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Note

Direct analysis of diflunisal ester and ether glucuronides by high-performance liquid chromatography

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Diflunisal (2′,4′-difluoro-4-hydroxy-3-biphenylcarboxylic acid) is metabolised by conjugation with glucuronic acid to form both an ester and an ether glucuronide [1]. Previous methods for the quantitative analysis of diflunisal ester and ether glucuronides have employed β-glucuronidase or acid hydrolysis and have measured the glucuronide concentration as the difference in diflunisal concentration before and after hydrolysis [1, 2]. Difficulties in documenting the specificity of these hydrolytic methods and their lack of sensitivity in the presence of high concentrations of aglycone, such as those that frequently occur in plasma after aglycone administration, have previously been discussed by us [3].

In the case of diflunisal glucuronides, enzymatic and acid hydrolysis will yield diflunisal with both the ester and ether glucuronides and thus such analysis cannot be used to specifically quantitate each compound. Mild alkaline hydrolysis can be used to selectively hydrolyse ester glucuronides in the presence of the ether glucuronide [4] but such an approach would require three measurements of diflunisal: before and after both acid and alkaline hydrolysis in order to quantitate both glucuronides in the presence of diflunisal. Such an approach is time-consuming and frequently inaccurate.

It was our aim to develop a direct analysis for diflunisal ester and ether glucuronides that would be specific and capable of measuring low concentrations of one glucuronide in the presence of high concentrations of the other, or the aglycone.

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EXPERIMENTAL

Preparation of diflunisal glucuronides

Urine was collected from a volunteer prior to drug administration and over a 6-h period after ingestion of 125 mg diflunisal 6-hourly for one week.

A high-performance liquid chromatograph (Model 6000A) with a Model U6K injector and a variable-wavelength detector operated at 254 nm (Model 450) together with a 10-μm particle size, reversed-phase column (μBondapak C_{18}) all from Waters Assoc. (Milford, MA, U.S.A.) were used to examine the urine for diflunisal metabolites. The output from the detector was recorded using a dual-pen recorder with 10- and 50-mV voltage spans. The mobile phase of acetonitrile—0.01 *M* citrate buffer, pH 3 (250:750) had a flow-rate of 2.0 ml/min. Using the above conditions, the chromatographic profiles of pre-dose urine and diflunisal containing urine were compared prior to and after the following pre-treatments: (a) with 2000 Fishman units of β-glucuronidase (Type VII, Sigma, St. Louis, MO, U.S.A.) as previously described [3]; (b) as above but in the presence of 30 mg/ml of D-saccharic acid 1,4-lactone; (c) an equal volume of 0.2 *M* sodium hydroxide at room temperature for 30 min. Treatment a was designed to identify peaks due to glucuronides and the specificity of this approach was confirmed by treatment b. The ester, but not the ether glucuronide was hydrolysed by treatment c [4].

The urine (50 ml) was acidified to pH 2 with sulphuric acid and extracted with diethyl ether (100 ml). The diethyl ether was removed at room temperature and reduced pressure and the residue was taken up in 1 ml of 0.01 *M* citrate buffer, pH 3. This buffer was washed with hexane, which removed an interfering peak. The hexane was discarded and the buffer was filtered through a 0.45-μm filter. The filtered extract was then purified by preparative chromatography using the conditions previously described for the identification of the glucuronide peaks and the fractions containing the glucuronides were collected, based on retention time. An aliquot of each fraction was re-chromatographed prior to pooling the fractions, in order to confirm chromatographic purity. The pure fractions were pooled, lyophylised as previously described [5] and stored at -20°C prior to their use.

Samples of each lyophylised fraction were dissolved in water and their identity and purity were assessed using the following criteria: (a) the glucuronide fraction chromatographed as a single peak; (b) the glucuronide peaks were removed by β-glucuronidase hydrolysis, while additionally, the ester glucuronide peak but not the ether glucuronide peak was removed by mild alkaline hydrolysis; (c) the hydrolysis by glucuronidase was inhibited by D-saccharic acid 1,4-lactone; (d) hydrolysis of the pure glucuronide fractions yielded only diflunisal.

Chromatographic analysis of the fractions after the above pretreatments was carried out using a reversed-phase column as previously described but with a mobile phase consisting of methanol—0.01 *M* citrate buffer, pH 3 (40:60).

Assay procedure

To a 1.5-ml disposable centrifuge tube containing 0.15 ml of internal standard solution 0.05 ml of plasma or urine was added. The internal standard

solution consisted of desmethylnaproxen (50 mg/l) dissolved in acetonitrile—20%, w/v aqueous trichloroacetic acid (1:3). The samples were mixed on a vortex mixer and centrifuged at 9500 *g* for 2 min. An aliquot of supernatant (0.01 ml) was injected into the high-performance liquid chromatograph with a mobile phase of acetonitrile—0.01 *M* citrate buffer, pH 3 (300:700) flowing at 2 ml/min. The effluent was monitored at a wavelength of 254 nm. A Schoeffel (FS970 LC) fluorometer, at an excitation wavelength of 235 nm with a 370-nm emission cut-off filter was also used to monitor low concentrations of the glucuronides.

Calibration

Lyophilised fractions containing diflunisal ester and ether glucuronides were reconstituted in distilled water and the concentrations were established by hydrolysis of a sample of each of the fractions with β-glucuronidase. The amount of diflunisal liberated on hydrolysis was determined using a method previously described [6]. The ester and ether glucuronide containing solutions were mixed and serially diluted to give glucuronide concentrations in the range of approximately 3—75 mg/l for both compounds. Duplicates of these solutions were then analysed. The reproducibility of the method was assessed by determining the coefficient of variation (C.V.) of the normalised peak height ratio (peak height ratio divided by concentration).

RESULTS AND DISCUSSION

Characterisation of diflunisal glucuronides

Fig. 1 shows chromatograms of the reconstituted lyophilised ester glucuronide fraction without pretreatment (Fig. 1A), after hydrolysis with sodium hydroxide (Fig. 1B), after treatment with β-glucuronidase (Fig. 1C) and after treatment with β-glucuronidase in the presence of its inhibitor, D-saccharic acid 1,4-lactone (Fig. 1D). Under these conditions the retention times of diflunisal ether glucuronide, diflunisal ester glucuronide and diflunisal were 3.3, 5.9 and 10.1 min, respectively. When pure extracts of diflunisal ester and ether glucuronide were hydrolysed both by β-glucuronidase and acid there was a maximum of 2.8% difference between these two methods in the amount of diflunisal liberated suggesting complete hydrolysis by both methods. Similarly Fig. 2 shows chromatograms of the reconstituted ether glucuronide fraction without pretreatment (Fig. 2A), after treatment with sodium hydroxide (Fig. 2B), after treatment with β-glucuronidase (Fig. 2C) and after β-glucuronidase treatment in the presence of its inhibitor D-saccharic acid 1,4-lactone (Fig. 2D). The pattern of peaks resulting from the above pretreatments is consistent with the interpretation that the fraction shown in Fig. 1 contains pure diflunisal ester glucuronide and the fraction shown in Fig. 2 contains pure ether glucuronide, using the criteria previously described (see Experimental). The retention time of diflunisal liberated by these pretreatments of 10.1 min was the same as that of authentic diflunisal under these conditions.

Quantitative analysis

Fig. 3A shows a chromatogram of urine containing diflunisal ether glu-

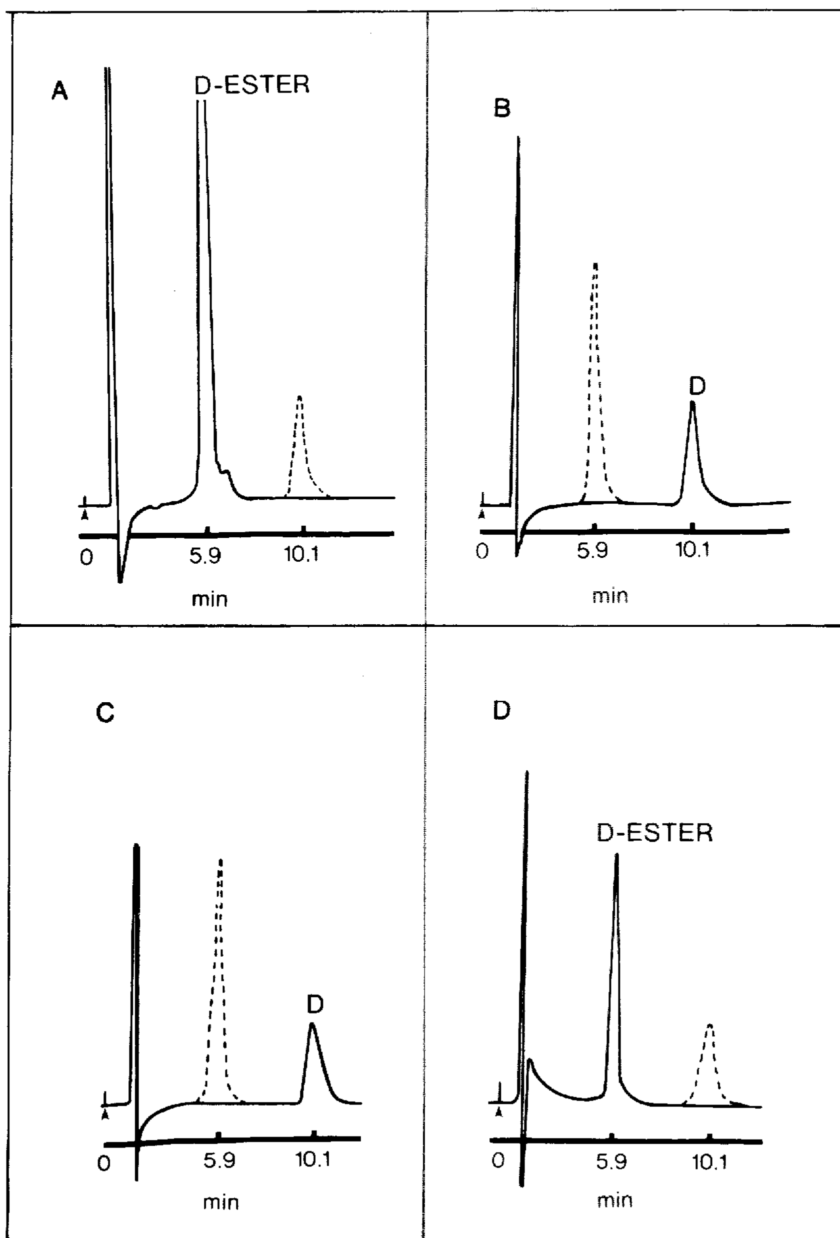

Fig. 1. Chromatograms of the reconstituted lyophilised ester glucuronide fraction. (A) No pretreatment; (B) treatment with 0.1 *M* sodium hydroxide; (C) treatment with β-glucuronidase; and (D) treatment with β-glucuronidase in the presence of D-saccharic acid 1,4-lactone. Peaks: D = diflunisal; D-ester = diflunisal ester glucuronide. In samples where no diflunisal or diflunisal ester glucuronide was detected, their retention times are indicated by a broken line.

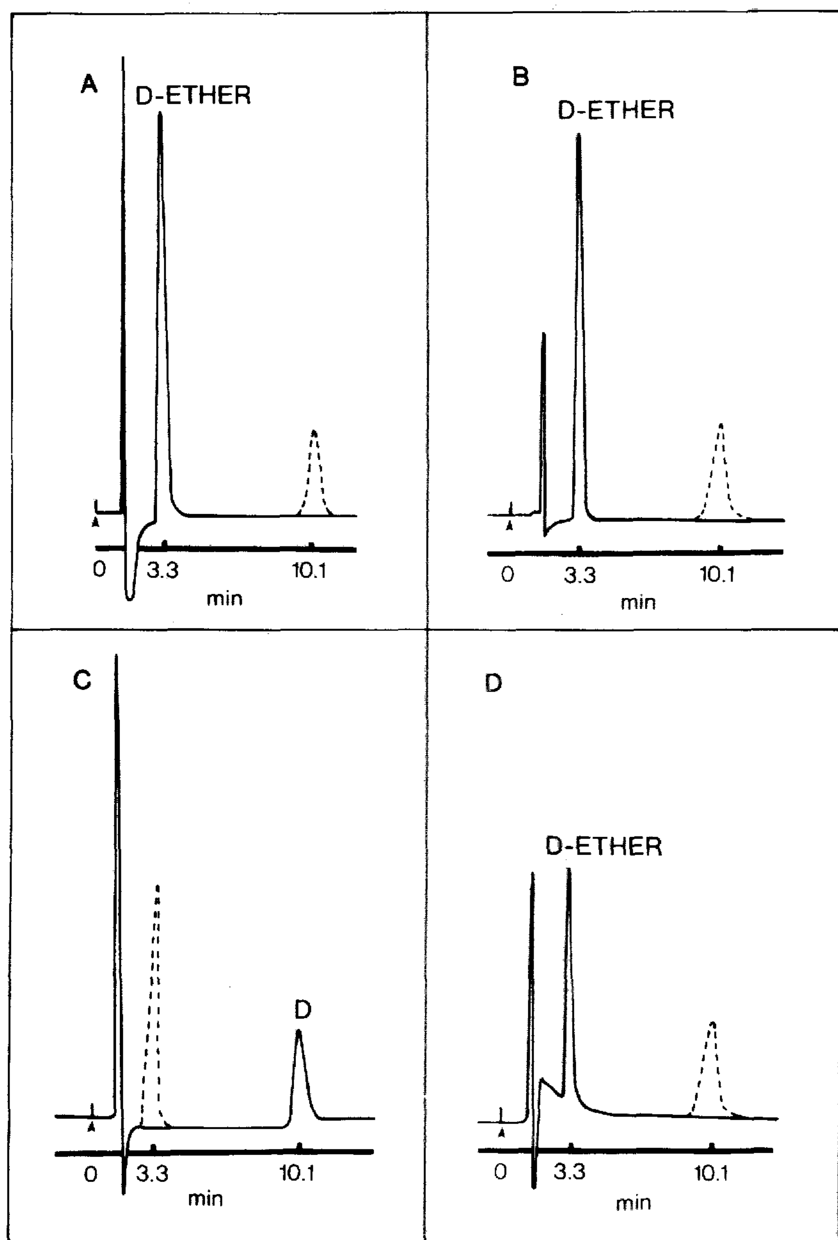

Fig. 2. Chromatograms of the reconstituted lyophylised ether glucuronide fraction. (A) No pretreatment; (B) treatment with 0.1 *M* sodium hydroxide; (C) treatment with β-glucuronidase; and (D) treatment with β-glucuronidase in the presence of D-saccharic acid 1,4-lactone. Peaks: D = diflunisal; D-ether = diflunisal ether glucuronide. In samples where no diflunisal or diflunisal ether glucuronide was detected, their retention times are indicated by a broken line.

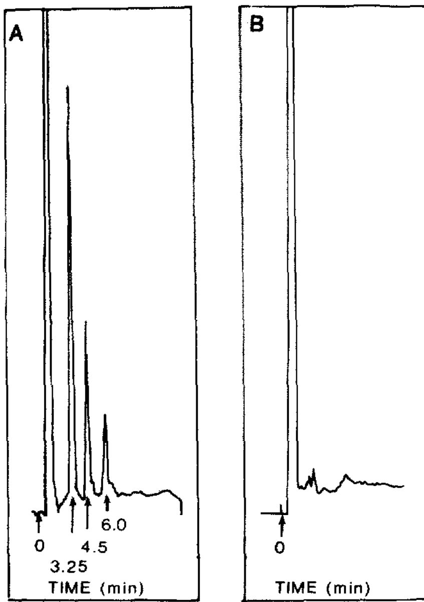

Fig. 3. Chromatograms of urine containing (A) ether glucuronide (3.25 min), internal standard (4.5 min) and ester glucuronide (6.0 min) and (B) control urine.

curonide (59 mg/l) with a retention time of 3.25 min, diflunisal ester glucuronide (20 mg/l) with a retention time of 6.0 min and the internal standard (50 mg/l) with a retention time of 4.5 min. A chromatogram of diflunisal-free urine is also shown (Fig. 3B). There were no peaks in control plasma or urine which coincided with those of the diflunisal glucuronides or the internal standard.

The reproducibility of the method over the calibrated range is shown in Table I. In the range 7.4—59.2 mg/l for the ether glucuronide and 2.8—22.0 mg/l for the ester glucuronide the coefficient of variation for the normalised peak height ratio is approximately 5%. In the range 4.7—75.2 mg/l for both ester and ether glucuronide in urine, the coefficient of variation is about 3%.

Fluorescence detection was used in a qualitative manner to examine the pre-

TABLE I

CALIBRATION DATA

Biological fluid	Concentration range (mg/l)	Mean C.V. of normalised peak height ratio (%)	
		Diflunisal ether glucuronide	Diflunisal ester glucuronide
Plasma (n = 20)	7.4—59.2	5.2	–
	2.8—22.00	–	5.1
Urine (n = 15)	4.7—75.2	2.9	3.2

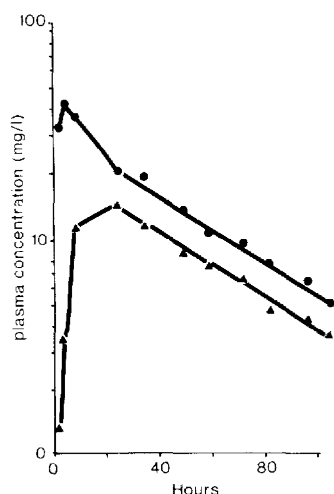

Fig. 4. The concentration of diflunisal (●) and diflunisal ether glucuronide (▲) in plasma of a patient who took a single 250-mg oral dose of diflunisal. No ester glucuronide was detected in plasma.

sence or absence of glucuronide in plasma but no calibration data are available for this mode of analysis. It is estimated that the minimum concentration detectable by fluorescence was in the order of 0.3 mg/l for the ester glucuronide and 0.1 mg/l for the ether glucuronide.

The method has been applied in the analysis of human samples (Fig. 4). Both diflunisal ether glucuronide and diflunisal, measured by a previously described method [6], are present in the plasma of a patient who took a single 250-mg dose of diflunisal, but no diflunisal ester glucuronide could be detected.

The data in Fig. 4 illustrate the difficulty of accurate glucuronide quantitation by indirect hydrolytic methods in the presence of high concentrations of aglycone. Hydrolysis of the glucuronides would result in an increase of approximately 5—10% in diflunisal concentration and if acid or β-glucuronidase and alkaline hydrolysis were carried out in order to estimate each glucuronide separately, this small increase would have to be partitioned between both compounds with a resulting accumulation of errors.

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