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# REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THE SIMULTANEOUS DETERMINATION OF DIFLUNISAL AND ITS GLUCURONIDES IN SERUM AND URINE

## REARRANGEMENT OF THE 1-O-ACYLGLUCURONIDE

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SUMMARY

A reversed-phase ion-pair high-performance liquid chromatographic assay for the simultaneous determination of diflunisal and its ester and ether glucuronide in urine and serum has been developed. The determination of the ester glucuronide in serum has not been previously reported. The genuine glucuronide conjugates isolated from urine were used as standards. The ester glucuronide is found to be unstable, especially in neutral and basic solutions, and special precautions therefore have to be taken during sampling and sample treatment. Nine rearrangement/degradation products of the ester glucuronide were detected.

## INTRODUCTION

Diflunisal (2′,4′-difluoro-4-hydroxy-3-biphenyl carboxylic acid) is an analgesic and antiinflammatory drug belonging to the salicylate group [1]. Diflunisal is metabolized in the liver to form an ether glucuronide and an ester glucuronide (Fig. 1). These are both excreted in the urine, together with a small amount of the parent compound [2]. Recently a new metabolite, the sulphate ester, has been reported after multiple dosage with diflunisal [3].

Different methods for quantitative determination of diflunisal have been reported. Among these are gas chromatographic, radiometric and fluorimetric methods [2]. Furthermore, several high-performance liquid chromatographic (HPLC) methods have been published [4–7]. Veenendaal and Meffin [8] have published an HPLC method for the separation of diflunisal and its metabolites

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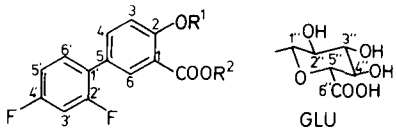


Fig. 1. Structures of diflunisal and its glucuronides. Diflunisal: $R^1 = H$ and $R^2 = H$; diflunisal ester glucuronide: $R^1 = H$ and $R^2 = GLU$, diflunisal ether glucuronide: $R^1 = GLU$ and $R^2 = H$.

in urine and plasma. The ester glucuronide was, however, not detected in plasma. For the determination of the glucuronides in urine these authors used the genuine metabolites, isolated from urine and purified by preparative chromatography.

Musson et al. [9] have developed an HPLC method for the assay of the ester and ether glucuronides in urine. This assay makes it possible to investigate the stability of the ester glucuronide, but no assay of diflunisal and its metabolites in plasma is reported. The assay of diflunisal and the ether glucuronide is made directly, whereas the ester glucuronide is measured as diflunisal liberated after hydrolysis with sodium hydroxide.

In this work, we present a reversed-phase ion-pair HPLC method for direct

TABLE I

ASSIGNMENT OF $^{13}C$ MAGNETIC RESONANCE

Spectra were collected from ca. 70 mg of glucuronide dissolved in 500 $\mu$l of [$^2H_6$]acetone. Spectra were obtained at 67.9 MHz.

| | Diflunisal ether glucuronide | | Diflunisal methyl ether | | Diflunisal ester glucuronide | | Diflunisal methyl ester | |
|---|---|---|---|---|---|---|---|---|
| | Chemical shift (ppm) | $^{13}C$-$^{19}F$ coupling (Hz) | Chemical shift (ppm) | $^{13}C$-$^{19}F$ coupling (Hz | Chemical shift (ppm) | $^{13}C$-$^{19}F$ coupling (Hz) | Chemical shift (ppm) | $^{13}C$-$^{19}F$ coupling (Hz) |
| C-1 | 99.60 | | 127.56 | | – | | – | |
| C-2 | 157.04 | | 158.62 | | 161.56 | | 161.37 | |
| C-3 | 118.96 | | 112.91 | | 118.00 | | 117.95 | |
| C-4 | 134.61 | | 134.55 | | 137.2 | | 136.38 | |
| C-5 | 121.41 | | 119.88 | | 126.43 | | 126.17 | |
| C-6 | 129.85 | | 132.56 | | 130.70 | | 130.25 | |
| C-7 | 169.42 | | 165.81 | | 167.2 | | 170.42 | |
| C-1′ | 123.84 | 13.7(F2′)<br>4.2(F4′) | 124.4 | | 124.3 | | 124.39 | |
| C-2′ | 159.71 | | 159.87 | 248.4(F2′)<br>11.9(F4′) | – | | – | |
| C-3′ | 104.38 | 27.4-26.3 | 104.40 | 26.4-26.3 | 104.36 | 27.4-26.3 | 104.3 | 27.3-26.3 |
| C-4′ | 162.60 | 248.4(F4′)<br>11.6(F2′) | 162.47 | 247.2(F4′)<br>11.3(F2′) | – | | – | |
| C-5′ | 112.07 | 20.0(F4′) | 112.01 | 24.0(F4′) | 111.95 | 14.7(F4′) | 111.95 | 21.0(F4′) |
| C-6′ | 132.04 | | 131.81 | | 131.9 | | 131.7 | |
| C-1″ | 103.36 | | | | 95.43 | | | |
| C-2″ | 75.58 | | | | 76.01 | | | |
| C-3″ | 75.47 | | | | 75.92 | | | |
| C-4″ | 73.48 | | | | 72.46 | | | |
| C-5″ | 71.59 | | | | 71.66 | | | |
| C-6″ | 166.37 | | | | 168.60 | | | |
| $-CH_3$ | | | 56.28 | | | | 52.38 | |

TABLE II

ACCURACY AND PRECISION OF SERUM ASSAY

| Analyte | Concentration (mg/l) | Assay concentration (mean ± S.D., $n=7$) (mg/l) | Recovery (%) | Coefficient of variation (%) | |
|---|---|---|---|---|---|
| | | | | Within-day | Between-day ($n=4$) |
| Ether glucuronide | 1.21 | 1.12 ± 0.12 | 92.5 | 9.1 | 7.2 |
| | 8.08 | 6.76 ± 0.21 | 84.0 | 3.0 | 3.2 |
| | 12.12 | 9.79 ± 0.17 | 81.0 | 2.0 | 3.5 |
| Ester glucuronide | 1.03 | 0.99 ± 0.03 | 96.5 | 3.6 | 9.8 |
| | 6.83 | 6.57 ± 0.21 | 96.2 | 3.2 | 4.3 |
| | 10.25 | 9.97 ± 0.15 | 97.3 | 1.5 | 7.2 |
| Diflunisal | 5.09 | 4.67 ± 0.04 | 91.7 | 0.8 | 13.0 |
| | 47.61 | 42.70 ± 0.36 | 89.7 | 0.8 | 8.5 |
| | 91.88 | 80.20 ± 0.93 | 87.3 | 1.2 | 7.7 |

determination of diflunisal and its glucuronides in urine as well as in serum. The genuine glucuronides were isolated from urine and used as standards for quantitation. The instability of the ester glucuronide has been investigated, and nine rearrangement/degradation products of the ester glucuronide were detected.

TABLE III

ACCURACY AND PRECISION OF URINE ASSAY

| Analyte | Concentration (mg/l) | Urine | Assay concentration (mean ± S.D., $n=7$) (mg/l) | Recovery (%) | Coefficient of variation (%) | |
|---|---|---|---|---|---|---|
| | | | | | Within-day | Between-day ($n=4$) |
| Ether glucuronide | 5.71 | A | 5.80 ± 0.04 | 101.6 | 0.7 | 2.9* |
| | | B | 5.71 ± 0.06 | 100.0 | 1.0 | 0.5 |
| | | C | 5.91 ± 0.06 | 103.5 | 1.7 | 1.8 |
| | 57.10 | A | 58.25 ± 0.76 | 102.0 | 1.3 | 1.8 |
| | | B | 58.04 ± 0.62 | 101.7 | 1.1 | 2.8 |
| | | C | 57.46 ± 0.82 | 100.8 | 1.4 | 1.5 |
| | 158.98 | A | 166.48 ± 2.28 | 104.7 | 1.1 | 4.4 |
| | | B | 166.98 ± 2.15 | 104.5 | 1.2 | 4.0 |
| Ester glucuronide | 6.40 | A | 6.44 ± 0.05 | 100.6 | 0.8 | 1.8* |
| | | B | 6.24 ± 0.21 | 97.5 | 1.2 | 2.1 |
| | | C | 6.48 ± 0.11 | 101.3 | 0.9 | 1.3 |
| | 88.84 | A | 93.08 ± 0.57 | 104.5 | 0.6 | 3.3 |
| | | B | 87.38 ± 1.13 | 101.7 | 1.3 | 2.0 |
| | | C | 89.57 ± 1.82 | 100.8 | 2.1 | 1.2 |
| | 161.54 | A | 164.93 ± 1.99 | 102.1 | 1.2 | 1.6 |
| | | B | 157.78 ± 3.68 | 97.7 | 2.3 | 1.4 |
| Diflunisal | 6.27 | A | 6.51 ± 0.07 | 103.8 | 1.1 | 3.3* |
| | | B | 6.47 ± 0.06 | 103.2 | 0.9 | 1.1 |
| | | C | 6.49 ± 0.04 | 103.5 | 0.6 | 1.1 |
| | 85.12 | A | 87.89 ± 1.70 | 103.3 | 1.9 | 4.2 |
| | | B | 85.99 ± 0.45 | 101.0 | 0.5 | 1.2 |
| | | C | 84.38 ± 1.14 | 99.1 | 1.3 | 1.8 |
| | 154.76 | A | 162.42 ± 2.13 | 104.9 | 1.3 | 5.4 |
| | | B | 156.78 ± 2.85 | 100.9 | 1.8 | 3.7 |

*$n=3$.

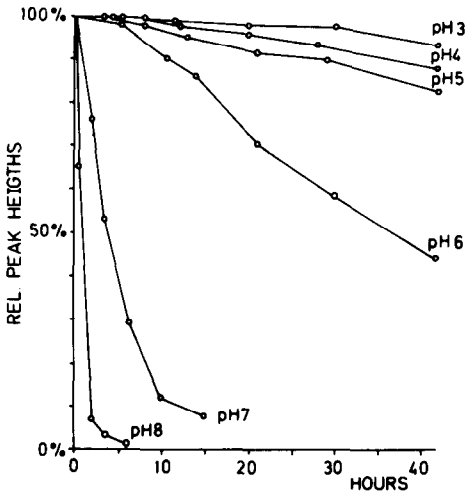


Fig. 2. Stability of diflunisal ester glucuronide at pH 3–8 and 20–25 °C; starting concentration ca. 10 mg/l.

## EXPERIMENTAL

### *Chemicals*

Diflunisal (purity greater than 99.5%) was a gift from Dumex (Copenhagen, Denmark). *Escherichia coli* β-glucuronidase (EC 3.2.1.31), containing 570 000 Fishman units/mg, was obtained from Sigma (St. Louis, MO, U.S.A.). Amberlite XAD-2 (0.2–1.3 mm) from BDH (Poole, U.K.) was purified by the method described by Dieterle et al. [10]. Methanol and acetonitrile were of HPLC grade. All other chemicals were of analytical grade.

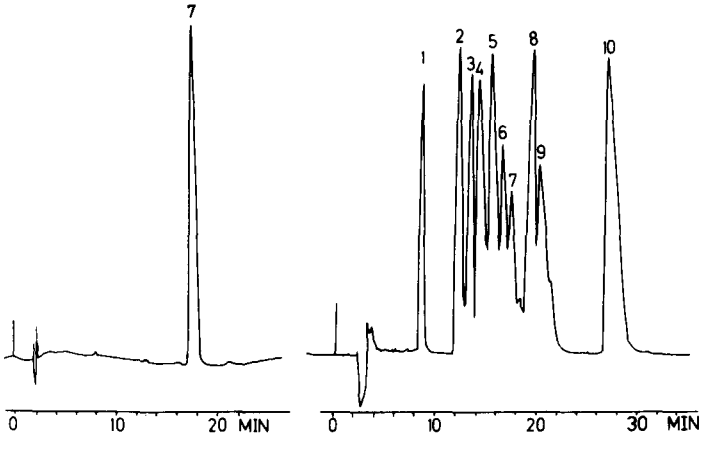


Fig. 3. Samples containing 100 mg/l diflunisal ester glucuronide after 0 and 2 h incubation in 0.05 *M* phosphoric acid buffer (pH 8.0) at 37 °C. Peak 1 corresponds to the ether glucuronide, peak 7 is the ester glucuronide and peak 10 diflunisal. Peaks 2–6 and 8–9 are isomers of the ester glucuronide. Column operated at 20–25 °C.

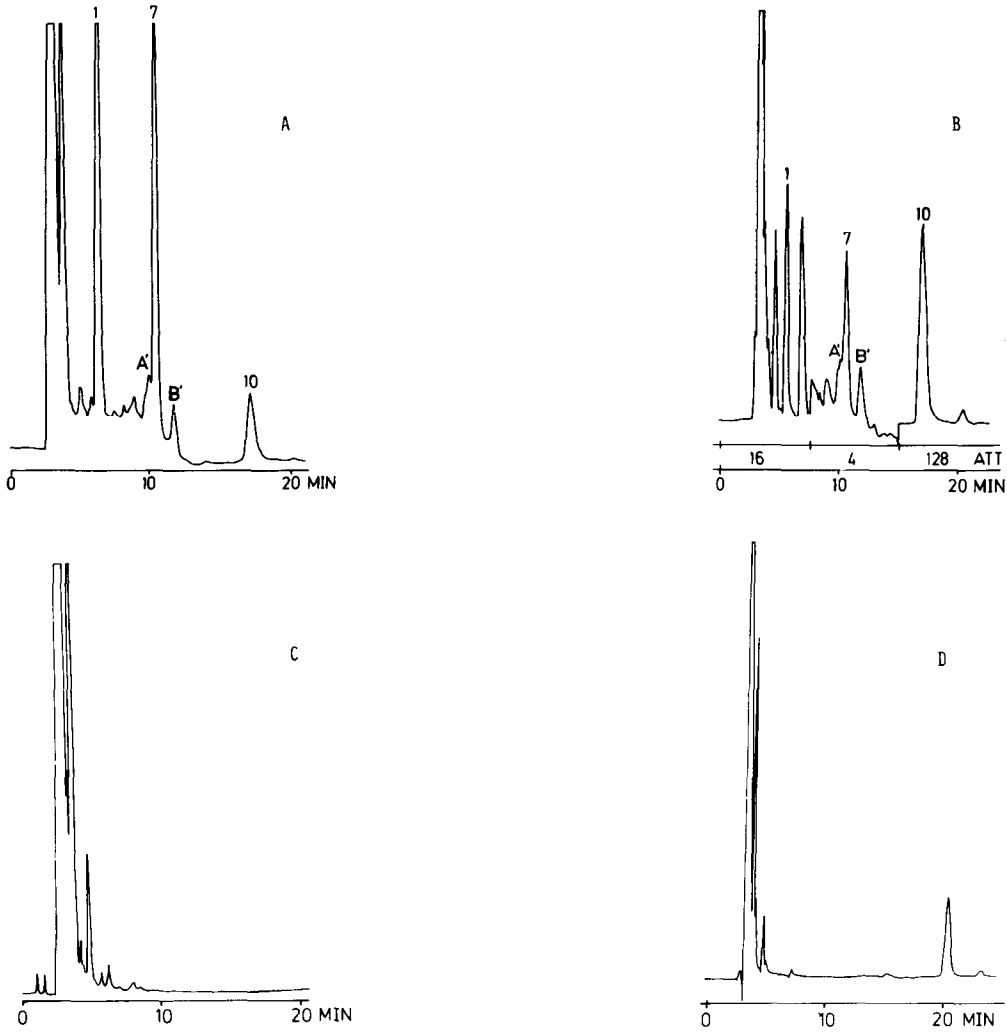


Fig. 4. Chromatograms of urine (A) and serum (B) collected at 4–8 and 4 h, respectively, after ingestion of 500 mg of diflunisal, and diluted with one part of acetonitrile. Chromatograms of blank urine (C) and serum (D). Peaks marked A′ and B′ have retention times equal to those of the isomers of diflunisal ester glucuronide. Peaks are assigned as in Fig. 3.

*Thin-layer chromatography (TLC)*

The mobile phase consisted of *n*-butanol–acetic acid–water (24:6:6, v/v/v). Silica gel 60 F-254 sheets (5×7.5 cm) from E. Merck (Darmstadt, F.R.G.) were used as the solid phase. The $R_F$ values obtained were 0.44 for the ether glucuronide, 0.58 for the ester glucuronide and 0.76 for diflunisal. When viewed under UV light (254 nm) all three compounds appeared as blue spots on a yellow background. The glucuronides appeared as blue spots on a pink background when the plates were sprayed with a mixture of 0.2% 1,3-naphthoresorcinol in ethanol and 85% orthophosphoric acid (5:1) and heated at 100°C for 10 min.

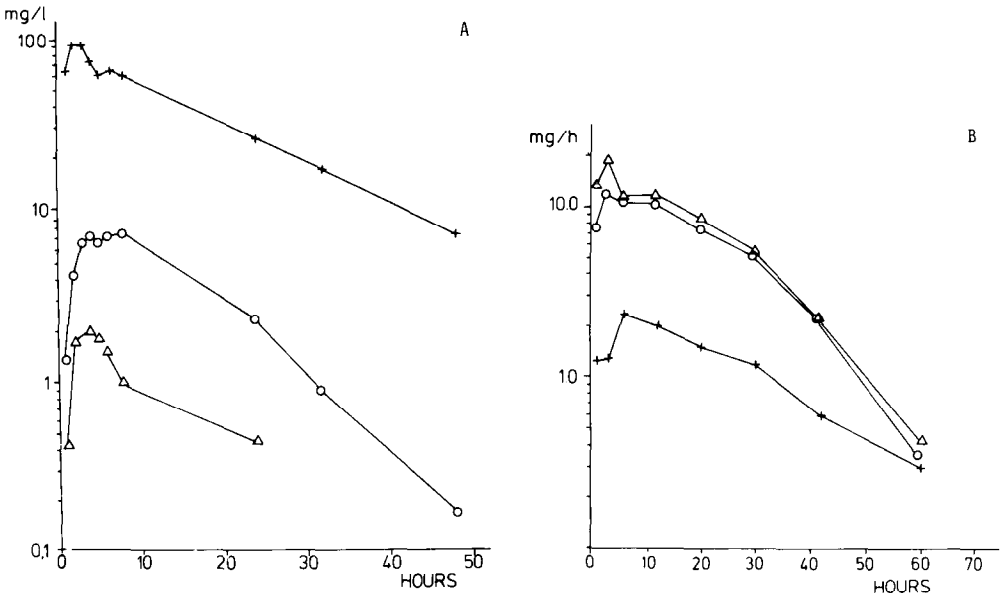


Fig. 5. (A) Serum concentration profile and (B) urine excretion rates of diflunisal and its glucuronides after ingestion of 500 mg of diflunisal, and diluted with one part of acetonitrile. Curves: + = diflunisal; ○ = diflunisal ether glucuronide; △ = diflunisal ester glucuronide.

*High-performance liquid chromatography*

An HPLC pump, Model 6000A from Waters Assoc. (Milford, MA, U.S.A.), fitted with a Rheodyne 7125 sample injector (Cotati, CA, U.S.A.), and an absorbance detector, Model 440 from Waters, operated at 254 nm were used. In all assays 10 μl of the sample were injected.

The analytical column (250 mm×4.6 mm I.D.) was packed with Spherisorb Octyl 5 μm (Phase Separations, Norwalk, CT, U.S.A.) and operated at 40°C.

The composition of the mobile phase was methanol (48%, v/v) and 20 m*M* potassium citrate (pH 3.6) with 0.02 m*M* of tetramethylammonium hydrogensulphate added. The flow-rate was 1.0 ml/min.

Standard curves (based on peak-height measurements) for the ester and the ether glucuronide were linear in the range 0.2–400 mg/l, and the curve for diflunisal was linear in the range 2–400 mg/l. None of the standard curves had a coefficient of correlation less than 0.99.

*Isolation of glucuronides from urine*

Two volunteers were given a dose of 500 mg of diflunisal each, and urine samples were collected for a period of 12 h after ingestion. Immediately after collection the urine was acidified with citric acid to pH ca. 4. After filtration the urine (1600 ml) was pumped through a column (700 mm×33 mm I.D.) packed with Amberlite XAD-2 at a flow-rate of 5 ml/min. By the use of an LKB (Bromma, Sweden) Ultrograd 11300 gradient mixing kit, the column was washed with 1% acetic acid for 4 h at 3 ml/min. The adsorbed components were eluted with a linear gradient from 1% acetic acid in water to 1% acetic acid in methanol over

12 h (3 ml/min). The fractions containing the glucuronides (investigated by TLC) eluted at ca. 80% methanol were evaporated at reduced pressure (water-bath, maximum temperature 40°C), until they could be frozen and lyophilized.

Each glucuronide was purified by preparative HPLC on a column (250 mm×8 mm I.D.) packed with Spherisorb Octyl (5 μm). The mobile phase consisted of 1% acetic acid and 50% methanol at a flow-rate of 5 ml/min. Detection was performed with a variable-wavelength UV spectrophotometer (Kontron Uvikon LCD 725, Zurich, Switzerland) at 310 nm. About 30 mg of lyophilized Amberlite XAD-2 extract dissolved in 500 μl of mobile phase was injected per run. The fractions were lyophilized as described above. The yield was 470 mg of the ether glucuronide and 330 mg of the ester glucuronide.

*Identification of the metabolites*

The identities of the glucuronides were confirmed by the use of $^{13}C$ NMR and $^{1}H$ NMR spectroscopy and fast atom bombardment (FAB) mass spectrometry (MS).

$^{1}H$ and $^{13}C$ spectra were obtained on a Brucker (Rheinstetten, F.R.G.) HX-270S operated at 270 and 67.9 MHz, respectively. In each sample ca. 70 mg of the glucuronides were dissolved in 500 μl of [$^{2}H_6$]acetone containing 2 μl of tetramethylsilane.

Data from the $^{13}C$ spectra are shown in Table I. Comparison of the spectra of the two glucuronides with those of the methyl ester and the methyl ether of diflunisal indicated that the two compounds were glucuronides of diflunisal. The assignment of the $^{13}C$ signals from the diflunisal part was in good agreement with published data [11].

The signals in the $^{1}H$ NMR spectrum for the ester glucuronide were assigned as follows: $\delta = 8.02$ ppm (m, 1H, H-6), $\delta = 7.72$ ppm (m, 1H, H-4), $\delta = 7.59$ ppm (m, 1H, H-6′), $\delta = 7.33$ ppm (m, 1H, H-3′), $\delta = 7.19$ ppm (m, 1H, H-5′), $\delta = 7.14$ ppm (d, 1H, H-3), $\delta = 5.71$ ppm (d, 1H, $J = 7.3$ Hz, H-1″), $\delta =$ ca. 4.2 ppm (broad, OH), $\delta = 3.93$ ppm (d, 1H, $J = 8.9$ Hz), $\delta = 3.94$–$3.41$ ppm (m, 3H). In the spectrum of the ether glucuronide, signals at $\delta = 8.03$ ppm (m, 1H, H-6), $\delta = 7.03$–$6.74$ ppm (m, 5H, H-3,4,3′,5′,6′), $\delta = 5.95$ ppm (broad, OH), $\delta = 5.16$ ppm (d, 1H, H-1″, $J = 8.0$ Hz), $\delta = 4.17$ ppm (d, 1H, $J = 8.0$ Hz, H-5″) and $\delta = 3.79$–$3.69$ ppm (m, 3H, H-2″,3″,4″) were found.

Proton signals originating from the diflunisal moiety are in good agreement with published data [11]. Coupling constants of 7.3 and 8.0 Hz for the anomer proton in the ester and the ether glucuronide, respectively, confirm that both glucuronides are β-glucuronides.

FAB mass spectra were recorded with a Varian (Palo Alto, CA, U.S.A.) MAT 311 A mass spectrometer fitted with an Ion Tech FAB-11 NF saddle field atom gun using argon atoms at 8 keV. Positive-ion FAB spectra were recorded into a Varian SS 200 data system, calibrated in the electron-impact mode. Samples were dissolved in glycerol.

Ions were detected in the mass spectrum of the ester glucuronide at *m/z* 427, 233, and 251. These peaks correspond to the molecular ion plus 1 (M+1), M − $C_6H_9O_7$ (base peak) and M − $C_6H_8O_6$ at 251. The spectrum of the ether glu-

curonide showed ions at $m/z$ 427, 251 and 233. These peaks correspond to the molecular ion plus 1 (M+1), $M-C_6H_8O_6$ (base peak) and $M-C_6H_9O_7$.

*Purity of glucuronides*

Several approaches were made to establish the purity of the metabolites.

*β-Glucuronidase.* A 1000-μl volume of a freshly prepared solution of each of the glucuronides, containing ca. 50 μg/ml glucuronide, was mixed with 1000 μl of 0.5 *M* phosphoric acid buffer (pH 7.0) containing 2 mg/ml (1 400 000 U/ml) β-glucuronidase. These samples were incubated at 37°C for 1 h.

*Perchloric acid.* A 1000-μl volume of an aqueous solution containing ca. 50 μg/ml glucuronide was mixed with 10 μl of 70% perchloric acid and heated for 2 h at 110°C. After cooling, 1000 μl of methanol were added.

An aqueous solution of diflunisal was treated in the same way. After 4 h, 98.5% of the diflunisal remained intact.

*Sodium hydroxide.* The ester glucuronide can be selectively cleaved by hydrolysis at high pH. This was done by mixing 2000 μl of a solution containing 50 μg/ml of ester glucuronide with 200 μl of 2 *M* sodium hydroxide. After 2 h incubation at 37°C, 100 μl of 4 *M* acetic acid were added.

An aqueous solution of diflunisal was treated in the same way for 4 h, and no decrease in the diflunisal concentration could be detected.

*Elemental analysis.* The calculated composition of both of the glucuronides is 53.52% C and 3.78% H. The composition was found to be 52.21% C and 4.21% H for the ether glucuronide. The ester glucuronide contained 51.68% C and 4.31% H. Both of the glucuronides were found to contain 0.3% N.

*Sampling and sample treatment*

Immediately after collection, the urine was acidified with citric acid to pH ca. 4 (3 g of citric acid per litre of urine) and frozen at −18°C. Blood samples were cooled and centrifuged. Serum was then acidified with citric acid (25 μl of 50% citric acid per 5 ml of serum) to pH ca. 4 and frozen.

Before analysis the samples were diluted with one part of acetonitrile and centrifuged at 18 000 *g* for 4 min.

## RESULTS AND DISCUSSION

*Chromatographic system*

Spherisorb Octyl was chosen as the stationary phase because it offers the best compromise between the small $k'$ values of the glucuronides and the large $k'$ value of diflunisal. The use of citric acid as buffering agent diminished the severe tailing of diflunisal. The addition of tetramethylammonium ions to the eluent gave a good retardation of both glucuronides, and by adjusting the pH of the mobile phase to 3.6 a separation of nine rearrangement/degradation products of the ester glucuronide could be obtained.

UV detection at 254 nm was a more convenient method than electrochemical (1000 mV) or fluorescence detection (excitation 320 nm, emission 420 nm). The ether glucuronide could not be detected by the electrochemical method, and the

fluorescence of the glucuronides was shown to be only one tenth of that of diflunisal.

*Assay validation*

Validation studies were conducted by adding known amounts of diflunisal and its glucuronides to drug-free serum or urine at three different concentrations. For the validation of urine assays three different types of drug-free urine were used.

Recoveries in serum were between 81.0 and 97.3% for all three analytes (Table II). The recoveries in urine were between 97.5 and 104.9% for the three analytes (Table III).

Within-day reproducibility was determined by assaying seven different samples at three concentrations. The coefficients of variation (C.V.) were 0.8–4.3% for the serum assay and 0.5–2.3% for the urine assay.

The C.V. for between-day assays were 3.2–13% for serum and 0.5–5.4% for urine.

*Purity of the parent glucuronides*

The contents of the ether and the ester glucuronide in the reference samples isolated were found to be 84.7% and 83.0%, respectively, using enzymic cleavage, and the contents found by cleavage using acid were 83.1 and 80.3%. Treatment of the ester glucuronide with sodium hydroxide yielded a content of 81.5%.

It is known [12–14] that it may be difficult to cleave glucuronides quantitatively. However, in these investigations we did not find any trace of the original glucuronides in any of the chromatograms obtained after cleavage with acid, base or $\beta$-glucuronidase. Degradation of diflunisal, treated in the same way, was found to be negligible.

The genuine glucuronides were used as reference compounds and the quantitative determination of the glucuronides in biological samples was based on the purity of the reference compounds found by enzymatic cleavage.

*Stability of the ester glucuronide*

The stability of the ester glucuronide was studied at different pH values. Aqueous solutions containing ca. 20 mg/l in 0.1 *M* phosphate buffers with pH values in the range from 3 to 8.8 were stored at ambient temperature. The results are shown in Fig. 2. When stored at pH 4 or below for 36 h, less than 10% of the ester glucuronide disappeared.

The literature contains several examples of drugs partly excreted as unstable ester glucuronides; among these drugs are zomepirac [15], clofibrate [16], probenecid [17], valproate [18], fenclofenac [19], isoxepac [20] and wy-18,251 (3-(*p*-chlorophenyl)thiazolo-[3,2-*a*]benzimidazole-2-acetic acid) [21]. Bilirubin [22] is an example of an endogenous compound excreted as an ester glucuronide. Under mildly alkaline conditions the diflunisal moiety starts to migrate from the first hydroxy group of the glucuronic acid moiety through an 1,2-ortho ester to the second hydroxy group and then to the third and fourth hydroxy groups [23,24], thereby giving the possibility of several isomers.

In the present work, nine isomers of the ester glucuronide were found (Fig. 3).

After isolation of each compound it was found that, after some time, all the fractions corresponding to peak numbers 2, 3, 4, 5, 6 and 9 contained two compounds. When the fractions corresponding to peak 2 and peak 3 were kept at 0°C for 48 h, it was found that the two fractions both contained approximately equal amounts of peak 2 and peak 3. The same observations were made for the two pairs 4/5 and 6/9. Peaks 1, 7 and 8 did not change when stored. This indicates that the peak pairs 2/3, 4/5 and 6/9 may be $\alpha$- and $\beta$-isomers of the same positional isomer. Peak 7 is the original ester glucuronide and peak 8 may be the $\alpha$-isomer of the ester glucuronide. By treating a mixture of peaks 2–9 with base, all peaks were converted into diflunisal. On treatment of the isomers with $\beta$-glucuronidase the original glucuronide and peak 1 disappeared. Peak 1 has the same $k'$ value as the ether glucuronide, but further studies are necessary to confirm the identity of this peak.

Stability studies on the ester glucuronide showed that it is possible to use it as a standard sample. An alternative method is a selective cleavage by base and quantification as the difference in diflunisal content before and after the cleavage. This method would not be suitable for assaying the ester glucuronide in serum because of the low concentration of this compound compared with that of diflunisal. The small changes in the diflunisal content are difficult to measure accurately. Another disadvantage is that each sample has to be assayed twice.

The instability of the ester glucuronide implies that special precautions have to be taken during sample collection. In this study it was found that acidifying the samples to pH 3–4 at the time of collection stabilizes the ester glucuronide, and only negligible amounts of the rearrangement products were detected in urine (Fig. 4A) and serum (Fig. 4B) samples. Peaks marked A′ and B′ have the same $k'$ values as peaks 6 and 8 of the isomers of the ester glucuronide.

*Application*

A dose of 500 mg of diflunisal was given to a healthy male volunteer aged 35 with a body weight of 68 kg. Urine was collected at the following intervals: 0–2 , 2–4, 4–8, 8–16, 16–24, 24–32, 32–48, 48–72 and 72–96 h. Blood samples were collected at the following hours after ingestion: 0, 1, 2, 3, 4, 5, 8, 24, 32, 48.

Fig. 4C and D shows chromatograms of blank urine and serum. The selectivity is good in both materials.

The serum concentration curves are shown in Fig. 5A. From the terminal phase of the diflunisal curve a serum half-life of 13.3 h could be calculated.

The urine excretion data are shown in Fig. 5B. Some 35% of the given dose is excreted as ether glucuronide and ca. 43% is excreted as ester glucuronide. Only 13.9% is excreted unmetabolized. A total amount of 92.5% of the dose was found in the urine. This is in good agreement with results published by Tocco et al. [2]; those authors, however, found that most of the drug was excreted as ether glucuronide. In the present study and in the study by Musson et al. [9], it is found that the ester glucuronide is the main metabolite. This discrepancy may be explained by the instability of the ester glucuronide.

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REFERENCES

1 C.A. Stone, C.G. Van Arman, V.J. Lotti, D.H. Minsker, E.A. Risely, W.J. Bagdon, D.L. Bokelman, R.D. Jensen, B. Medlowski, C.L. Tate, H.M. Peck, R.E. Zwickey and S.E. McKinney, Br. J. Clin. Pharmacol., 4 (Suppl. 1) (1977) 19-29.
2 D.J. Tocco, G.O. Breault, A.G. Zacchei, S.L. Steelman and C.V. Perrier, Drug Metab. Disp., 3 (1975) 453-466.
3 G.R. Loewen, G. McKay and R.K. Verbeeck, Drug Metab. Disp., 14 (1986) 127-131.
4 J.W.A. van Loenhout, H.C.J. Ketelaars, F.W.J. Gribnau, C.A.M. van Ginneken and Y. Tan, J. Chromatogr., 182 (1980) 487-491.
5 M. Balali-Mood, I.S. King and L.F. Prescott, J. Chromatogr., 229 (1982) 234-240.
6 C. Midskov, J. Chromatogr., 278 (1983) 439-444.
7 J.E. Ray and R.O. Day, J. Pharm. Sci., 72 (1983) 1403-1405.
8 J.R. Veenendaal and P.J. Meffin, J. Chromatogr., 307 (1984) 432-438.
9 D.G. Musson, J.H. Lin, K.A. Lyon, D.J. Tocco and K.C. Yeh, J. Chromatogr., 337 (1985) 363-378.
10 W. Dieterle, J.W. Faigle and H. Mory, J. Chromatogr., 168 (1979) 27-34.
11 K. Florey, Analytical Profiles of Drug Substances, Vol. 14, Academic Press, London, 1985.
12 G.J. Dutton, Glucuronic Acid, Academic Press, London, 1966.
13 M.D. Saunders, Dissertation, State University College of Foresty Syracuse University, Syracuse, NY, 1967.
14 S. Goenechea, W. Wessling and K.J. Goebel, Fresenius' Z. Anal. Chem., 315 (1983) 359-360.
15 J. Hasegawa, P.C. Smith and L.Z. Benett, Drug Metab. Disp., 10 (1982) 469-473.
16 K.A. Sinclair and J. Caldwell, Biochem. Pharmacol., 33 (1982) 953.
17 N.J. Eggers and K. Doust, J. Pharm. Pharmacol., 31 (1981) 123-124.
18 R.G. Dickinson, W.D. Hooper and M.J. Eadie, Drug Metab. Disp., 12 (1984) 247-252.
19 M.V. Marsh, J. Caldwell, T.P. Sloan and R.L. Smith, Xenobiotica, 13 (1983) 233-240.
20 H.P.A. Illing and I.D. Wilson, Biochem. Pharmacol., 30 (1981) 3381-3384.
21 F.W. Janssen, S.K. Kirkman, C. Fenselau, M. Stogniew, B.R. Hofmann, E.M. Young and H.W. Ruelius, Drug Metab. Disp., 10 (1982) 599-604.
22 N. Blanckaert, F. Compernolle, P. Leroy, R. Van Houtte, J. Fevery and K.P.M. Heirwegh, Biochem. J., 171 (1978) 203.
23 W.A. Bonner, J. Org. Chem., 24 (1959) 1388-1390.
24 F. Compernolle, G.P. Van Hees, N. Blackaert and K.P.M. Heirwegh, Biochem. J., 171 (1978) 185-201.