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Note

Determination of diflunisal in human plasma and urine by fast high-performance liquid chromatography

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Diflunisal is a salicylic acid derivative with similar analgesic and anti-inflammatory properties to aspirin. Several assays have been reported in the literature for the analysis of this compound in biological fluids [1, 2]. A gas chromatographic assay [3] was used, but it involved solvent extraction and derivatization prior to analysis. Some high-performance liquid chromatographic (HPLC) assays have been reported using solvent extraction [4—9] and ultraviolet detection [6, 8]. Fluorescence detectors have also been used and gave higher sensitivity than ultraviolet detectors [10—12]. The present method uses reversed-phase HPLC with fluorescence detection. The sample clean-up involves protein precipitation and subsequent direct injection for both plasma and urine. The method is sensitive and there is no interference from endogenous compounds. The drug (I) and the internal standard (II) both give excellent peak shapes and the total HPLC analysis time is less than 2 min.

I II

EXPERIMENTAL

Materials

HPLC-grade methanol and acetonitrile were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Ammonium dihydrogen phosphate, phosphoric acid (85%) and perchloric acid (70%) were either reagent grade or HPLC grade and supplied by Fisher (Pittsburgh, PA, U.S.A.). Sodium hydroxide was obtained from Mallinckrodt (Paris, KY, U.S.A.).

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Equipment

In this study, a Varian 5000 high-performance liquid chromatograph with a built-in column heater was connected to a Perkin-Elmer 650-10S fluorometer, a Spectra-Physics SP4270 integrator, and a Waters WISP 710B autosampler. The analytical column used was a 3 μm particle size, 5 cm × 4.6 mm Sepralyte C_{18} from Analytichem International (Harbor City, CA, U.S.A.).

Chromatographic conditions

The aqueous part of the mobile phase was ammonium dihydrogen phosphate (0.05 *M*) and phosphoric acid (0.01 *M*), producing a final pH of 3.5. The organic modifier used was methanol. The ratio of aqueous—organic in the mobile phase was 42:58. The column was maintained at 65°C at a flow-rate of 1.5 ml/min. The injection volumes used were 10 μl for plasma and 12 μl for urine. The fluorometer excitation and emission wavelengths were set at 260 nm and 418 nm, respectively. The slits were set at 15 nm and the sensitivity range set at 0.1.

Standard and internal standard solutions

Diflunisal and the internal standard, a chloro analogue of diflunisal, were supplied by Merck Sharp & Dohme Research Labs. (Rahway, NJ, U.S.A.). A stock standard solution of diflunisal (1 mg/ml) was prepared in acetonitrile—water (80:20). This stock solution was further diluted in the same solvent to give a series of working standards. The concentrations were 200, 150, 100, 50, 25, 12.5 and 5 μg/ml for plasma and 400, 300, 200, 100, 50, 25 and 10 μg/ml for urine. A stock solution of internal standard (1 mg/ml) was prepared in the same solvent as diflunisal. The stock solution was further diluted to give working standard solutions for plasma (100 μg/ml) and urine (150 μg/ml).

Plasma samples

The plasma standard line was prepared using blank human plasma (100 μl, Sera-Tec Biologials, North Brunswick, NJ, U.S.A.), diflunisal working standard (100 μl), and working internal standard (50 μl). To this mixture phosphoric acid (250 μl, 0.1 *M*) and acetonitrile (750 μl) were added. The tube was then vortex mixed for 10 s and centrifuged (15 min at 2000 *g*). The supernatant was decanted into an autosampler vial and injected for HPLC analysis.

The plasma samples were prepared in the same way as the standards, substituting patients' plasma (100 μl) for blank plasma and acetonitrile—water (80:20, 100 μl) for working diflunisal standard.

Urine samples

The urine standard line was prepared using blank human urine (50 μl), diflunisal working standard (50 μl) and working internal standard (100 μl). Perchloric acid (70%, 150 μl) was added and the mixture heated on a dry block heater (1 h at 90°C). The mixture was cooled to room temperature and sodium hydroxide (5 *M*, 500 μl) added followed by acetonitrile (200 μl). The solutions were then vortex mixed and centrifuged (15 min at 2000 *g*). The supernatant was decanted into an autosampler vial and injected for chromatographic analysis.

The samples were prepared in the same way as the standards, substituting patients' urine (50 μl) for blank urine and acetonitrile—water (80:20, 50 μl) for working diflunisal standard.

RESULTS AND DISCUSSION

Diflunisal and the internal standard, a chloro analogue of diflunisal, are both naturally fluorescent. They were easily detected in plasma and urine at therapeutic concentrations with no interference from endogenous compounds. Excellent chromatography could be achieved on a C_{18} reversed-phase HPLC column and the total analysis time was 2 min. The column temperature may be reduced to below 65°C with a corresponding increase in total analysis time. The only limitation in the choice of mobile phase was that methanol had to be used as the organic modifier rather than acetonitrile; poor peak shapes were obtained with acetonitrile. Typical chromatograms for plasma and urine are shown in Figs. 1 and 2, respectively. Diflunisal had a retention time of 1.4 min and the internal standard 1.9 min. The peak shapes were excellent in both cases and there was no endogenous interference from plasma or urine.

Preparation of plasma samples for analysis was extremely simple. Acetonitrile was added to precipitate proteins, the sample centrifuged, and the supernatant injected. No further clean-up was required. The recovery of diflunisal and the internal standard was ≥ 98% throughout the range of the standard line. This was checked by spiking the standards into water and carrying them through the same sample preparation procedure as plasma. The volume of acetonitrile added to the 100-μl volume of plasma was found to be critical. A smaller volume gave incomplete protein precipitation. A larger volume

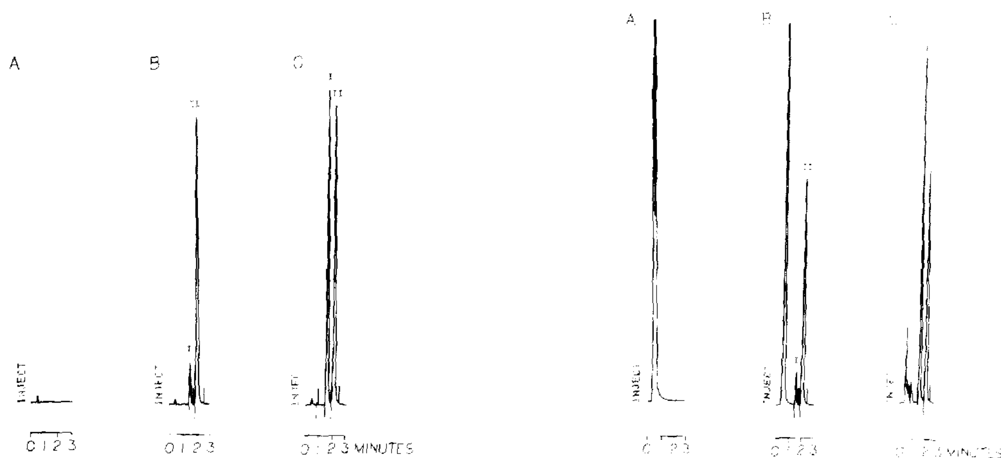

Fig. 1. Typical chromatograms for diflunisal in plasma. (A) Blank plasma; (B) blank plasma containing 5 μg/ml diflunisal (I) and 50 μg/ml internal standard (II); (C) patient plasma containing 37 μg/ml diflunisal and 50 μg/ml internal standard following an oral dose of 250 mg of diflunisal.

Fig. 2. Typical chromatograms for diflunisal in urine. (A) Blank urine; (B) blank urine containing 10 μg/ml diflunisal (I) and 150 μg/ml internal standard (II); (C) patient urine containing 106 μg/ml of diflunisal and 150 μg/ml internal standard.

increased the ratio of organic—aqueous in the injection solution and gave poor peak shapes. This has been a general observation in our laboratory for assays involving protein precipitation with acetonitrile. The concentration of water in the injection solution should be as high as possible. When acetonitrile supernatants have been injected that have given poor peak shapes the problem was usually rectified by the addition of an equal volume of water to the acetonitrile solution, and then increasing the injection volume to compensate for the dilution of the sample.

Urine samples were first hydrolyzed with perchloric acid and then assayed by HPLC. This procedure ensured that the ether- and ester-linked glucuronide metabolites of diflunisal were converted back to diflunisal. Other workers [8, 13] have reported assays for all three species simultaneously, but in most of the clinical studies involving diflunisal the total concentration of diflunisal in urine has been required. Diflunisal and its internal standard were both stable to this acid hydrolysis procedure.

The linearity of the standard lines (5—200 μg/ml for plasma and 10—400 μg/ml for urine) was confirmed. The method used no solvent extraction or solid phase extraction procedures in the sample preparation stages and consequently high correlation coefficients (> 0.9999) could easily be achieved for the standard lines. The limits of detection for this method were 2 μg/ml for both plasma and urine. This was adequate to measure plasma and urine levels of diflunisal following therapeutic doses of the drug (250—500 mg). The sensitivity of the assay could easily be increased if necessary. The procedure described only used 100 μl of plasma and a 10-μl injection volume. Both of these volumes could be increased with a corresponding increase in sensitivity of the assay. The precision of the assay was confirmed by running interday variation tests. This

TABLE I

INTERDAY VARIATION OF DIFLUNISAL IN PLASMA AND URINE ANALYSIS

Concentration (μg/ml)	Mean ratio drug/internal standard (n = 4)	S.D.	R.S.D. (%)
Plasma			
5	0.0574	0.0011	1.9
12.5	0.1703	0.0022	1.3
25	0.3438	0.0043	1.3
50	0.6769	0.0126	1.9
100	1.3617	0.0128	0.9
150	2.0699	0.0262	1.3
200	2.7501	0.0341	1.2
Urine			
10	0.0359	0.0012	3.3
25	0.1054	0.0009	0.9
50	0.2120	0.0016	0.7
100	0.4227	0.0034	0.8
200	0.8367	0.0097	1.2
300	1.3094	0.0088	0.7
400	1.6959	0.0109	0.6

TABLE II

QUALITY CONTROL SAMPLES FOR DIFLUNISAL IN PLASMA AND URINE

	Plasma		Urine	
	Low (target = 12.5 μg/ml)	High (target = 150 μg/ml)	Low (target = 25 μg/ml)	High (target = 300 μg/ml)
Mean (μg/ml)	13.1	153.3	26.1	310.1
S.D.	0.3050	2.4199	0.2510	3.0655
R.S.D. (%)	2.3	1.6	1.0	1.0
n	9	10	5	5

was carried out by running standard lines on five separate days. The ratio of drug to internal standard was computed for each point on the standard line to give the mean, standard deviation and percent relative standard deviation. The results are given in Table I. As can be seen, the relative standard deviation (R.S.D.) values were very low for all points on the plasma and urine standard lines ($\leqslant$ 1.9% for plasma and $\leqslant$ 3.3% for urine). The accuracy of this method was checked by running quality control samples of known concentration throughout the assay of patients' samples. The results are summarized in Table II. The values obtained were correct and the R.S.D. very low in each case (all $\leqslant$ 2.3%).

This assay has proved to be extremely simple and rugged. In excess of 1000 clinical samples have been run on a single HPLC column with no loss of chromatographic resolution. The 2-min analysis time has also proved to be useful for the assay of a large number of samples in a short time. The method is particularly suitable for routine analysis of diflunisal in clinical samples, but could also be extended to other applications.

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