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Short communication

An alternative high-performance liquid-chromatographic method for the determination of diclofenac and flurbiprofen in plasma

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1. Introduction

Diclofenac sodium {sodium [*o*-(2,6-dichlorophenyl)amino]phenyl acetate} and flurbiprofen [(±)-2-(2-fluorobiphenyl-4-yl)propionic acid] are non-steroidal anti-inflammatory (NSAIDs), analgesic, antipyretic drugs. Both NSAIDs are well absorbed orally with peak concentrations occurring within 1–2 h (flurbiprofen) and 2–3 h (diclofenac) after oral administration; both are extensively metabolized by hydroxylation and conjugation in the liver [1–4] and both are extensively bound to plasma proteins (99%) [3,5]. Several high-performance liquid-chromatographic (HPLC) assays, using UV detection, have been reported over the last 16 years for the determination of flurbiprofen or diclofenac in serum/plasma either alone (flurbiprofen [6–8]; diclofenac [9–20]), or together with their metabolites (diclofenac [21,22]), or, in the case of flurbiprofen, in its enantiomeric form [23,24]. The combination of extracting agent, column type, mobile phase and wavelength in each assay differ. Improved reproducibility and precision at diclofenac plasma concentrations between 5 and 2000 ng ml^{-1} have been achieved with detection limits as low as 2.5 ng ml^{-1} [17,20]; one assay in particular reported a detection limit of 1 ng ml^{-1} and linearity between 10 and 10000 ng ml^{-1} [15]. Because of the interferences evident in the chromatograms from UV detection, fluorescence has become the preferred detection technique for flurbiprofen [2,25,26], although stereoselective assays utilize either UV [23,24] or fluorescence detection [27], with detection limits as low as 50 ng ml^{-1} and 10 ng ml^{-1}, respectively.

In the present study, an alternative, validated HPLC procedure employing UV detection for the analysis of diclofenac and flurbiprofen in a 225 μl plasma sample is presented.

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2. Experimental

2.1. Apparatus

The HPLC equipment used comprised a single piston pump (Spectra Physics, San Jose, CA), a UV variable wavelength detector (Spectra Physics) set at 278 nm (AUFS 0.002 (internal standard flurbiprofen), 0.005 (internal standard Diclofenac)), a 20 μl injection loop (Rhoedyne, Cotati, CA) and a chart recorder (SP 4400; Spectra Physics). Chromatographic separations were performed using a C-18 Spherisorb 5 μm column (25 cm × 4.6 mm; Phase Separations, Deeside, UK).

2.2. Chromatographic conditions

The mobile phase was pumped isocratically at a flow rate of 1 ml min^{-1} (inlet pressure 1700 p.s.i.) at ambient temperature. The chart speed was 0.25 cm min^{-1}.

2.3. Reagents

Diclofenac sodium and its metabolites—4-hydroxydiclofenac, 5-hydroxydiclofenac, 3-hydroxydiclofenac, 4,5-dihydroxydiclofenac and 3-hydroxy-4-methoxydiclofenac—were kindly supplied by Ciba Geigy (Basel, Switzerland); flurbiprofen was from Eli Lilly (USA) and 4′-hydroxyflurbiprofen from Vianex (Greece). All solvents used were of HPLC grade. All other chemicals and reagents were of spectroquality or analytical grade. Acetonitrile, methanol, hexane and isopropyl alcohol were obtained from Labscan (Dublin, Ireland); *o*-phosphoric acid was from Merck (Darmstadt, Germany), sodium acetate from Riedel-De Haen (Seelze-Hanover, Germany) and glacial acetic acid from Erfar (Greece). The degassed mobile phase consisted of acetonitrile–0.1 M sodium acetate (35:65, v/v) adjusted to pH 6.3 with glacial acetic acid.

2.4. Standard solutions

Stock solutions (1000 μg ml^{-1}) of diclofenac and flurbiprofen, using either drug as internal standard, were prepared in methanol. The stock solutions were diluted ten-fold in methanol to give the working standard solutions (100 μg ml^{-1}).

2.5. Extraction procedure

In a disposable 10 ml culture tube, the diclofenac sodium or flurbiprofen working standards (100 μg ml^{-1}) were added in appropriate volumes to blank plasma so as to provide calibration standards of 20, 50, 100, 200, 500, 1500, 3000, 4000 and 5000 ng ml^{-1} for Diclofenac, or 100, 500, 1000, 2000, 4000, 8000, 16000, 32000, 40000 ng ml^{-1} for flurbiprofen. A 25 μl aliquot of the internal standard solution (either 3000 ng ml^{-1} flurbiprofen or 10000 ng ml^{-1} diclofenac) was added to 225 μl of plasma–drug mixture in a 10 ml glass tube. Subsequently, 500 μl of a 2.5 M *o*-phosphoric acid solution were added and the tube was vigorously shaken on a vortex mixer for 20 s. After agitation, 1.5 ml of hexane–isopropyl alcohol (80:20) were added to the mixture, which was shaken again on the vortex mixer for 2.5 min, and centrifuged for 10 min at 3000 rev min^{-1} at room temperature. The organic layer was transferred to a 10 ml centrifuge tube and evaporated to dryness under a stream of dry nitrogen at 37°C. The residue was reconstituted in 250 μl of mobile phase. An appropriate aliquot was then injected directly into the loop injector.

3. Results and discussion

3.1. Chromatography

Liquid–liquid extraction achieved a remarkable reduction in the solvent front and in the interference from normal plasma constituents. Coupled with the chosen mobile phase (UV detection at 278 nm), which was preferred because of its adjustment to a relatively neutral pH, excellent selectivity and resolution of both diclofenac and flurbiprofen were achieved. An optimum flow rate of 1 ml min^{-1} for the mobile phase resulted in a retention time of 5.6 min for diclofenac and 4.8 min for flurbiprofen. Representative chromatograms of extracted standards and a plasma

sample from a healthy male volunteer after oral administration of 100 mg of diclofenac sodium (Voltaren; enteric coated tablets) are shown in Fig. 1.

3.2. Linearity and reproducibility

The linear detector response for the assay was tested for plasma diclofenac ($n = 8$) and flurbiprofen ($n = 7$) concentrations, using either of the drugs as internal standard, between 20 and 5000 ng ml^{-1} and 100 and 40000 ng ml^{-1}, respectively. Peak height ratios (referred to the internal standard) and analyte concentrations were found to be reproducibly linear over these ranges. Regression statistics from the calibration standard curves studied are presented in Table 1.

3.3. Limits of detection and quantification

The limit of detection was defined as the lowest concentration of an analyte that the analytical process can reliably differentiate from background levels [28]. Detection limits for the determination of diclofenac and flurbiprofen in human plasma were 1 ng ml^{-1} and 10 ng ml^{-1}, respectively. The limit of quantitation, defined as the lowest concentration of the standard curve that can be measured with acceptable accuracy, precision and variability, was 20 ng ml^{-1} for the diclofenac assay and 100 ng ml^{-1} for the flurbiprofen assay.

3.4. Precision and accuracy

To determine intra- and inter-day precision of the assay, replicate ($n = 6$ and $n = 8$, respectively) sets of calibration samples were analysed. The percentage relative standard deviation [R.S.D. (%) = (Standard deviation × 100)/(Average concentration)] of the assay results were determined (see Table 2). The intra-day R.S.D. was less than 8% for diclofenac and less than 7.0% for flurbiprofen standard concentrations, whereas inter-day precision was 11% or less for both drugs.

Extraction efficiency was determined by comparing replicate ($n = 6$) peak heights of extracted plasma samples versus unextracted water stan-

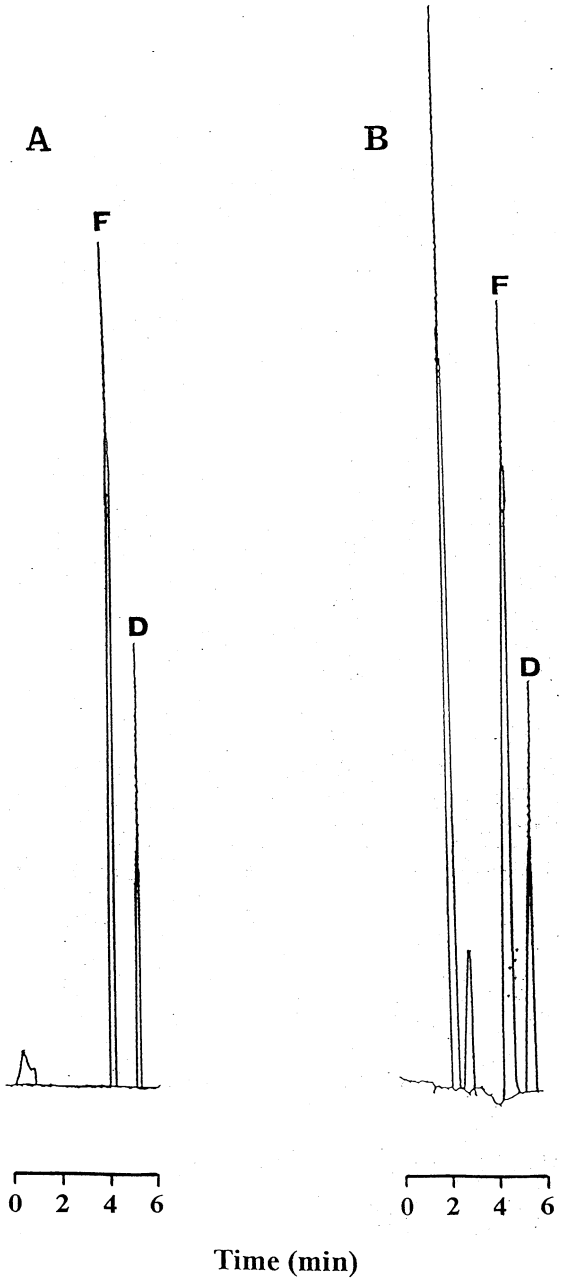

Fig. 1. Chromatograms corresponding to extracts of (A) a 225 μl sample of a standard solution and (B) a 225 μl plasma sample 3.0 h after administration of 2 × 50 mg enteric coated tablets of diclofenac sodium (concentration 1100 ng ml^{-1}). Peaks: D, diclofenac; F, flurbiprofen (internal standard).

Table 1
Regression statistics for diclofenac and flurbiprofen

Analyte	Range (ng ml^{-1})	n	Slope	Intercept	Correlation coefficient
Diclofenac[a]	20–5000	8	0.00054 ± 0.000032	0.0212 ± 0.0186	0.99957 ± 0.0002
Flurbiprofen[b]	100–40000	7	0.00035 ± 0.000018	0.1021 ± 0.0044	0.99993 ± 0.00003

[a] Internal standard flurbiprofen.
[b] Internal standard diclofenac.

dards for the same concentrations used to validate the precision of the assay. The percentage recoveries [Recovery (%) = (Peak height of plasma standard)/(Peak height of water standard) × 100] were 91.2, 91.0, 93.7, 95.2, 95.0, 93.0 for the diclofenac concentrations 20, 100, 500, 1500, 3000, 4000 ng ml^{-1} and 90.9, 92.5, 93.0, 93.0, 94.5, 91.0, 92.4 for the flurbiprofen concentrations 100, 1000, 4000, 10000, 16000, 20000 and 40000 ng ml^{-1}.

The analytical recovery (AR) was used to assess the accuracy of the assay. AR values were calculated by comparing the concentrations obtained from spiked plasma samples with the actual added concentrations [AR (%) = (Average concentration × 100)/(Amount of analyte added)]. The average accuracy was 98.8% for diclofenac (internal standard flurbiprofen) and 99.0% for flurbiprofen (internal standad diclofenac) (see Table 2). The acceptance criteria, as described by Shah et al. [28], were not more than 15% RSD for precision and not more than 15% deviation from the nominal value for accuracy. At the limit of quantitation 20% was considered acceptable for precision and accuracy.

3.5. Interference from metabolites

The following metabolites of diclofenac and flurbiprofen did not interfere with their assay: 4-hydroxydiclofenac, 5-hydroxydiclofenac, 3-hydroxydiclofenac, 4,5-dihydroxydiclofenac, 3-hydroxy-4-methoxydiclofenac and 4-hydroxyflurbiprofen.

Table 2
Intra-day and inter-day precision for diclofenac and flurbiprofen in plasma

Concentration (ng ml^{-1})	Precision (R.S.D., %)		Accuracy (AR, %)
	Intra-day	Inter-day	Intra-day
Diclofenac			
20	8.0	11.0	99.0
100	5.7	7.1	99.1
500	3.0	5.2	100.3
1500	3.3	5.3	99.2
3000	1.8	3.8	98.0
4000	3.0	5.6	97.2
Flurbiprofen			
100	7.0	11.0	99.0
1000	6.5	8.1	99.4
4000	3.5	6.1	99.0
10000	2.6	5.5	98.9
16000	3.0	5.8	99.6
20000	2.1	3.6	99.4
40000	3.2	5.0	98.0

R.S.D., relative standard deviation; AR, analytical recovery.

3.6. Clinical application

The assay was applied in an on-going pharmacokinetic–pharmacodynamic study of NSAIDs. Plasma sampling was continued for 12 h following administration of diclofenac. All samples were frozen immediately and stored at −20°C until the time of analysis, in accordance with indications from previous stability studies [10,24]. The range of plasma diclofenac concentrations measured after administration of 50 mg or 2 × 50 mg enteric coated tablets of the sodium salt of the drug (Voltaren) to healthy volunteers was 23–743 ng ml^{-1} and 25–1430 ng ml^{-1}, respectively.

4. Conclusions

The proposed HPLC method for the determination of diclofenac and flurbiprofen has been demonstrated to be simple, to have short retention times, excellent limits of detection and comparable, or in most cases greater, linearity and reproducibility over a broader range of concentrations than those methods of analysis reported most recently using UV detection (diclofenac [16–20]; flurbiprofen [23,24]), with the exception of the method described by Santos (linearity with 10–10000 ng ml^{-1} of diclofenac) [15]. The assay may therefore be considered a useful alternative method for routine bioavailability/bioequivalence studies of these NSAIDs. Furthermore, the assay method has been used successfully for the analysis of a large number of plasma samples obtained during a pharmacokinetic–pharmacodynamic study of diclofenac, providing valuable information on the dose–response relationships of this NSAID.

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