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Biosynthesis, characterisation and direct high-performance liquid chromatographic analysis of gemfibrozil 1-O-β-acylglucuronide

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Abstract

Gemfibrozil 1-O-β-acylglucuronide was purified from the urine of a volunteer administered gemfibrozil, and an isocratic reversed-phase HPLC method was developed for its direct measurement. Quantitation of gemfibrozil and gemfibrozil 1-O-β-acylglucuronide was carried out from plasma, following extraction from acidified specimens into ethyl acetate, on a 5-μm CN reversed-phase column with a mobile phase (pH 3.5) containing acetonitrile, tetrabutylammonium sulphate and distilled water, using fluorescence detection at 284 nm excitation and 316 nm emission. Calibration curves were linear for both compounds over a concentration range of 0.1 to 40 mg/l, with intra-assay coefficients of variation $< 5\%$ at concentrations of 20.0, 2.0 and 0.2 mg/l, and inter-assay coefficients of variation $< 10\%$. No degradation of gemfibrozil 1-O-β-acylglucuronide was detected as a result of the analytical procedure. However, a preliminary application of the method indicates that gemfibrozil acylglucuronide is chemically unstable undergoing intra-molecular rearrangement and hydrolysis under physiological conditions.

1. Introduction

Gemfibrozil ($pK_a = 4.7$) is a fibrate hypolipidaemic agent demonstrated to lower the incidence of coronary heart disease in humans [1,2]. In both animals and humans it is extensively metabolised [2] to four oxidised metabolites and an acylglucuronide conjugate whose chemical structure is shown in Fig. 1. In humans up to 50% of an oral dose has been recovered in urine as gemfibrozil 1-O-β-acylglucuronide [3]. It is now well documented that, unlike most other glucuronide conjugates, acylglucuronides are electrophilic chemically reactive metabolites [4]. Due to their electrophilicity they (i) readily hydrolyse reforming the parent acid and free glucuronic acid, (ii) undergo intra-molecular rearrangement where the xenobiotic moiety migrates from the 1-O-β position to the 2-, 3- and 4-C positions on the glucuronic acid ring and (iii) bind covalently to nucleophilic sites on proteins, both in vivo and in vitro [4].

This chemical reactivity has important analytical, pharmacokinetic and toxicological consequences. Most obvious, the facile hydrolysis of acylglucuronide conjugates can result in analytical errors when quantitating concentrations in biological fluids, if samples are not adequately stabilised prior to analysis [4]. Consequently, many early studies may have greatly underesti-

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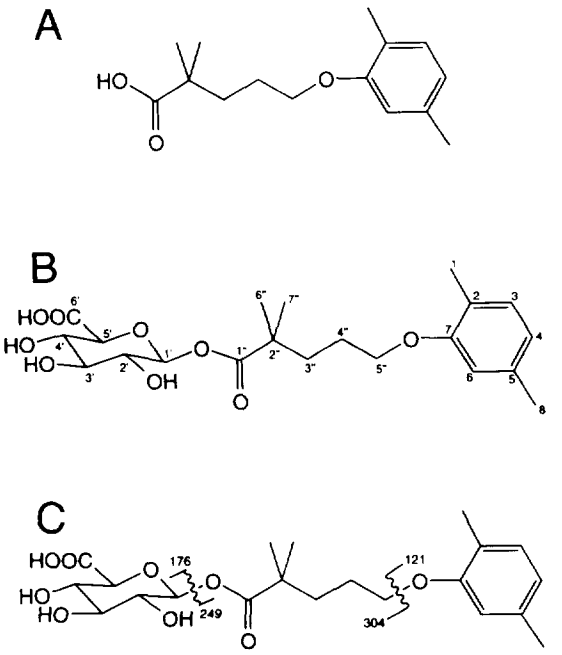

Fig. 1. Chemical structures of (A) gemfibrozil; (B) gemfibrozil 1-O-β-acylglucuronide, showing coding used in NMR analysis; and (C) MS fragmentation of gemfibrozil 1-O-β-acylglucuronide.

mated the contribution of acylglucuronidation to drug metabolism. Such underestimation of acylglucuronidation may also have occurred as a consequence of intra-molecular rearrangement, since rearrangement isomers do not act as substrates for the enzyme β-glucuronidase [4]. More importantly, the in vivo hydrolysis of acylglucuronides is an example of reversible metabolism, thus as a group, acylglucuronides are pharmacokinetically distinct from most other glucuronide conjugates [4,5]. To date very few studies have adequately addressed the reversible nature of acylglucuronidation and much of the available data on the pharmacokinetics of carboxylic acid drugs and their conjugates should be re-evaluated in light of the unique kinetic properties of acylglucuronides. Finally, the role of acylglucuronide conjugates in mediating covalent binding of xenobiotics to proteins is gaining increasing toxicological interest. The covalent binding of xenobiotics to tissue macromolecules has long been associated with drug toxicity, and the potential toxicity of covalently bound protein adducts formed from acylglucuronide conjugates is now beginning to be investigated [4].

Several methods are currently available for the quantitation of gemfibrozil and its oxidative metabolites from plasma [6–8]. To our knowledge, only one previous publication has attempted to identify gemfibrozil glucuronide [8]. HPLC was used with linear gradient elution to qualitatively identify gemfibrozil glucuronide in urine from a human volunteer. However, no attempt was made to quantitate the conjugate in urine or determine the method's suitability for measuring low concentrations of gemfibrozil glucuronide, such as those likely to be present in vivo in plasma. The present paper describes the synthesis and characterisation of gemfibrozil 1-O-β-acylglucuronide, and the development of a direct isocratic HPLC analytical method to quantitate gemfibrozil and gemfibrozil 1-O-β-acylglucuronide in a plasma matrix. An isocratic system was possible due to the use of the ion-pairing agent tetrabutylammonium sulphate in the mobile phase. The ion-pairing agent retards the elution of the glucuronide conjugate and has previously been applied to the quantitation of various other acylglucuronide conjugates [4,9–13].

2. Experimental

2.1. Reagents

Gemfibrozil and its oxidative metabolites were kindly provided by Parke-Davis Australia (NSW, Australia), flurbiprofen and β-glucuronidase were purchased from Sigma (NSW, Australia). Tetrabutylammonium sulphate was purchased from Eastman Fine Chemicals (Kodak, Rochester, NY, USA). Acetonitrile (HiperSolv) and ethyl acetate (HiperSolv) were purchased from BDH Australia (Vic., Australia). All other solvents and chemicals were of analytical grade, and all aqueous solutions were prepared in glass distilled water.

2.2. Synthesis and characterisation of gemfibrozil 1-O-β-acylglucuronide

Gemfibrozil glucuronide was biosynthesised from a volunteer administered 600 mg gemfibrozil (Lopid, Parke Davis). Urine was collected every 2 h, immediately acidified to pH 5.0 with H_3PO_4 and stored at −20°C.

Prior to extraction urine was further acidified to pH 3.0 with H_3PO_4 and extracted with two volumes of ethyl acetate. The organic layer was collected, transferred into a series of 5-ml disposable culture tubes and evaporated to dryness in an evacuated centrifuge (SpeedVac, Savant Instruments, NY, USA). The residue was redissolved in mobile phase and purified using semi-preparative HPLC with UV detection at 235 nm, on a cyano preparative column (Econosil CN 10 μm, 250 × 10 mm I.D., Alltech Associates, IL, USA) using a mobile phase of 30% (v/v) acetonitrile in 5 m*M* tetrabutylammonium sulphate, and the mixture was adjusted to a final apparent pH of 3.5.

The identity of the gemfibrozil glucuronide peak was based on its presence or absence before and after mild alkaline or β-glucuronidase hydrolysis of the urine. Eluate corresponding to the gemfibrozil glucuronide peak was collected and extracted with two volumes of ethyl acetate. The organic layer was separated, transferred into a series of 5-ml disposable culture tubes in 4-ml aliquots, and evaporated to dryness in an evacuated centrifuge. These stock sample tubes were capped and stored dry at −20°C.

To initially confirm the identity and purity of the prepared gemfibrozil glucuronide, one of the stock sample tubes was prepared in 1.0 ml of 0.1 *M* sodium acetate buffer (pH 5) and sample was injected for HPLC analysis before and after hydrolysis at 37°C with 1 *M* NaOH or β-glucuronidase. The purity of the gemfibrozil glucuronide was calculated as the amount of gemfibrozil liberated following β-glucuronidase hydrolysis as a percentage of the gemfibrozil liberated following NaOH hydrolysis. The amount of gemfibrozil glucuronide contained in each stock sample tube was calculated from the amount of gemfibrozil recovered following β-glucuronidase hydrolysis. Confirmation that the purified compound was indeed a glucuronide conjugate was also obtained by TLC analysis using a cellulose plate (No. 5716, Merck, Darmstadt, Germany) spotted with authentic D-glucuronic acid, glucose, gemfibrozil and samples of the putative gemfibrozil glucuronide stock solution before and after β-glucuronidase or NaOH hydrolysis. The plate was developed with a mobile phase of ethyl acetate–pyridine–water–acetic acid (36:36:21:7, v/v) and the spots were made visible by spraying with 0.5% (w/v) silver nitrate in acetone, followed by 0.1 *M* NaOH in methanol, and finally 0.5% (w/v) aqueous sodium thiosulphate.

To further confirm the identity of the putative gemfibrozil 1-O-β-acylglucuronide, specimens were also subject to NMR and MS analysis. Prior to analysis of gemfibrozil glucuronide, the conjugate was dissolved in a small volume of ethyl acetate, followed by addition of hexane in a layering procedure. After 24 h a clear oil had developed which, following separation from the solvents and drying under vacuum, produced a thick brown oil. This oil was then used in NMR, MS and MS–MS analysis. NMR spectra were recorded on a Bruker ACP-300 Fourier transform NMR spectrometer operating at 300 Hz. All spectra were recorded as dilute solutions of deuterochloroform, using tetramethylsilane as the internal standard. Negative-ion mass spectra (MS and MS–MS) were recorded on a V.G. ZAB 2HF mass spectrometer by fast atom bombardment in a glycerol matrix.

2.3. Preparation of calibration and quality control standards

Stock solutions of gemfibrozil (200 mg/l) and flurbiprofen (200 mg/l) were prepared in 30% (v/v) acetonitrile in distilled water. These were then diluted in acetonitrile–distilled water to give gemfibrozil stocks of 20 and 2.0 mg/l, and a flurbiprofen stock of 2.0 mg/l. Gemfibrozil glucuronide stock solutions of 200, 20 and 2.0 mg/l were prepared by making appropriate dilutions of the stock sample tubes in 0.1 *M* sodium acetate buffer (pH 5.0). Calibration standards

spanning a concentration range of 0.1 to 40 mg/l for gemfibrozil and gemfibrozil glucuronide were prepared fresh on each assay day by adding the appropriate amounts of stock solutions to drug free plasma previously adjusted with H_3PO_4 to a pH value between 4 and 5 (approximately 30 μl of H_3PO_4 per 4.0 ml of plasma).

A series of quality control specimens were prepared in acidified plasma using separate stock solutions. Three separate batches of quality control specimens were prepared at concentrations of 10, 2.0 and 0.2 mg/l of both gemfibrozil and gemfibrozil glucuronide. Samples were stored at −20°C in 1.0-ml aliquots. Calibration curve standards and quality control samples were stable for at least 3 months under the storage conditions described.

2.4. Sample analysis

To a 5-ml borosilicate glass culture tube were added 500 μl of acidified plasma sample (calibrator, QC or unknown), 100 μl of 2.0 mg/l flurbiprofen and 1.3 ml of acetonitrile. Samples were immediately vortex-mixed, centrifuged (1000 *g* for 15 min) and the supernatant transferred into a 15-ml borosilicate glass culture tube. To these tubes 800 μl of 1.0 *M* glycine buffer (pH 3.0) and 4.0 ml of ethyl acetate were added. The tubes were capped (PTFE lined) and mixed on a horizontal shaker at 70 rpm for 15 min. Following centrifugation (1000 *g* for 15 min) the organic layer was transferred to clean 5-ml disposable glass culture tubes and evaporated to dryness in an evacuated centrifuge. The samples were reconstituted in 250 μl of mobile phase (see below) and 10–100 μl were injected onto the HPLC system.

Chromatographic separation was carried out on a cyano column (Beckman Ultrasphere, 5 μm, 250 × 4.6 mm I.D., Alltech Associates, IL, USA) with a mobile phase consisting of 28% (v/v) acetonitrile in 10 m*M* tetrabutylammonium sulphate adjusted to a final apparent pH of 3.5, pumped at a flow-rate of 1.0 ml/min. Detection was carried out using a variable-wavelength fluorescence detector (Spectra Physics FL2000, CA, USA) at excitation and emission wavelengths of 284 and 316 nm, respectively. Detector sensitivity was set at range = 50, rise time = 2.0, and peaks were recorded on a dual-pen chart recorder at 10 and 50 mV.

Quantitation of gemfibrozil and gemfibrozil glucuronide in unknown samples was based on a calibration curve constructed, for each analytical run, by plotting the peak-height ratio of each compound to internal standard, against the corresponding spiked concentration of the calibration standard. Due to the very large range of calibration concentrations (400-fold), a line of best fit was calculated by linear regression forced through the origin. Calibration curves were accepted according to previously defined criteria [14].

Intra-assay reproducibility was determined by assaying five replicates of three standards at 10, 2 and 0.2 mg/l concentrations of gemfibrozil and gemfibrozil glucuronide. Inter-assay reproducibility was assessed over 4 analytical runs by comparing the concentrations calculated for the quality control samples assayed in duplicate in each analytical run.

2.5. Stability of gemfibrozil glucuronide in human plasma and buffered solutions

The stability of gemfibrozil glucuronide in acidified plasma and in mobile phase solution was tested over a 22-h period by direct HPLC analysis to determine the extent of hydrolysis to gemfibrozil, and also by comparison of NaOH or β-glucuronidase hydrolyses to determine the extent of intra-molecular rearrangement.

The stability of gemfibrozil 1-O-β-acyl-glucuronide was examined by incubating gemfibrozil glucuronide at 37°C for up to 48 h with either (i) human plasma buffered to pH 7.4 with 10% (v/v) of 1.0 *M* phosphate buffer pH 7.1, or (ii) 0.1 *M* phosphate buffer pH 7.4. Samples were immediately acidified (pH 5.0), snap-frozen using a dry ice–ethanol bath and stored at −20°C until analysis.

3. Results and discussion

3.1. Characterisation and purity of gemfibrozil glucuronide

Chromatograms of the purified gemfibrozil glucuronide, before and after NaOH or β-glucuronidase hydrolyses, are shown in Fig. 2. The mean purity, as calculated by release of gemfibrozil, was 101.6%. No rearrangement isomers or parent gemfibrozil were detectable in the pure gemfibrozil glucuronide stock samples. However, there appeared to be a small amount of contamination with "non-gemfibrozil" material in the region of the gemfibrozil glucuronide peak, but this was consistently less than 3.85% of the total gemfibrozil glucuronide peak height (Fig. 2), and final purity was determined as within 96%.

Following TLC analysis the R_F values for D-glucuronic acid and glucose were 0.14 and 0.29 respectively. Spots with the same R_F value as D-glucuronic acid were visible following NaOH or β-glucuronidase hydrolysis of the stock sample, but were not present in unhydrolysed sample or in pure gemfibrozil samples. Thus confirming the identity of the purified conjugate as a glucuronic acid conjugate.

Both ^1H and ^{13}C NMR were performed with gemfibrozil glucuronide. All multiplicities were abbreviated as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Chemical shifts are quoted in δ ppm, and assignments are given in accordance with standard practice [15–17] and coded as shown in Fig. 1B.

Unfortunately residue solvent complicated the ^1H spectrum, particularly in the 0–3.0 ppm range, so that only limited identification could be performed. ^1H NMR (δ, ppm): 7.29 (s, 1H, $C_{6'}$), 7.00 (d, 1H, C_3, $J = 7.4$ Hz), 6.66 (d, 1H, C_4, J distorted), 6.64 (s, 1H, C_6), 5.64 (d, 1H, $C_{1'}$, $J = 8.1$ Hz), 3.93 (m, 2H, $C_{5''}$, $J = 6.9$, 9.6 Hz), 3.27 (broad s, 3H, OH's on sugar ring), 2.31 (s, 3H, C_1), 2.18 (s, 3H, C_8), 1.49 (m, 2H, $C_{4''}$, $J = 7.3$ Hz), 1.26 (t, 6H, $C_{6''}$ and $C_{7''}$, $J = 2.9$ Hz), 1.02 (t, 2H, $C_{3''}$, $J = 7.3$ Hz). Providing

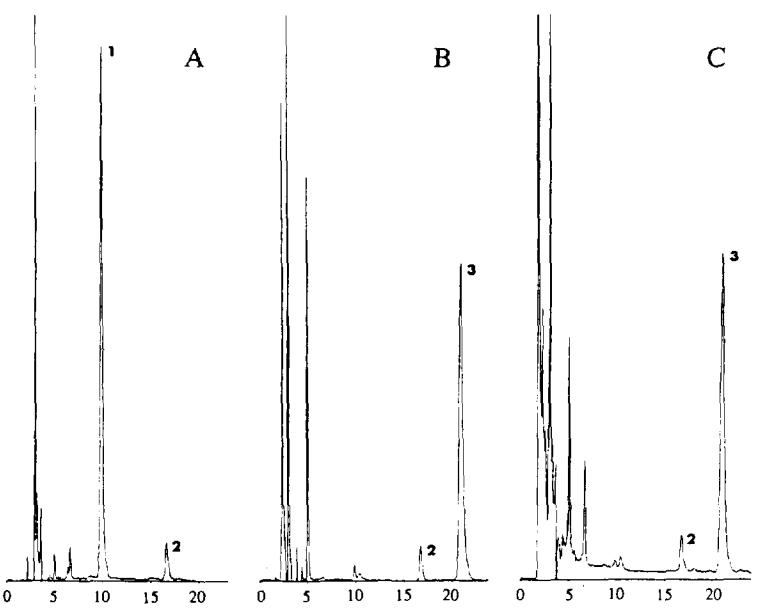

Fig. 2. Chromatograms of samples containing the putative pure gemfibrozil 1-O-β-glucuronide, (A) before, and after (B) NaOH or (C) β-glucuronidase hydrolysis. Peaks: 1 = gemfibrozil 1-O-β-acylglucuronide, 2 = flurbiprofen (internal standard) and 3 = gemfibrozil. Chromatographed material eluting between 0–7 min was also present in control specimens not containing gemfibrozil 1-O-β-acylglucuronide.

evidence for the aromatic ring in the gemfibrozil moiety and the anomeric hydrogen on the sugar ring at 5.64 with a coupling constant of 8 Hz indicating that the conjugate is indeed in the β-position [15,17].

The ^{13}C spectrum was clearer and more conclusive. ^{13}C NMR (δ, ppm): 176.27 ($C_{6'}$), 169.2 ($C_{1''}$), 156.9 (C_7), 136.5 (C_2), 130.2 (C_6), 123.5 (C_5), 120.7 (C_3), 112.3 (C_4), 93.8 ($C_{1'}$), 73.9, 72.1, 71.5 ($C_{2'}$, $C_{3'}$, $C_{4'}$), 68.1 ($C_{5''}$), 42.2 ($C_{2''}$), 36.9 ($C_{5'}$), 25.2, 24.9 ($C_{3''}$, $C_{4''}$), 24.5, 24.0 ($C_{6''}$, $C_{7''}$), 21.4 (C_1), 15.8 (C_8). Providing conclusive proof for the ester and free acid on the sugar ring.

The negative-ion mass spectrum of gemfibrozil glucuronide contained the following peaks: 425 ([M − H$^-$], 100%), 249 (48%) and 175 (43%), corresponding to gemfibrozil glucuronide, gemfibrozil and a fragment of the sugar ring (Fig. 1C). In order to show that 249 was a fragment of 425, and not a contaminant in the sample, MS–MS was performed on the 425 ion. Fragmentation of the 425 ion resulted in peaks at 249, 175 and 121, indicating cleavage of the ester bond releasing gemfibrozil and further fragmentation of the gemfibrozil molecule (121 peak) as shown in Fig. 1C.

3.2. Sample analysis

The mean (S.D., $n = 9$) absolute extraction recoveries for gemfibrozil, gemfibrozil glucuronide and flurbiprofen were 83.4 (5.3)%, 72.6 (3.2)% and 77.9 (4.5)%, respectively. Extraction recoveries were constant over the range of calibration concentrations (0.1–40 mg/l). Typical chromatograms of plasma extracts are shown in Fig. 3. There was no interference from endogenous compounds near any of the 3 peaks of interest. Retention times for gemfibrozil 1-O-β-glucuronide, flurbiprofen and gemfibrozil were approximately 11.4, 17.6 and 20.9 min, respectively. Potential chromatographic interference by the four oxidative metabolites of gemfibrozil was

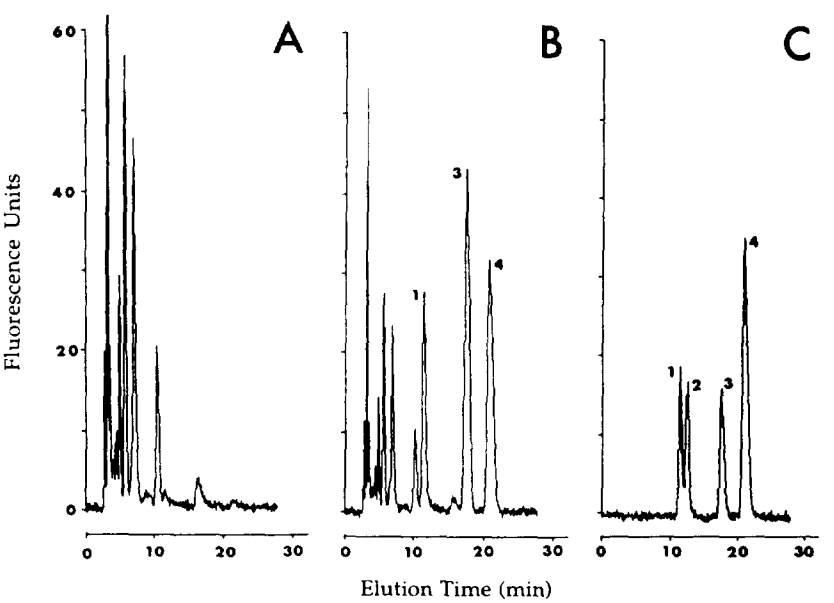

Fig. 3. Chromatograms of extracted plasma samples showing (A) 100-μl injection of drug free plasma, (B) 50-μl injection of plasma spiked with gemfibrozil glucuronide (1.5 mg/l), flurbiprofen and gemfibrozil (2.0 mg/l) and (C) spiked aqueous sample. Peaks: 1 = gemfibrozil 1-O-β-acylglucuronide, 2 = rearrangement isomer of gemfibrozil glucuronide, 3 = flurbiprofen (internal standard), 4 = gemfibrozil.

also investigated. The oxidative metabolites of gemfibrozil [8], metabolites I, II and IV all had retention times less than 10 min, and only metabolite III eluted near any of the peaks of interest, with a retention time of 10.5 min and was resolved from gemfibrozil 1-O-β-glucuronide.

Calibration curves were linear over the concentration range 0.1–40 mg/l. A total of 4 calibration curves gave a mean slope for gemfibrozil and gemfibrozil glucuronide of 0.3822 and 0.4195 respectively, with coefficients of variation of 5.6% and 3.9%, respectively. Calibration curves showed consistently better accuracies, as assessed by % bias, at all calibrator concentrations when the line of best-fit was forced through the origin, and this was routinely carried out for all assays. Intra-assay and inter-assay reproducibilities and accuracies are shown in Tables 1 and 2. The limit of detection of the assay was 0.1 mg/l based on a signal-to-noise ratio of 4.

3.3. Stability of gemfibrozil glucuronide in human plasma and buffered solutions

Gemfibrozil glucuronide appeared to be relatively stable in both acidified plasma and mobile phase at room temperature. No gemfibrozil was detectable up to 22 h in either acidified plasma or mobile phase as a result of hydrolysis of gemfibrozil glucuronide. Approximately 16.7% of the 1-O-β-glucuronide had undergone intramolecular rearrangement in acidified plasma by 22 h as determined by β-glucuronidase hydrolysis. There was no evidence of rearrangement or hydrolysis of gemfibrozil glucuronide as a result of the extraction procedure or sample reconstitution as determined by β-glucuronidase hydrolysis. No rearrangement isomers were detected up to 1 h in acidified plasma at room temperature, this being the maximum handling time of samples during analysis.

Table 1
Intra-assay accuracy and reproducibility obtained for the quantitation of gemfibrozil and gemfibrozil glucuronide (n = 5)

Concentration added (mg/l)	Concentration measured (mean) (mg/l)	C.V. (%)	Bias (%)
Gemfibrozil			
20.0	20.7	3.5	3.5
2.00	1.88	1.4	−6.0
0.200	0.208	4.4	4.0
Gemfibrozil glucuronide			
20.0	19.6	2.5	−2.0
2.00	1.94	1.4	−3.0
0.200	0.213	3.6	6.5

Table 2
Inter-assay accuracy and reproducibility obtained for the quantitation of gemfibrozil and gemfibrozil glucuronide (n = 8)

Concentration added (mg/l)	Concentration measured (mean) (mg/l)	C.V. (%)	Bias (%)
Gemfibrozil			
10.0	10.0	6.1	0.0
2.00	2.05	5.3	2.5
0.200	0.214	8.6	7.0
Gemfibrozil glucuronide			
10.0	8.9	5.9	−11.0
2.00	1.94	4.4	−3.0
0.200	0.201	9.4	0.5

The stability of gemfibrozil 1-O-β-glucuronide in human plasma and 0.1 M phosphate buffer pH 7.4 is shown in Fig. 4. Under both incubation conditions, the amount of gemfibrozil 1-O-β-glucuronide gradually decreased with time, accompanied by a corresponding increase in gemfibrozil and a second peak on the chromatograms with a retention time of 12.1 min (Fig. 3). This second peak was susceptible to NaOH hydrolysis releasing gemfibrozil, but was not altered by β-glucuronidase hydrolysis, and on this basis has been identified as a rearrangement isomer of gemfibrozil glucuronide and quantitated assuming similar fluorescent properties as the 1-O-β-acylglucuronide. It appeared to be the only rearrangement product of the 1-O-β-acylglucuronide and, throughout the incubation periods, gemfibrozil 1-O-β-acylglucuronide, its rearrangement isomer and gemfibrozil accounted for approximately 100% of total "gemfibrozil" content. When incubated in buffer at pH 7.4 the

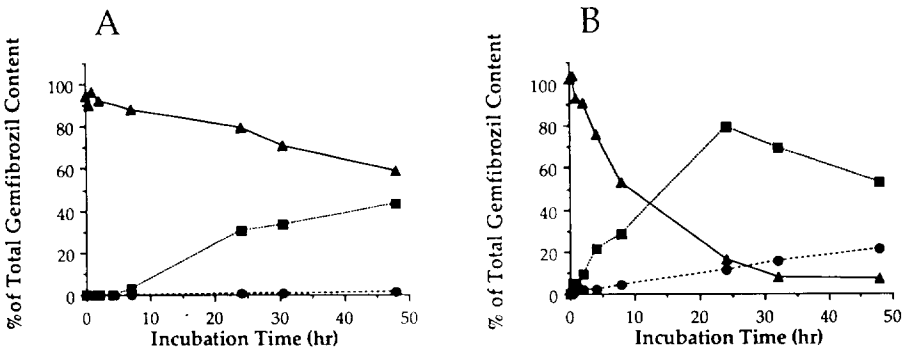

Fig. 4. Degradation of gemfibrozil 1-O-β-acylglucuronide (▲), and formation of rearrangement isomers (■) and gemfibrozil (●) following incubation of the 1-O-β-acylglucuronide in (A) 0.1 *M* phosphate buffer (pH 7.4) and (B) buffered human plasma (pH 7.4) at 37°C over 48 h.

1-O-β-glucuronide concentrations declined slowly with a half-life of approximately 74 h. Negligible degradation took place for the first 4 h, after which the predominant degradation pathway was intra-molecular rearrangement with only negligible hydrolysis taking place (Fig. 4A). Only one major rearrangement isomer peak was detectable as confirmed by β-glucuronidase hydrolysis. In contrast, when incubated in buffered human plasma the degradation of the 1-O-β-glucuronide was significantly increased with a half-life of approximately 9 h, and a greater proportion of the conjugate being hydrolysed to gemfibrozil by 48 h (Fig. 4B).

Thus, gemfibrozil 1-O-β-acylglucuronide displays the characteristic reactivity previously described for other acylglucuronides under physiological conditions [4]. Endogenous substances in plasma, such as albumin, appear to increase the degradation of gemfibrozil glucuronide, consistent with previous reports for other acylglucuronides [4]. The present method minimises acylglucuronide degradation as a result of analytical procedures, and provides an accurate and sensitive technique for the direct quantitation of gemfibrozil glucuronide. In addition to the precautions required during analysis, biological specimens must be immediately stabilised and appropriately stored prior to analysis to ensure accurate quantitation of the 1-O-β-acylglucuronide conjugate.

This method is currently being applied to in-vitro studies examining the mechanisms and consequences of the gemfibrozil 1-O-β-acylglucuronide reactivity, and its pharmacokinetics in various isolated organ perfusion studies. The concentration ranges over which the method has been validated may also be suitable for the quantitation of gemfibrozil and gemfibrozil glucuronide in plasma following administration of the drug in both animals or humans. However, this would require further validation to ensure suitability for such an application.

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