 yrs breast carcinoma (1 year)	calcichew D3 forte, folic acid, methotrexate, omeprazole, prednisolone, tamoxifen, zoladex
N08 (male, 63 yrs)	RA, >20 yrs insulin-dependent diabetes	adcal D3, alendronate, aspirin, atenolol, beclomethasone, hydroxychloroquine, insulin, paracetamol, ramipril, rosuvastatin, sulfasalazine, tadalafil, thyroxine
N09 (male, 48 yrs)	ankylosing spondylitis, 34 yrs Wegener's granulomatosis, left hemisphere infarction (2009)	azathioprine, lansoprazole, paracetamol, simvastatin
N10 (female, 77 yrs)	generalised nodal osteoarthritis, 1yr hypertension, migraine	co-codamol, lansoprazole

[a]RA= rheumatoid arthritis.

Supplemental Table 4. Parameters of degradation of diclofenac 1-β acyl glucuronide in vitro[a]

Incubation[b]	Half-life (min)	k (min^{-1})	R^2
Phosphate buffer[c]	46.5	0.015	0.995
HSA solution (2 mM AG)[d]	56.8	0.012	0.999
HSA solution (400 μM AG)[d]	31.9	0.022	0.999
Human plasma (2 mM AG)[e]	7.0	0.099	0.993
Human plasma (400 μM AG)[e]	5.4	0.128	0.995

[a]Diclofenac 1-β acyl glucuronide remaining in the incubations (n=3 replicates) was estimated by LC-UV analysis (Fig. 2B-F). Parameters of the AG's degradation through acyl migration and hydrolysis were derived from these data by non-linear regression analysis using the equation of a first-order reaction, $C=C_0\exp^{(-kt)}$, where C_0 is the estimated initial fractional UV peak area of the AG and k is the degradation rate constant. The half-life was the time taken for the initial fractional peak area to fall to $C_0/2$. Computations were performed with SigmaPlot Version 11 software (Systat Software, San Jose, CA).

[b]16 h at 37°C.

[c]Potassium phosphate, 0.1 M, pH 7.4.

[d]HSA solutions were buffered with 0.1 M potassium phosphate, pH 7.4.

[e]Pooled plasma from healthy unmedicated male volunteers.

Supplemental Table 5. Modified peptides detected by LC-MS/MS (AB Sciex 5500 Qtrap) in a tryptic digest of HSA that was isolated from plasma of diclofenac patient N08 (male, 63 yrs, rheumatoid arthritis).

The MRM survey scans were set up to search for acylated and glycated HSA peptides with missed trypsin cleavages, i.e. covalent modifications, at lysine residues.

The listing shows the cation exchange fractions in which the five acylated, three acyl-glucuronide glycated (diclofenac carboxyl and glucuronyl residues) and one glucuronylated/hexuronylated (GLUC) peptides were recovered. Note, the MS/MS analysis of peptide ^{525}KQTALVELVK^{534} was not able to differentiate between isomeric hexuronyl residues.

Cation Exchange Fraction	Modified Tryptic Peptide[a]	Acylated	Glycated	Peptide Precursor Ion *m/z* and Charge[b]
39-40	^{198}LK*CASLQK^{205}		√	700.8, 2+
39-40	^{198}LK*CASLQK^{205}		√ (-H_2O)	691.9, 2+
39-40	^{525}K*QTALVELVK^{534}	√		469.2, 3+
41-42	^{198}LK*CASLQK^{205}	√		612.8, 2+
41-42	^{429}NLGK*VGSK^{436}		√	628.3, 2+
41-42	^{539}ATK*EQLK^{545}	√		547.7, 2+
43-44	^{191}ASSAK*QR^{197}		√	600.7, 2+
43-44	^{191}ASSAK*QR^{197}	√		512.7, 2+
43-44	^{433}VGSK*CCK^{439}	√		559.2, 2+
51-52	^{525}K*QTALVELVK^{534}		√ (GLUC)	435.6, 3+

[a]Asterisk indicates modified lysine on the mis-cleaved peptide. Cysteine residues of isolated and reduced HSA were carboxyamidomethylated before trypsin digestion.

[b]These are the *m/z* values of the doubly charged precursor ions selected for fragmentation. Product ions of MRM transitions: *m/z* 215.1 and *m/z* 250.1.

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