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Note

Rapid reversed-phase high-performance liquid chromatographic assay of diflunisal in biological fluids

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Diflunisal (2′,4′-difluoro-4-hydroxy-3-biphenylcarboxylic acid) (Fig. 1) is a prostaglandin synthetase inhibitor [1] and a potent analgesic and anti-inflammatory drug whose chief use is in the treatment of rheumatoid arthritis and osteoarthritis [2—4]. The bioavailability is almost 100% after oral administration; after doses of 250—500 mg once or twice a day it is reported to be eliminated from the blood circulation with a half-life of 10–12 h [5], which is longer than that of other salicylates. However, recent reports claim that diflunisal, like salicylic acid itself, has a limited capacity in the elimination processes [6, 7]. Suspected intoxication may therefore arouse greater concern even though the therapeutic index is several times that of aspirin [8].

Different methods for assaying diflunisal in biological fluids have been published. For some years the methods of Tocco et al. [7] were used, but the gas chromatographic, radiometric and fluorimetric techniques described are time-consuming and laborious, or lack specificity. High-performance liquid chromatographic methods with UV detection have since been reported [9—11] and they are all quicker and simpler. However, the methods of Van Loenhout et al. [9] and Balali-Mood et al. [11] have the disadvantage of using other analgesics (naproxen and flufenamic acid, respectively) as internal standards, which may unintentionally confound their therapeutic use Wåhlin-Boll et al. [10] used 6-methoxy-2-naphthylacetic acid as internal standard, which is not a drug. Furthermore, this method [10] employed a water-immiscible organic sample clean-up unlike the procedure of Balali-Mood et al. [11], in which a potential of column pressure build-up was accepted due to injection of a sample supernatant obtained from an incomplete acetone precipitation of plasma proteins.

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This report deals with a new high-performance liquid chromatographic assay of diflunisal in plasma and urine and a pilot study carried out to judge its practical application in conjunction with the development of the method.

MATERIALS AND METHODS

Materials and reagents

Diflunisal and 1-hydroxy-2-naphthoic acid (internal standard) (Fig. 1) were supplied from the Synthesis Laboratories of Dumex. All other chemicals were of analytical reagent grade. Standard solutions of diflunisal and internal standard were prepared separately; concentrations were 10 mg and 1.5 mg per 100 ml methanol, respectively. As an auxiliary to the extraction agent (see below) 10 g of concentrated sulphuric acid were added to an aqueous solution of 14.2 g disodium sulphate per l. The water was glass distilled. The mobile phase consisted of volume fractions of 50% of methanol, 33.3% of 0.1 *M* phosphoric acid, and 16.7% of tetrahydrofuran. To a mixture of 1 l, about 9 ml of 1 *M* aqueous sodium hydroxide were added until a pH value of 3.0 was reached. The mobile phase was filtered and placed in an ultrasonic bath before use.

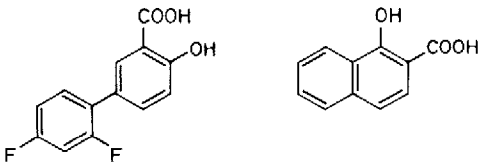

Fig. 1. The chemical structure of diflunisal (left) and plasma internal standard 1-hydroxy-2-naphthoic acid (right).

Instruments and equipment

Chromatographic separations were performed on a Waters Model 6000A constant-flow solvent delivery system, a Waters U6K injection unit and a Waters Model 440 UV detector equipped with a 254-nm filter. Detector response was monitored with a Hewlett-Packard Model 3380 integrator. A water-bath was used to maintain a column temperature of 35°C. A stainless-steel column (Knauer, Taunus, F.R.G.), 25 cm × 4 mm I.D., was filled with 5-μm Spherisorb ODS (Phase Separations, Queensferry, U.K.) using a slurry technique.

Analytical procedure

A 1-ml volume of the auxiliary extraction solution was added to samples of 100 μl (or another suitable volume) of serum, plasma or urine. Except for urine analysis, 20 μl of the internal standard solution were then added and the mixture was shaken vigorously. After adding 3 ml of diethyl ether and gently rotating for 3 min the tubes were centrifuged at 10°C. Then 2-ml portions of the organic phase were transferred to conical tubes and evaporated under nitrogen jet streams at ambient temperature. The residues were dissolved in 200 μl of the mobile phase and 50-μl aliquots were injected onto

the column. The mobile phase was delivered at a rate of 1 ml/min and the column was maintained thermostatically at 35°C.

Calibration of the method was performed by means of the peak areas (or peak area ratios) stemming from diflunisal (and the internal standard) in standards. The peak area ratios were only used in the analysis of serum and plasma diflunisal. The standards were prepared as drug-free samples to which were added known amounts of the compound(s).

Quantification of diflunisal was possible from the peak areas (or peak area ratios) of the samples by reference to a linear standard curve determined by least-squares linear regression.

RESULTS AND DISCUSSION

Method development

UV detection was selected because of previous reports of its usefulness [9—11].

The chromatographic phases were the next factors to be examined. Van Loenhout et al. [9] described ion pair elution involving methanol and tetramethylammonium as the ion pairing agent, the latter alkalinized with Tris [tris(hydroxymethyl)aminomethane]. However, this system had a tendency to peak tailing and a remarkably low detector response. In keeping with this the selective principle of ion pair elution was tried with other systems, such as tetrabutylammonium hydrogen sulphate buffered to pH 9 by means of aqueous borate and modified with methanol. However, the chromatographic performance was still not considered satisfactory, because of severely tailing peaks.

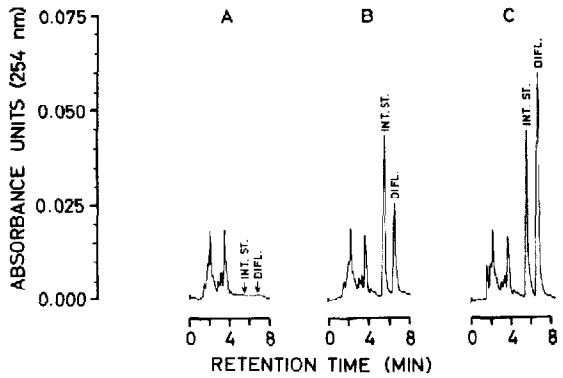

Fig. 2. Representative chromatograms of (A) blank serum extract; (B) serum standard extract of 10 μg/ml diflunisal and 0.3 μg/ml internal standard (the peaks correspond to approximately 0.1 and 1.4 μg, respectively); and (C) volunteer serum extract containing about 20 μg/ml diflunisal (the peak of diflunisal corresponds to approximately 2.8 μg).

Chromatography was then performed at an acid pH on a C_{18} column, which improved the peak shape. The final choice of mobile phase was a mixture of tetrahydrofuran, an aqueous phosphate buffer solution, and methanol, which at room temperature was found to be useful for the analysis of serum and plasma diflunisal (Fig. 2) but not for urine. Analysis of diflunisal in urine

was accomplished by increasing the column temperature from ambient to 35°C. This brought about a more efficient retention of the urinary diflunisal peak compared to the unresolved front at the beginning of the chromatogram (Fig. 3). The elevated temperature was maintained during the serum and plasma analyses, because of improved chromatography (Fig. 2). The number of theoretical plates was about 7000 for diflunisal and about 9000 for the plasma internal standard. The capacity factors were 3.3 and 2.7, respectively, and the separation factor appeared to be 1.2.

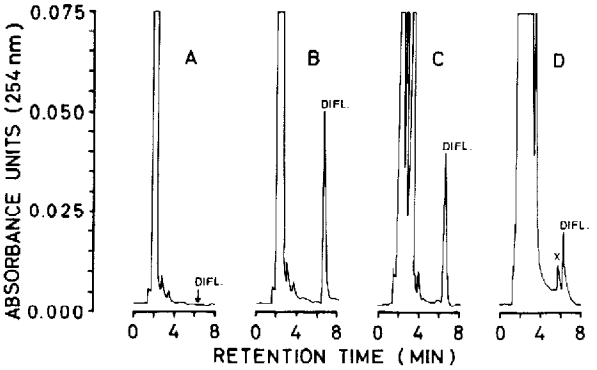

Fig. 3. Representative chromatograms of (A) blank urine extract; (B) urine standard extract of 10 μg/ml diflunisal (the peak corresponds to approximately 2.4 μg); (C) volunteer urine extract of approximately 8 μg/ml diflunisal (the peak corresponds to approximately 2 μg); and (D) volunteer urine extract showing unidentified peak (X), which appeared 48 h after administration (see text). The peak of diflunisal corresponds to approximately 0.1 μg. Extraction volume of urine was in case D ten times the normal, i.e. 1000 μl.

The extraction procedure and the choice of a possible internal standard were the final factors to be explored. Ion suppression by acidification and shifting of the partition equilibrium in favour of the analyte was found to be profitable for the extraction procedure. The final extraction technique was accomplished by adding an aqueous solution of sulphuric acid and sodium sulphate to the sample before extraction with diethyl ether.

The selection of a proper internal standard can be a matter of great import to the assay precision, especially when analysing routine samples. The choice for the serum and plasma assay was 1-hydroxy-2-naphthoic acid (Fig. 1). The impact of the same functional groups as found in diflunisal proved beneficial to the extraction and to the reversed-phase chromatography (Fig. 2). With regard to the urine assay the compound mentioned was left out because an interfering peak turned up in the chromatograms of urine samples from the two days following dosing (see application study below).

The extraction yield of diflunisal and the internal plasma standard were estimated as the ratios of the slopes of the calibration curves applying to processed and unprocessed (i.e. absolute standards) compounds. Calculation of the plasma yields provided a mean of 72% for diflunisal and 90% for the internal standard. For the urine assay the extraction yield of diflunisal was found to be essentially complete. In all cases the S.E.M. was approximately 1% with $n = 10$. The linearity of the standard curves (range 0—20 μg/ml)

was satisfactorily verified for both plasma and urine by the determination coefficient, which appeared to be $r^2 = 0.99$ with a coefficient of variation of less than 1% ($n = 10$) in both cases. The detection limit (signal-to-noise ratio of 2) was found to be approximately 1 ng of diflunisal per 1 ml of sample.

Freezing to −20°C did not cause deterioration of diflunisal in serum, plasma or urine. The assay demonstrated no interference with the following other analgesics: salicylic acid, phenacetin and paracetamol. When applied to unprocessed compounds, the capacity factors were 1.2, 0.9 and 0.6, respectively.

Quality control samples were included in each analytical series in order to estimate the precision of the method. These pools were prepared by mixing drug-free sample material and diflunisal to an appropriate concentration and dividing into 100-μl aliquots. The pools were stored at −20°C until analysis. From ten consecutive runs the inter-assay analytical variation appeared to be approximately 7% (relative standard deviation) at a concentration level of 10 μg/ml serum.

A pilot application study

In order to demonstrate the usefulness of the method, analyses have been performed on serum and urine samples obtained from a healthy volunteer, who had ingested a 250 mg tablet of Diflonid® (Dumex).

The serum concentration–time relationship found (Fig. 4) indicates an anomaly within the 7–24 h time interval. However, the overall elimination rate from the circulation was within the normal range: in the time interval 5–30 h the elimination half-life was about 10 h. Consistent with the anomaly indicated, the curve of the urinary excretion rate (Fig. 4) decreases about 10 h after medication, i.e. within the same period.

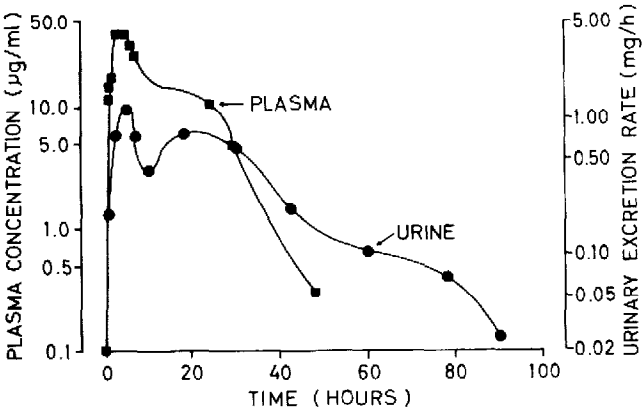

Fig. 4. Plasma concentration profile (■) and mean urinary excretion rate (●) of diflunisal after a single oral administration of 250 mg of Diflonid to a healthy volunteer. The mean urinary excretion rate for each collection period was related to the midpoints of the time intervals.

An unidentified compound with a retention time of 5.9 min (i.e. capacity factor of 2.9) occurred in chromatograms of urine samples collected after 48 h (Fig. 3). The finding of an extra peak seems to agree well with the observation of Balali-Mood et al. [11]. The size of the peak appeared intact relative to diflunisal after enzymatic hydrolysis with β-glucuronidase and sulphatase. Furthermore, treatment with 70% aqueous perchloric acid [7] did not give any clarification, instead it generated new interfering peaks in the chromatograms. The peak could be a metabolite of diflunisal, the data suggesting either the ether glucuronide or that of an unknown, similar to a previous report [11].

CONCLUSION

A rapid and sensitive method for the determination of diflunisal in serum, plasma and urine is presented. Reversed-phase liquid chromatography was applied to diflunisal after a single extraction step. The assay is simple and quick, well suited for routine purposes such as processing large numbers of samples.

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