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Note

Rapid estimation of diflunisal in plasma and urine by high-performance liquid chromatography and a comparison with a fluorometric method

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Diflunisal (2′,4′-difluoro-4-hydroxy-3-biphenyl-carboxylic acid) is a recently introduced derivative of salicylic acid with similar analgesic and anti-inflammatory properties [1, 2].

Gas chromatographic, radioisotopic and fluorometric methods have been used for quantitation of diflunisal [3], but all have disadvantages. The gas chromatographic assay necessitates lengthy sample preparation (extraction, evaporation and derivatisation), while the radioisotopic and fluorometric methods are non-specific.

Since the present work was started, high-performance liquid chromatographic (HPLC) methods for the determination of diflunisal in plasma have been reported [4, 5]. In both, diflunisal and naproxen (internal standard) were extracted from plasma into organic solvents which were evaporated to dryness. In one report [4] the chromatography was inefficient as judged by broad tailing peaks and the limit of detection was only 5 μg/ml.

The present simple method does not require extraction and can be completed in a fraction of the time with a detection limit of 0.5—1.0 μg/ml.

EXPERIMENTAL

Materials

Diflunisal was obtained from Thomas Morson Pharmaceuticals (Division of Merck Sharp & Dohme, Hoddesdon, Great Britain) and flufenamic acid (the internal standard) from Parke Davis & Company (Pontypool, Great Britain). All solvents and reagents were of Analar grade and obtained commercially.

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Standard solutions

Diflunisal (50 mg) was dissolved in 1.0 ml of methanol in a 100-ml volumetric flask and brought to volume with 1/15 *M* phosphate buffer (pH 7.2). Further dilution was made with water, urine or plasma to produce final concentrations of 1—10 and 10—100 μg/ml. Flufenamic acid (50 mg) was dissolved in 1.0 ml methanol in a 100-ml volumetric flask and made up to volume with 0.001 *M* sodium bicarbonate. The stock solutions could be stored for at least eight weeks at 4° C.

Chromatography

The HPLC system consisted of a Pye Unicam Model LC3 variable-wavelength UV detector set at 251 nm, an Orlita DMP AE 10.4 pump, a loop injector (Rheodyne Model 7120) and a recorder (Bryans Model 28000). The column was 150 × 4.5 mm I.D. internally polished stainless steel, slurry packed with 5-μm Hypersil ODS (Shandon, Runcorn, Great Britain).

The mobile phase was a mixture of 0.08 *M* potassium nitrate in 2% acetic acid—isopropanol—ethyl acetate (55:25:20) which was degassed under reduced pressure prior to use. The solvent flow-rate was 1.3 ml/min at room temperature with a working pump pressure of 110 bar (1600 p.s.i.). The detector sensitivities were 0.08 and 0.02 a.u.f.s. for the plasma and urine assays, respectively.

Estimation of diflunisal in plasma

To 0.5 ml of plasma containing 10—100 μg/ml of diflunisal in a disposable polypropylene tube was added 0.5 ml of flufenamic acid solution (250 μg) followed by 0.5 ml of acetone to precipitate the proteins. After mixing, the sample was centrifuged and 25-μl aliquots of the clear supernatant injected directly into the chromatograph. For lower concentrations, 50 μl of the flufenamic acid solution was used. Plasma containing diflunisal in concentrations of more than 100 μg/ml was diluted appropriately with 0.9% saline (dilution with saline or blank plasma gave similar results). Two standards of diflunisal (5 and 50 μg/ml) in plasma were assayed with each set of unknown samples. Diflunisal concentrations were calculated from the regression of these standards using the peak-height response ratio of diflunisal to internal standard.

Estimation of diflunisal in urine

To 1.0 ml of urine containing 1—10 μg/ml diflunisal in a disposable polypropylene tube were added 100 μl of flufenamic acid solution (500 μg/ml). After mixing, 25 μl were injected directly into the chromatograph. Urine containing more than 10 μg/ml of diflunisal was diluted appropriately with distilled water.

Two standards of diflunisal (1 and 10 μg/ml) in urine were assayed with each set of unknown samples. Diflunisal concentrations in urine were calculated as described above for plasma.

RESULTS AND DISCUSSION

Typical chromatograms of plasma and urine obtained from a healthy volunteer 3 h after ingestion of 750 mg of diflunisal (three Dolobid tablets) are illustrated in Figs. 1 and 2. The retention time of diflunisal was 6 min in both assays and samples could be injected every 9 min. No other interfering peaks were observed.

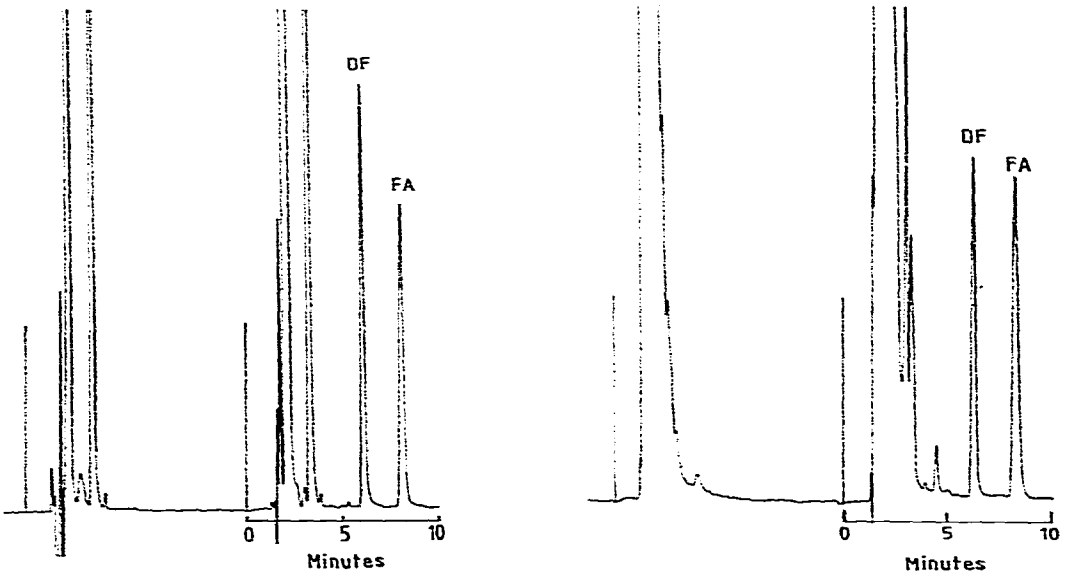

Fig. 1. Chromatogram of plasma from a healthy volunteer before (left) and 3 h after ingestion of 750 mg of diflunisal (right). Peaks: DF = diflunisal, FA = flufenamic acid (internal standard).

Fig. 2. Chromatogram of urine from a healthy volunteer before (left) and 3 h after ingestion of 750 mg of diflunisal (right). Peaks: DF = diflunisal, FA = flufenamic acid (internal standard).

The standard calibration graphs for both assays were linear and passed through the origin [6]. The precision and reproducibility of the assays are shown in Table I as the result of five replicate analyses of diflunisal in plasma and urine. The overall recovery (drug found) of diflunisal was 100.4 ± 3.6% (S.D.) for plasma and 100.2 ± 5.3% (S.D.) for urine. The respective limits of detection were 1.0 and 0.5 μg/ml. Diflunisal was stable in plasma and urine at −20° C for more than 6 months and at −4° C for at least 2 months.

The results of analyses of 17 plasma and 13 urine samples from five patients with diflunisal overdosage are given in Table II and mean plasma diflunisal concentrations in six healthy volunteers following a single oral dose of 750 mg are shown in Fig. 3.

Naproxen was initially chosen as the internal standard because it eluted before diflunisal with good separation. However, an interfering peak appeared in the urine of healthy volunteers following ingestion of diflunisal. The peak did not change after hydrolysis of urine with β-glucuronidase and arylsul-

TABLE I

REPLICATE ANALYSES OF DIFLUNISAL

Five replicate analyses were carried out at each concentration.

Plasma			Urine		
Drug added (μg/ml)	Mean concentration found (μg/ml)	Coefficient of variation (%)	Drug added (μg/ml)	Mean concentration found (μg/ml)	Coefficient of variation (%)
10	10.2	7.3	1	1.06	9.4
20	20.2	2.1	2	1.94	4.6
40	40.5	2.4	4	4.11	3.9
60	58.6	2.5	6	5.91	5.1
80	80.0	1.8	8	7.87	1.6
100	100.6	1.0	10	10.09	2.3

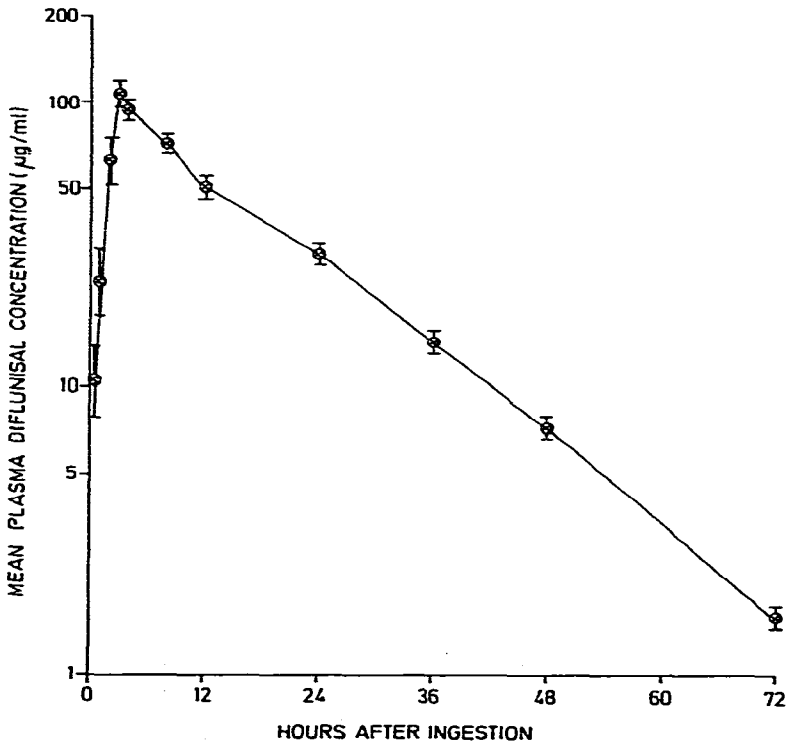

Fig. 3. Plasma concentrations of diflunisal in six healthy volunteers following an oral dose of 750 mg (means ± S.E.).

TABLE II

PLASMA AND URINE CONCENTRATIONS FOLLOWING DIFLUNISAL OVERDOSAGE MEASURED BY THE HPLC METHOD

Patient	Alleged number of Dolobid tablets	Other drugs taken	Time after ingestion (h)	Plasma diflunisal concentration (μg/ml)	Time after ingestion (h)	Urine diflunisal concentration (μg/ml)
RH	50	30 prochlorperazine	3	348	N.A.*	2.2
		30—40 dihydrocodeine	7	251	N.A.	0.9
			26	123	N.A.	0
SG	30	20 aspirin/codeine	3	103	—	—
			25	39	—	—
AD	70	—	37	173	37	57
ML	30	—	12	131	13—19	82
			18	103	19—25	50
			24	80	23—31	38
			30	70	31—43	26
			36	44	43—55	30
			48	19	55—67	12
			60	6	67—79	5
			72	2	79—81	2
			84	trace	81—93	trace
JL	?	? paracetamol plus	?	134	—	—
		d-propoxyphene	?+8	300	—	—

*N.A. = not available.

phatase whereas thin-layer chromatography showed that the glucuronide conjugates of diflunisal had disappeared. This interfering peak appeared to be an unknown metabolite of diflunisal.

Comparison of fluorometric and HPLC methods

The HPLC method was compared with the following modification of the fluorometric assay described by Tocco et al. [3].

To 100 μl of plasma containing 1—30 μg/ml diflunisal in a 15-ml round-bottomed glass tube were added 900 μl of pooled blank plasma. Appropriate dilutions were made for higher diflunisal concentrations. After mixing, 1 ml of 5 *N* hydrochloric acid was added and the drug extracted into 5 ml of chloroform. Following centrifugation, 2 ml of the chloroform phase was extracted with 3 ml of 0.1 *M* phosphate buffer (pH 8.0). The fluorescence of the aqueous phase was measured in an Aminco-Bowman spectrophotofluorometer set at 260 nm (activation) and 425 nm (emission).

Concentrations were determined by reference to a previously constructed calibration graph of per cent transmission minus the blank value plotted

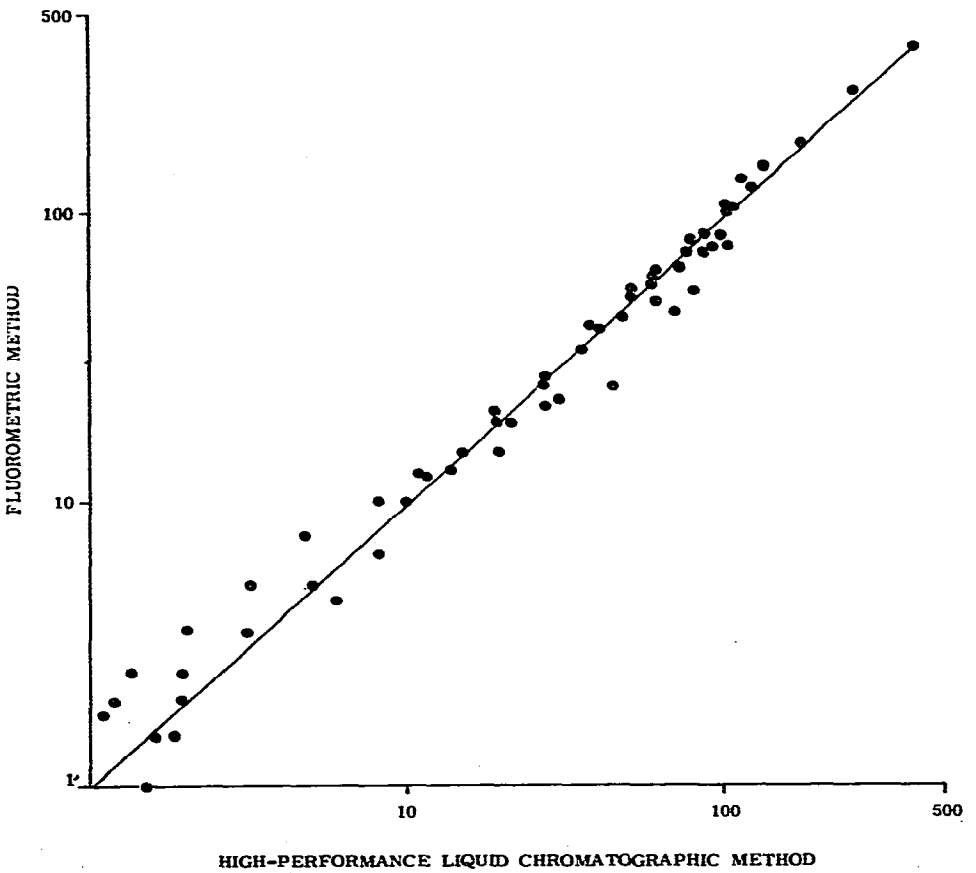

Fig. 4. Comparison of HPLC and fluorometric methods for the estimation of diflunisal in plasma. Individual measurements (μg/ml) were plotted for 59 samples and the line of identity is shown.

against diflunisal concentrations obtained from five different sets of plasma standards taken through the procedure.

Unchanged diflunisal cannot be measured in the urine by this method because of interference from the glucuronide conjugates. However, total unconjugated and conjugated diflunisal can be measured after hydrolysis with perchloric acid [3].

The plots of percentage transmission (minus the blank value) versus plasma concentrations of diflunisal passed through the origin and were linear up to 30 μg/ml. The limit of sensitivity was about 1.0 μg/ml.

Plasma concentrations in three healthy volunteers after oral administration of 750 mg diflunisal and three patients with diflunisal overdosage were measured by both methods. There was excellent agreement with concentrations above 10 μg/ml (Fig. 4).

CONCLUSION

Both the HPLC and fluorometric methods are suitable for the estimation of diflunisal in plasma. However, the HPLC method has the advantages of simplicity, specificity and may also be used to measure diflunisal in urine.

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