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ASSAY METHODOLOGY FOR QUANTIFICATION OF THE ESTER AND ETHER GLUCURONIDE CONJUGATES OF DIFLUNISAL IN HUMAN URINE

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SUMMARY

Diflunisal is a salicylate derivative with analgesic and anti-inflammatory properties. It is excreted in the urine as an ether glucuronide, a 1-O-acyl glucuronide and as unchanged drug. The 1-O-acyl glucuronide rearranges to isomeric esters of glucuronic acid under neutral to alkaline pH conditions. The development of a urine assay for the conjugates enables the elucidation of diflunisal non-linear pharmacokinetics. The assay quantitates the ether and ester glucuronides and free diflunisal in urine at 0.5—1.0 μg/ml. Analysis of the glucuronides does not require authentic standards.

INTRODUCTION

Diflunisal (2′,4′-difluoro-4-hydroxy-3-biphenylcarboxylic acid) is a salicylic acid derivative with analgesic and anti-inflammatory activity [1]. The elimination of diflunisal from the human circulatory system is concentration-dependent. Its major route of metabolism is glucuronidation to ether and ester conjugates (Fig. 1) [2—4]. A reliable assay method for the glucuronides is necessary to study the pharmacokinetics of the drug. Several assay methods are available for determining total and free levels of diflunisal in biological fluids [2, 5—10]. The assay method reported earlier [3] for the glucuronides did not achieve sufficient selectivity and specificity since it was based on solvent extraction and cross-contamination could not be avoided. A recently reported assay [11] using the isolated ether and ester glucuronides to calibrate concentrations did not consider the instability of the ester glucuronide and the unavailability of the standards.

The development of the assay methodology for the ether and ester glucuronides of diflunisal has resulted in different approaches of analysis due to

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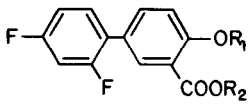


Diflunisal: $R_1 = H, R_2 = H$

Ether Conjugate: $R_1 = Glu, R_2 = H$

Ester Conjugate: $R_1 = H, R_2 = Glu$

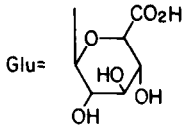


Fig. 1. Structures of diflunisal and its ether and ester glucuronide conjugates.

the lack of availability and the instability of the standards, and the acyl rearrangement of the ester glucuronide. Similar rearrangements have been reported for other drugs [12, 13]. The assay described herein requires no glucuronide standards. The concentration of the ether glucuronide conjugate (EtG) is extrapolated from a diflunisal calibration curve and a molecular weight conversion factor. The ester glucuronide (EsG) is initially hydrolyzed to diflunisal, its value is corrected for free diflunisal and the EsG concentration is determined from a diflunisal curve and an appropriate conversion factor. The EtG and EsG have been isolated for validation and stability data.

MATERIALS AND METHODS

Chemicals

Diflunisal and 5-(4′-fluorophenyl)salicylic acid were synthesized at Merck Sharp & Dohme Research Labs. [14]. Radioactive diflunisal* was synthesized at Merck Sharp & Dohme with ^{14}C in the carboxyl group [2]. Bovine liver β-glucuronidase, Type B-10, was obtained from Sigma.

Isolation of diflunisal glucuronides from human urine

A flow diagram in Fig. 2 depicting the isolation procedure indicates (a) an initial extraction of the urine with diethyl ether to remove diflunisal and waste, (b) urine pH adjustment to 1.0 with 1 *M* hydrochloric acid and (c) a second solvent extraction removing the EtG and EsG, plus some diflunisal. This latter organic extract (organic phase B) was further purified by extracting it with a sodium phosphate buffer (0.1 *M*) at pH 6.0 (2 vols.), readjusting the extract pH value to 1.0 with 1 *M* hydrochloric acid and finally back-extracting the analytes into diethyl ether (2 vols). The ether extracts (organic phase B′)* were concentrated to a thin oil-film and kept in the freezer.

The extracted glucuronide conjugates of diflunisal and diflunisal in the

*The glucuronide metabolites of diflunisal were not successfully synthesized at Merck Sharp & Dohme. The glucuronides were isolated from the urine of several healthy male subjects who received [^{14}COOH]diflunisal in 250-mg capsules in an earlier clinical study. Urine samples were collected in 6- or 12-h intervals for a maximum of fourteen days.

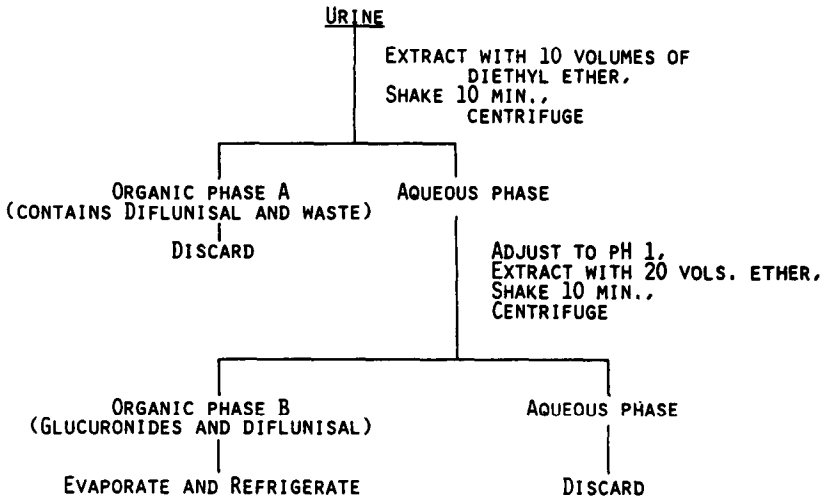


Fig. 2. Flow diagram for the procedure to isolate the two conjugates from urine.

organic phase B′ were further separated on thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) (see *HPLC conditions*). On TLC silica gel (250 μm) the components were resolved from each other by the solvent mixture acetone—toluene—*n*-butanol—glacial acetic acid—water (6:6:5:4:4). The relative retentions of the glucuronides on TLC were determined by radioautography: for the EtG, R_F = 0.43; EsG, R_F = 0.57; diflunisal, R_F = 0.94. Authenticity of the EtG and EsG was determined by mass spectrometry (MS) and co-chromatography (TLC and HPLC) with previously reported standards [2].

The radioactive glucuronides of diflunisal used in this investigation as analytical standards were isolated by extraction and TLC from clinical human urine samples*. The isolated standards were not purified further. The amount of glucuronide present in the isolates was determined from their radioactivity (^{14}C) and the specific activity of diflunisal (14.8 μCi per 250 mg). A typical TLC isolate (organic phase B′) was reconstituted in 1.0 ml of acetonitrile, and an aliquot (100 μl) was mixed with 10 ml of scintillation cocktail Aquassure from New England Nuclear and counted for 4.0 min on a Packard Tricarb liquid scintillation spectrometer.

HPLC conditions

Separation and quantification of analytes was performed on a reversed-phase Hypersil ODS column (30 cm × 5 mm I.D.) from Shandon Southern and a guard column Co:Pell ODS from Whatman. Two pumps Model 6000A and a system controller Model 720 from Waters Assoc. performed a linear gradient elution at 2 ml/min. Solvent A consisted of 30% methanol and 70% of dilute aqueous acetic acid (2%). Solvent B consisted of 65% methanol and 35% of dilute acetic acid (2%). The composition of the mobile phase changed from 60% solvent A and 40% B initially to 0% A and 100% B in 25 min. It flowed

*See footnote on p. 364.

at 100% B for an additional 6 min. Re-equilibration of the mobile phase back to 40% of B took 3 min.

Detection and integration involved an ultraviolet (UV) detector Model 441 (fixed-wavelength filter at 254 nm a.u.f.s.) and an integrator Model 730 (0.5 cm/min) from Waters Assoc. For comparison detection by fluorometry using a Schoeffel GM 970 (Fig. 7) was operated at λ_{ex} 215 and λ_{em} 440 nm (range 1.0 μA).

Procedure for quantification of EsG, EtG and free diflunisal in urine with an EtG standard and with the hydrolysis of EsG to diflunisal

Sample preparation. (1) Incubate a 1.0-ml urine sample with 1.0 ml of 0.25 *M* sodium hydroxide at ambient temperature for 1.5 h; (2) a second 1.0-ml aliquot of the above sample is mixed with 1.0 ml of water and 3 drops of glacial acetic acid from a pasteur pipet; (3) after 1.5 h of incubation 3 drops of glacial acetic acid are added to the above sample in step No. 1; (4) the internal standard 5-(4′-fluorophenyl)salicylic acid (30 μg/ml of urine) is added to both above samples in steps Nos. 2 and 3; (5) the above samples are centrifuged (657 *g*) and analyzed by HPLC.

Standards for calibration curves. A 1.0-ml aliquot of control urine is mixed with an appropriate volume of the EtG stock solution and of the diflunisal stock solution. The standard is prepared for analysis as discussed in the above steps Nos. 2—5.

Calculations for the ether, ester and free acid concentrations. Concentrations of the ether conjugate and the free acid of diflunisal are calculated from the chromatogram generated from the pre-hydrolyzed urine aliquot (in step No. 2). Peak area ratios are measured and the concentrations are extrapolated from the above standard curves. Concentration of the ester conjugate is the difference of the diflunisal concentration measured from the hydrolyzed urine aliquot (step No. 1 above) and the prehydrolyzed urine (No. 2). The difference is multiplied by 1.79.

Procedure for quantification of EsG, EtG and free diflunisal in urine without an EtG standard and without EsG hydrolysis

Sample preparation. (1) A 1.0-ml aliquot of a urine sample is mixed with 1.0 ml of water and 3 drops of glacial acetic acid; (2) the internal standard is added, and the sample is mixed, centrifuged and analyzed, as discussed above.

Standards for calibration curves. A 1.0-ml sample of control urine is mixed with an appropriate volume of the diflunisal stock solution. The standard is prepared for analysis as discussed above.

Calculations. The concentration of EtG is calculated from its peak area ratio (EtG/internal standard), a diflunisal standard curve and the molecular weight factor 1.79. The free diflunisal concentration is determined from its peak area ratio and the diflunisal standard curve. The EsG concentration is determined from the sum of the peak areas of EsG and rearrangement products divided by the peak area of the internal standard, a diflunisal curve and 1.79.

Chemical rearrangement

An aliquot (1 ml) from human clinical urine sample* was mixed 1:1 with

*Human clinical urine sample, 6—12 h collection interval, 500-mg dose of unlabeled diflunisal, p.o.

0.2 *M* potassium phosphate buffer, pH 7.5 giving a final pH of 7.4. The sample was placed in an autosampler vial and chromatographed repetitively over time at ambient temperature.

Similarly, an aliquot from the above urine sample was mixed 1:1 with 0.17 *M* potassium acetate buffer, pH 4.0, final pH 4.3. The sample was monitored as above over time.

Enzymatic hydrolysis

An aliquot from a clinical urine sample* was mixed with 0.17 *M* sodium acetate buffer, pH 4.0 (1:1, v/v). An aliquot from the same sample was mixed with the buffer and β-glucuronidase (2 mg/ml). The two samples were incubated at 37° C over 12 h.

Stability studies

(A) An aliquot from a clinical urine sample* was mixed with 1.0 ml of 0.25 *M* sodium hydroxide. An aliquot from the same sample was mixed with 0.17 *M* sodium acetate buffer, pH 4. The two samples were allowed to stand at room temperature over 4.28 h.

(B) The TLC-isolated EtG and EsG of diflunisal were incubated (70 μg/ml and 90 μg/ml, respectively) at ambient temperature in acetonitrile, methanol, ethanol, mobile phase (solvent B) and aqueous buffers at pH 1.19 (hydrochloric acid—potassium chloride buffer, 0.2 *M*), pH 2.77 (citrate—phosphate buffer, 0.1 *M* and 0.2 *M*), pH 4.88 and pH 6.93. The incubates were monitored by HPLC at 3, 6, 9, and 12 h.

(C) Aliquots (1 ml) from a clinical urine sample* were adjusted to pH 4.0, 5.8, 6.9 and 7.6 with a sodium acetate buffer (pH 4.0, 0.17 *M*) and potassium phosphate buffers (pH 5.9, 7.0 and 8.0, 0.2 *M*) (1:1, v/v). The 1-O-acyl glucuronide (89.3 μg/ml) was monitored by HPLC over time at room temperature.

Comparison of ultraviolet and radioactivity chromatograms

A human urine collection sample (6—12 h)** was extracted (organic phase B′, discussed earlier) concentrated and reconstituted in mobile phase. A 30-μl aliquot was injected into the HPLC system and fractions were collected every 15 sec up to 30 min. The 15-sec fractions were mixed with 10 ml of scintillation cocktail and counted for 4.0 min. The UV chromatogram was monitored at 254 nm.

RESULTS AND DISCUSSIONS

The development of an assay for the EtG and EsG glucuronides of diflunisal in urine required their initial isolation [2], characterization and stability studies. Characterization involved co-chromatography by TLC and HPLC of the glucuronide standards isolated by Tocco et al. [2]. Additional mass spectral (MS) data were obtained to substantiate previous results and identify rearrange-

*See footnote on p. 366.

**See footnote on p. 364.

ment products of the EsG. Stability data dictated the direction of the assay development for the EsG. Poor stability of the conjugates suggested the development of an assay without standards.

Stability studies

Room temperature (22—24°C) stability studies of the isolated EtG reveal less than 5% loss over 12 h in all aqueous buffers and organic phases tested, except in neat methanol and ethanol (Tables I and II). The EsG shows similar results except in a pH 6.93 phosphate buffer. The degradation of glucuronide conjugates in neat alcohols has been reported for other drugs [12, 13, 15]. For the ester conjugate, losses may result from nucleophilic substitution during acyl

TABLE I

SOLVENT STABILITY OF THE EtG AND EsG AT ROOM TEMPERATURE

Time (h)	Percent remaining			
	Aceto-nitrile	Mobile phase*	Methanol	Ethanol
EtG				
3	100.0	100.0	99.4	97.2
6	97.6	97.6	94.1	95.0
9	95.2	96.4	96.0	94.1
12	96.4	95.7	92.0	93.0
EsG				
3	98.3	98.3	97.3	94.6
6	98.1	100.0	96.5	93.4
9	97.6	100.1	94.7	91.4
12	93.0	100.1	93.0	91.3

*Solvent B, see Materials and methods.

TABLE II

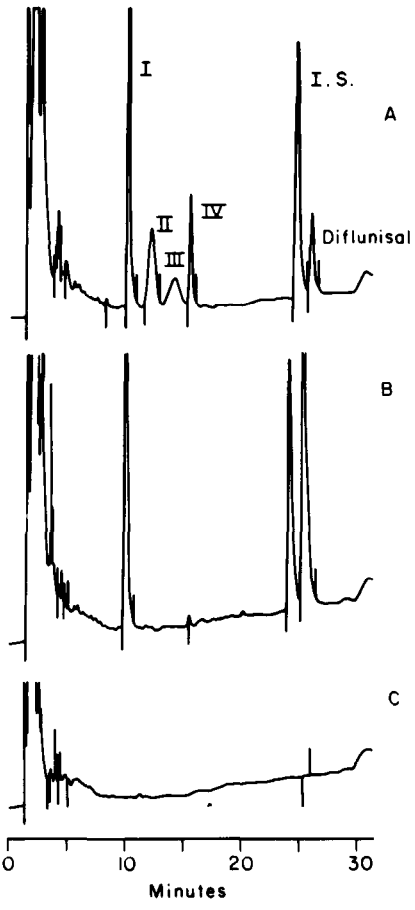
pH STABILITY OF THE EtG AND EsG AT ROOM TEMPERATURE

Time (h)	Percent remaining			
	pH* 1.19	pH 2.77	pH 4.88	pH 6.93
EtG				
3	97.0	98.1	100.0	100.0
6	97.0	100.0	100.0	96.8
9	96.0	100.0	98.6	97.6
12	98.2	100.0	96.4	95.6
EsG				
3	99.0	97.3	100.0	74.0
6	97.2	97.6	100.0	48.0
9	97.2	100.0	98.0	39.0
12	97.5	97.8	95.0	24.0

*pH of aqueous buffers; see Materials and methods.

rearrangement. In the mobile phase, however, which contains methanol, acetic acid and water (solvent B, pH 3.4) observed losses are less than 5%.

In pH 6.93 phosphate buffer only 24% of the EsG remains after 12 h. In urine with pH adjustments, the half-life of the EsG changes dramatically from 300 h at pH 4.0 to 39.5 min at pH 7.6, respectively, at room temperature. At pH 5.8 the half-life is 37.5 h, and at pH 6.9 it is 2.42 h. Without pH adjustment of the above urine matrix (pH 5.2—5.3), 23% of the EsG is lost over 43 h (89.3 μg/ml). The extrapolated half-life is 135 h. Over the same period with addition of citric acid (50 mg/ml) or glacial acetic acid (3 drops/ml), less than 5% is lost.



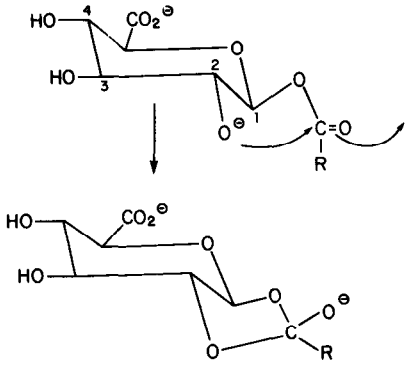


Fig. 3. (A) Chromatogram (before hydrolysis) of a urinary clinical sample containing the EtG of diflunisal (I), EsG rearrangement products (II and III), 1-O-acyl-glucuronide (IV), internal standard and diflunisal. The ether conjugate and free diflunisal are quantitated from this sample. (B) Chromatogram of the above urine sample subjected to alkaline hydrolysis. It contains the ether conjugate (I), internal standard and diflunisal. The ester conjugate is quantitated from this diflunisal peak minus the free diflunisal concentration (from A). (C) Chromatogram of a urine blank before hydrolysis.

Fig. 4. 1,2-*Ortho* acid ester intermediate, proposed mechanism of ester rearrangement [16].

Characterization of the conjugates

The EtG and EsG of diflunisal have been previously isolated from human urine by TLC and characterized by MS [2]. The isolation techniques have been slightly modified (see flow diagram, Fig. 2) resulting in a procedure that clearly separates the EtG and EsG and free diflunisal from urine and each other (TLC: R_F = 0.43, 0.57 and 0.94, respectively). HPLC methodology has been developed that similarly separates the above components (t_R = 10.1, 15.4 and 25.8 min, respectively). Co-chromatography of the standards isolated by Tocco et al. [2] in the above systems supports our assignment of the TLC spots and HPLC peaks. In addition, there exist in the chromatograms (Fig. 3A) of human urine samples from volunteers administered diflunisal other HPLC peaks between the ether and ester conjugates that did not exist in the predose samples (Fig. 3C). Reports from the literature of EsG rearrangements occurring

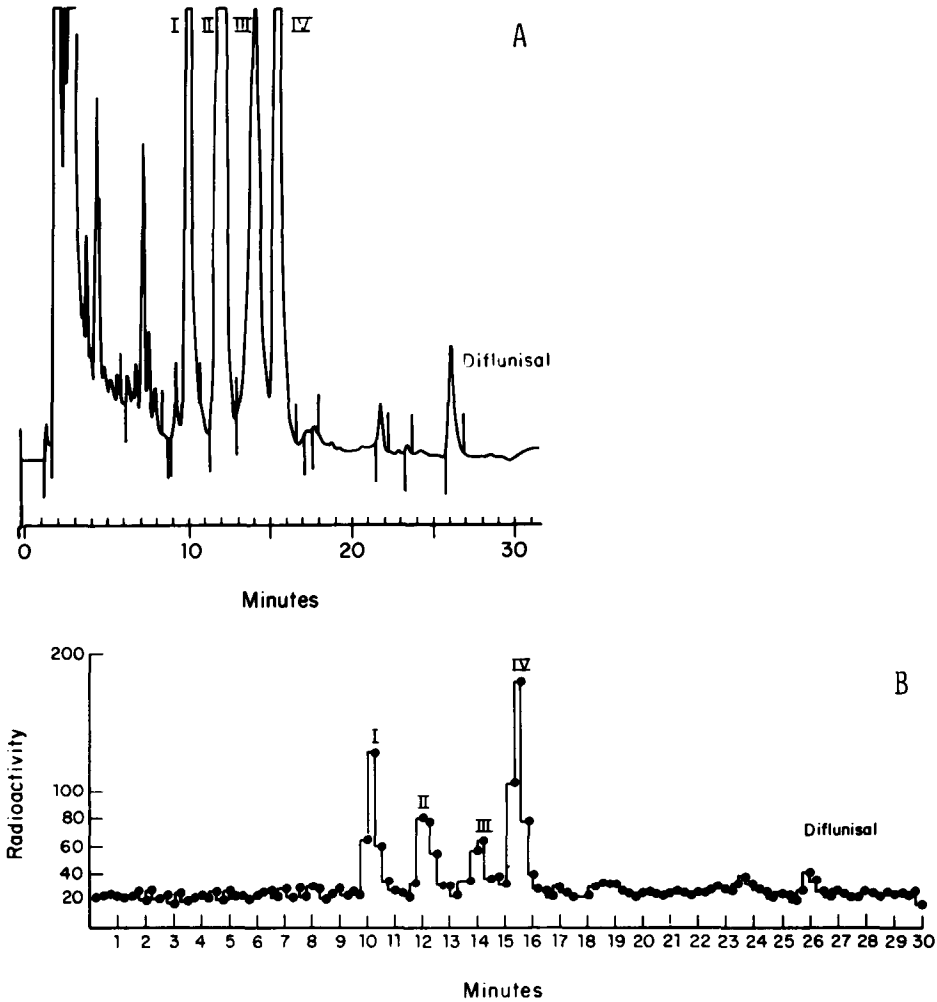


Fig. 5. UV chromatogram (A) versus a radioactivity chromatogram (B) of a human urine collection (6—12 h) post dose (a 250-mg, oral administration of radiolabeled diflunisal). Peaks: 1 = ether conjugate, IV = ester, II & III = ester rearrangements.

with other drug conjugates [12, 13] suggest a similar phenomenon may be happening for the ester conjugate of diflunisal. The 1-O-acyl conjugate would pass through a 1,2-*ortho* acid ester intermediate [13, 16] going to 2-, 3- and 4-O-esters of glucuronic acid (Fig. 4).

Confirmation of the rearrangement products of diflunisal in the urine of volunteers given radiolabeled diflunisal is depicted in a close comparison of a UV chromatogram and a radioactivity chromatogram (Fig. 5). The residue of a human urine extract (organic phase B′) is chromatographed with 15-sec eluent fractions collected and counted. The chromatographic peaks in Fig. 5 are denoted (henceforth) as I, II, III, and IV. I represents the EtG and IV represents the EsG, isolated and identified by Tocco et al. [2]. II and III represent the acyl rearrangements of IV.

The isomerization is further supported by the observation that the loss of the ester peak (IV) corresponds to the appearance of two broad peaks (II and III) overtime (Fig. 6) in a human urine sample. The loss of IV seems to be irreversible to II and III with slow hydrolysis to diflunisal.

Additionally, the literature [12, 13, 17] reports that only the 1-O-acyl

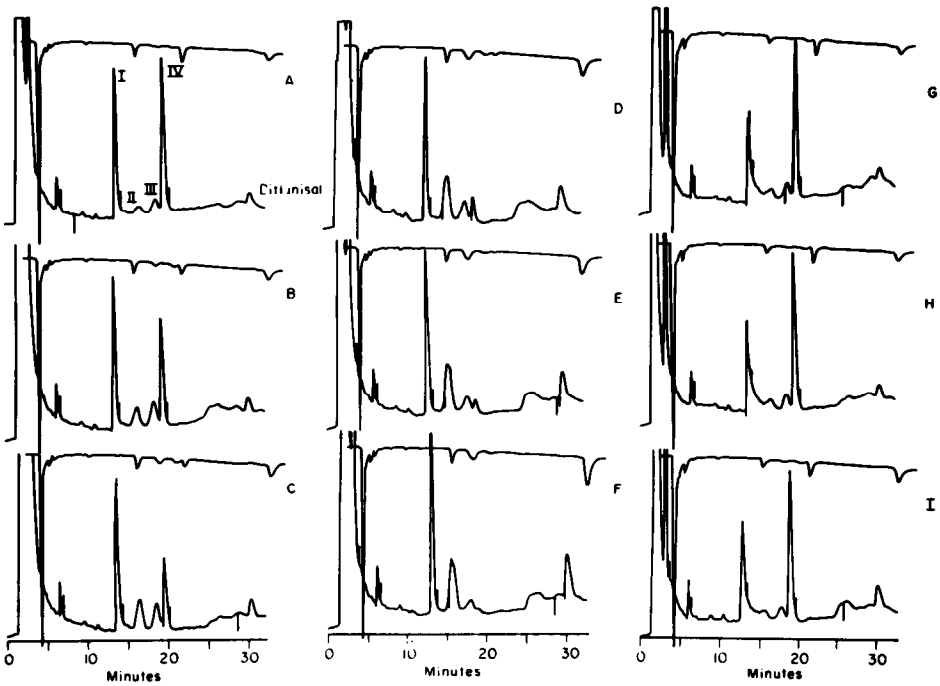


Fig. 6. Chemical rearrangement and hydrolysis of the EsG. (A) Chromatogram of a clinical human urine sample at pH 7.4 (0.2 *M* potassium phosphate buffer at pH 7.5, and urine, 1:1, v/v) at T = 0 min at room temperature; (B) chromatogram of the same sample at T = 77 min; (C) T = 154 min; (D) T = 353 min; (E) T = 505 min; (F) T = 1360 min; (G) chromatogram of the above sample at T = 0 min, pH 4.3 (0.17 *M* sodium acetate buffer at pH 4.0, and urine, 1:1, v/v) at room temperature; (H) T = 78 min; (I) T = 1323 min. The 1-O-acyl glucuronide (IV) rearranges to peaks II and III. The ester isomers slowly hydrolyze to diflunisal. The top tracing accompanying each UV chromatogram is the fluorometer response. The comparison shows similar detector response for diflunisal, but much less for the glucuronide conjugates.

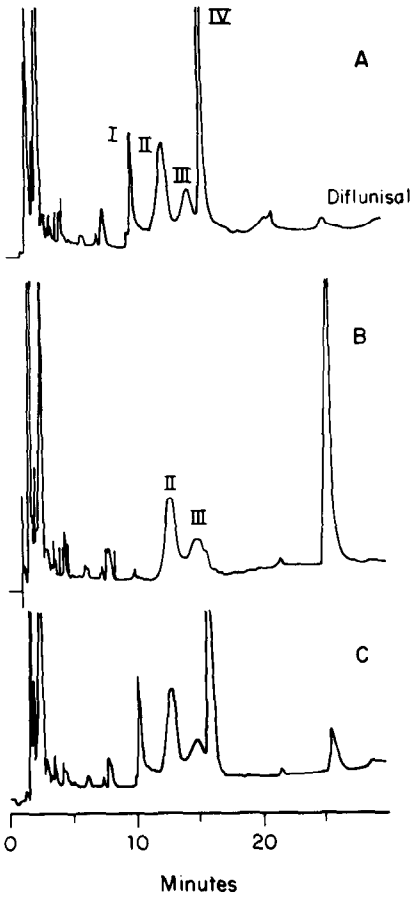


Fig. 7. Enzymatic hydrolysis of the EsG and EtG by β-glucuronidase. (A) Chromatogram of a clinical human urine sample, at pH 4.3 (0.17 *M* sodium acetate buffer at pH 4.0, and urine, 1:1, v/v); (B) chromatogram of the above sample incubated over 12 h at 37°C with β-glucuronidase; (C) chromatogram of the above sample incubated without β-glucuronidase over 12 h at 37°C. The study suggests (supports previous findings [17]) that peaks I and IV are naturally forming glucuronides of diflunisal. Peaks II and III (may be peak clusters) are rearrangements of the ester conjugate (migration of diflunisal from C_1 to C_2, C_3, C_4 on the sugar moiety).

glucuronides can be enzymatically hydrolyzed. The enzymatic hydrolysis was performed on a clinical sample at pH 4.0 containing 1—IV (Fig. 7). Only peaks I and IV completely disappear within an hour. A control at pH 4.0 incubated without β-glucuronidase is not affected. The results suggest peaks I and IV are C_1-O-conjugates and II and III are rearrangements of IV. Since the samples are incubated for 24 h at 37°C and there is no loss of II and III, the migration of diflunisal from C_1 to C_2, C_3 and C_4 appears to be irreversible. Peaks II and III are broader than I and IV and suggest the presence of multiple isomers ($\geqslant 2$) of IV (2-, 3- and 4-O-esters of glucuronic acid).

Final evidence for peaks II and III involved MS analysis following their isolation by HPLC from organic phase B′ and concentration by high vacuum to residues. Electron-impact mass spectra of underivatized II, III and IV gave

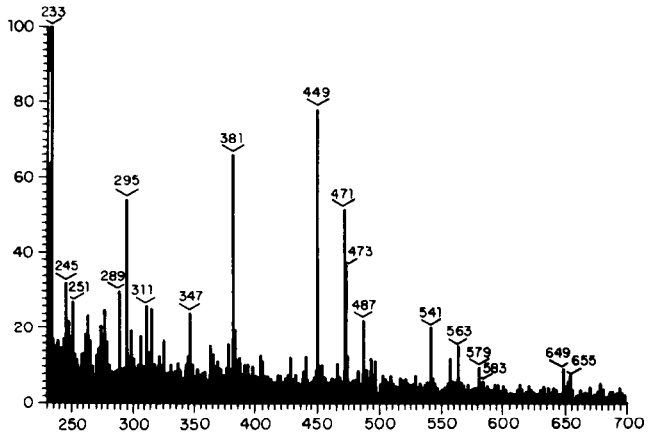


Fig. 8. Fast atom bombardment mass spectrum of IV. HPLC peaks II—IV were collected individually and concentrated under vacuum to a residue. The residue was analyzed by electron-impact and FAB MS.

identical mass fragmentation ions. Fast atom bombardment (FAB) MS (MS analysis was performed on a Varian Mat 731 mass spectrometer with a FAB ion source using a glycerol matrix) gave mass ions at *m/z* 427, 449, 471 and 487 which correspond to the molecular weight plus one (M + H), molecular weight plus sodium (M + Na), M + Na_2 and M + Na + K for II, III and IV, respectively (Fig. 8).

Assay methodology

During the development of an assay for the glucuronides three approaches were considered: (a) the use of the isolated EtG and EsG (1-O-acyl) as standards (as described in the Materials and methods); (b) the use of the isolated EtG only as a standard and hydrolyzing the EsG to diflunisal; and (c) calibrating the ether and ester peak areas (ratios) against a diflunisal standard curve. The first approach was abandoned because of the ester's vulnerability to hydrolysis and rearrangement (human clinical samples contained II and III).

TABLE III

STABILITY OF DIFLUNISAL'S CONJUGATES IN DILUTE BASE

Human clinical sample (6—12 h after dose) is mixed with 0.25 *M* sodium hydroxide (1:1).

Time (h)	Percent remaining				
	I	II	III	IV	Diflunisal
0*	100	100	100	100	100
0.12	98.4	106.8	112.5	26.4	152.7
0.73	96.2	36.0	25.0	7.1	409.1
1.35	98.4	18.0	0	0	490.9
2.08	100.7	0	0	0	518.2
2.82	96.2	0	0	0	463.6
4.28	96.2	0	0	0	477.3

*Clinical sample (above sample) mixed with 0.17 *M* sodium acetate buffer, pH 4.0.

The second was developed and applied to samples. The third was compared to the second.

The second approach involves the hydrolysis of the ester IV and the rearrangement products to diflunisal without affecting the EtG. The effects of 0.125 *M* sodium hydroxide on the five components in urine is summarized in Table III. By 1.5 h the ether conjugate has not hydrolyzed and peaks II—IV are completely gone. Also, the area ratio of the summation of peaks II—IV to I before hydrolysis and the area ratio of diflunisal with I after hydrolysis of the same samples are 1.177 ± 0.117 (n = 3) and 1.179 ± 0.036 (n = 3), respectively. A ratio of the above two numbers 0.9983 points to equal molar absorptivity for the EsGs (peaks II, III and IV) and diflunisal. Since the assay for the EsG involves quantitating diflunisal before and after hydrolysis, the integrity of diflunisal in 0.125 *M* sodium hydroxide was tested further in urine. Less than 3% degradation was observed over 9.6 h.

A typical urine sample collection would be split: one aliquot would be treated with 0.25 *M* sodium hydroxide (Fig. 3B); the second with glacial acetic acid or citric acid (Fig. 3A). After 1.5 h the alkaline aliquots would be treated with acid. The internal standard would be added at this stage and particulates centrifuged. A chromatogram of the second aliquot would yield data on the EtG and free diflunisal. The first would give a diflunisal concentration consisting of the ester equivalent and free diflunisal. The EsG concentration is the difference times a molecular weight factor. A standard calibration curve would consist of the EtG, diflunisal and internal standard. Sensitivity, precision and linearity are presented in Table IV. Urines from two subjects who received 500 mg of diflunisal orally were analyzed by the second method. The EtG and EsG concentrations and free diflunisal are listed in Table V. Accumulative

TABLE IV

ASSAY CHARACTERIZATION

Analyte	Minimum detection limit (μg/ml)	Precision		Linear regression analysis
		Concentration (μg/ml)	Coefficient of variation (%)	
EtG*	0.5	1.88	6.0	$r = 0.9978$
		3.77	6.8	$y = 0.218x - 0.010$
		18.9	5.2	
		70.3	3.7	
EsG**	1.0	3.2	8.0	$r = 0.9994$
		6.4	8.1	$y = 0.0324x - 0.010$
		32.0	3.14	
Diflunisal	0.5	1.7	5.8	$r = 0.9966$
		8.5	7.4	$y = 0.0512x - 0.0645$
		34.0	4.9	

*Based on standards containing the isolated ether conjugate. The concentration of the ether is calculated from the specific activity of the labeled conjugate.

**Based on standards containing the hydrolyzed ester conjugate. The concentration of the ester is caluclated from the specific activity of the labeled conjugate.

TABLE V

REPRESENTATIVE HUMAN CONCENTRATION LEVELS (μg/ml) OF THE EsG AND EtG OF DIFLUNISAL AND FREE DIFLUNISAL

Subjects received a single oral dose of 500 mg of diflunisal.

Time interval (h)	EtG based on standards	EtG based on diflunisal*	EsG based on diflunisal after hydrolysis	EsG based on diflunisal before hydrolysis**	Free diflunisal
Subject 1					
0	0	0	0	0	0
0—3	200.7	200.1	446.8	388.3	32.3
3—12	458.2	459.4	560.1	547.6	62.6
12—24	199.4	199.3	144.1	144.6	19.7
24—36	81.0	82.1	119.6	121.2	14.2
36—48	10.2	12.1	15.9	19.8	0
48—72	6.15	8.0	13.8	11.49	0
72—96	0	0	0	0	0
Subject 2					
0	0	0	0	0	0
0—3	137.3	146.1	243.9	251.9	13.2
3—6	78.7	83.4	149.8	121.7	7.11
6—12	53.5	56.6	89.2	70.9	7.68
12—14	124.6	132.5	146.1	111.70	12.2
24—36	19.6	20.4	29.5	28.3	3.19
36—48	30.7	32.2	51.0	49.1	0
48—72	7.83	7.74	12.1	11.8	0
72—96	0	0	0	0	0

*Concentrations are determined from peak area ratios of the ether, a diflunisal calibration curve and a conversion factor (1.79).

**Concentrations are determined from the peak area ratios of the ester peaks (includes rearrangements), a diflunisal curve and a conversion factor.

urinary levels of subject 2 are plotted in Fig. 9. Dose recovery is 95.7% for subject 1 and 77.7% for subject 2.

The third approach for the assay methodology involves extrapolating a conjugate concentration (with an appropriate peak area ratio) from a diflunisal curve and a molecular weight factor. From the area of peak I, the EtG concentration is determined; from the sum of the areas of peaks II—IV, the ester is determined. The approach requires the chromatography of only one stabilized urine sample. No standards are needed. The methodology (e.g. relative response factors, linearity, etc.) has been evaluated with the EtG initially by comparing concentrations of prepared standards (see Materials and methods) with respective concentrations calculated from the peak area ratios of the EsG standards, a diflunisal standard curve and 1.79 (Table VI). The maximum difference over the linear concentration range 1.88—70.3 μg/ml is 27.7% at 1.88 μg/ml. The average ratio of standard concentrations (based on radioactivity) to extrapolated over the concentration range is 1.005 ± 0.156. Table V compares clinical sample concentrations of subjects 1 and 2 (discussed

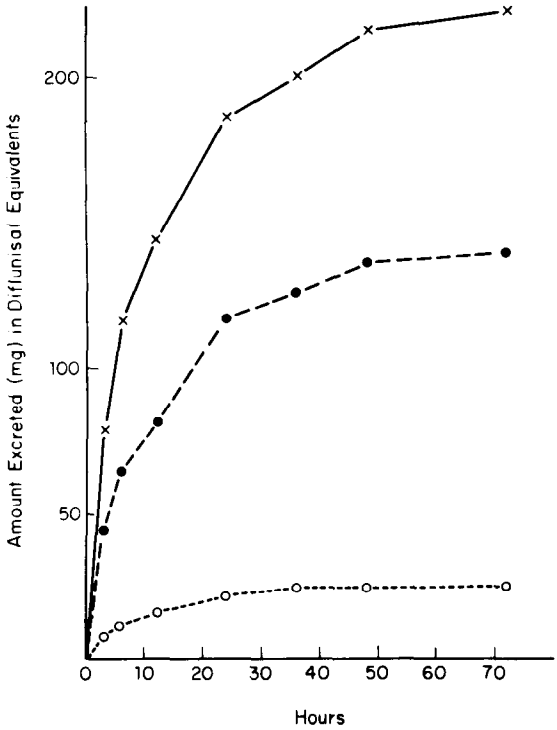


Fig. 9. Accumulative urinary levels of the ether and ester conjugate and free diflunisal of a single subject given a 500-mg oral dose of diflunisal. (•) EtG; (×) EsG; (○) free diflunisal.

TABLE VI

EtG: A COMPARISON CONCENTRATIONS BASED ON RADIOACTIVITY AND ON DIFLUNISAL

x: Concentration based on radioactivity* (μg/ml)	y: Concentration based on diflunisal** (μg/ml)	y/x
1.88	2.4	1.28
3.77	3.86	1.02
9.43	7.75	0.82
18.9	17.6	0.93
37.7	35.4	0.94
70.3	73.4	1.04

Mean ± S.D.: 1.005 ± 0.156

r^2 = 0.9978

y = 0.0218x—0.010

*Standards were prepared with the isolated, ^{14}C-labeled ether conjugate.

**The concentrations were extrapolated from the peak areas of the ether standards, a diflunisal calibration curve and the molecular weight factor 1.79.

earlier) based on an EtG standard and on a diflunisal calibration curve. The average concentration difference of both approaches over all time intervals for both subjects is 6.6 ± 8.6%.

A similar evaluation has been made of the EsG assay over the concentration range 2.4—96.2 μg/ml. The average ratio of standard concentrations (based on radioactivity) to extrapolated before hydrolysis (Table VII) is 1.15 ± 0.4. Concentrations extrapolated after hydrolysis have an average ratio of 0.99 ± 0.09.

TABLE VII

EsG: A COMPARISON OF CONCENTRATIONS BASED ON RADIOACTIVITY AND ON DIFLUNISAL

x: Concentration based on radioactivity*	y: Concentration based on diflunisal before hydrolysis**	y/x	z: Concentration based on diflunisal after hydrolysis***	z/x
2.4	4.7	1.96	2.41	1.0
4.81	5.8	1.21	5.44	1.13
12.0	11.4	0.95	11.9	0.99
24.0	22.6	0.94	21.0	0.87
48.0	45.2	0.94	44.8	0.93
96.2	84.2	0.88	95.5	0.99
Mean ± S.D.		1.15 ± 0.40		0.99 ± 0.09

*Standards were prepared with the isolated, labeled EsG.
**The concentrations were extrapolated from the sum of the areas of peaks II—IV, a diflunisal curve and the conversion factor 1.79.
***The concentrations were extrapolated from the areas of diflunisal peaks after hydrolysis, a diflunisal curve conversion and the factor 1.79.

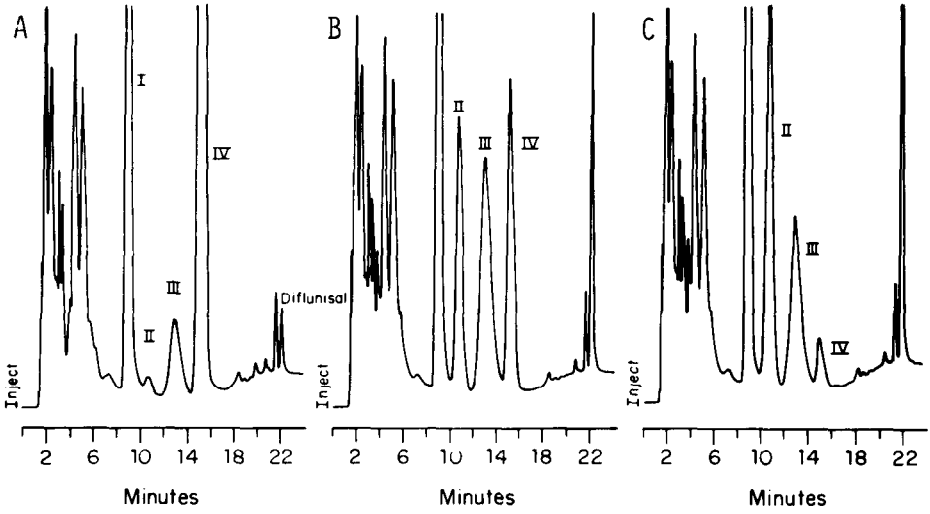


Fig. 10. Rearrangement of the 1-O-acyl glucuronide of diflunisal in rat bile. Chromatograms of (A) a rat bile sample, injected into the HPLC 5 min after collection; (B) the same sample injected 1 h later; (C) the same sample injected 2 h later. The pH of this sample is 6.8.

Table V compares clinical sample concentrations of the EsG based on diflunisal after hydrolysis and on diflunisal before hydrolysis. The average concentration difference for both subjects is 10.3 ± 9.3%.

The preferred assay should be the latter discussed above. However, if the rearrangement products of the EsG exist in the urine samples, summing the areas of peaks II—IV for the EsG may compromise the assays minimum sensitivity and its accuracy at the lower concentrations. A combination of parts of the second and third approach might be more appropriate. The EtG concentration would be extrapolated from a diflunisal curve and the EsG would be hydrolyzed to diflunisal for quantification. The rearrangement of the 1-O-acyl glucuronide conjugate in general and of diflunisal in particular has not been determined to be exclusively a chemical isomerization (versus enzymatic). Although stabilization of diflunisal's 1-O-acyl glucuronide in urine after collection can be accomplished with acid treatment, stabilization of the biological samples before (in vivo) acyl rearrangement would not be possible. The rearrangement products are observed in the urine and bile of rats administered diflunisal (30 mg/kg intravenously) collected in the presence of acetic acid. The acyl rearrangement occurs quickly in rat bile (pH 6.8) not treated with acid (Fig. 10). With the acid, there is no change over the same time period.

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