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High-performance liquid chromatographic determination of naproxen, ibuprofen and diclofenac in plasma and synovial fluid in man

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ABSTRACT

High-performance liquid chromatographic assay procedures have been developed for naproxen, ibuprofen and diclofenac in human plasma and synovial fluid samples. A single liquid–liquid extraction procedure was used to isolate each compound from acidified biological matrix prior to the quantitative analysis. A Spherisorb ODS column (12.5 cm × 4.6 mm I.D.) was used for all the chromatography. Naproxen was eluted with a mobile phase of methanol–Sörensen's buffer at pH 7 (37:63, v/v). Ibuprofen and diclofenac were eluted using mobile phases of methanol–water at pH 3.3 (65:35, v/v and 63:37, v/v, respectively). Diphenylacetic acid was used as the internal standard for the assay of naproxen and flurbiprofen was used in the analysis of ibuprofen and diclofenac. Inter- and intra-day coefficients of variation were less than 7%. The assays were used in clinical studies of the three drugs in osteo- and rheumatoid arthritis patients.

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are frequently used as first-line therapy in the management of the major arthritic diseases of osteo- (OA) and rheumatoid (RA) arthritis [1].

Naproxen, (*S*)-6-methoxy-α-methyl-2-naphthaleneacetic acid (Fig. 1a), is a potent anti-inflammatory agent with analgesic activity. It is widely used in the treatment of OA and RA and for the relief of mild to moderate pain. Activity resides mainly, if not exclusively, in the *S*-enantiomer, and it is formulated exclusively as this enantiomer. Therapeutic activity has been demonstrated in OA and RA at daily dose rates of 500–1500 mg, based mainly on clinical indices which include symptomatic pain relief and improvement of joint function [2]. Ibuprofen, α-methyl-4-(2-methylpropyl)benzeneacetic acid

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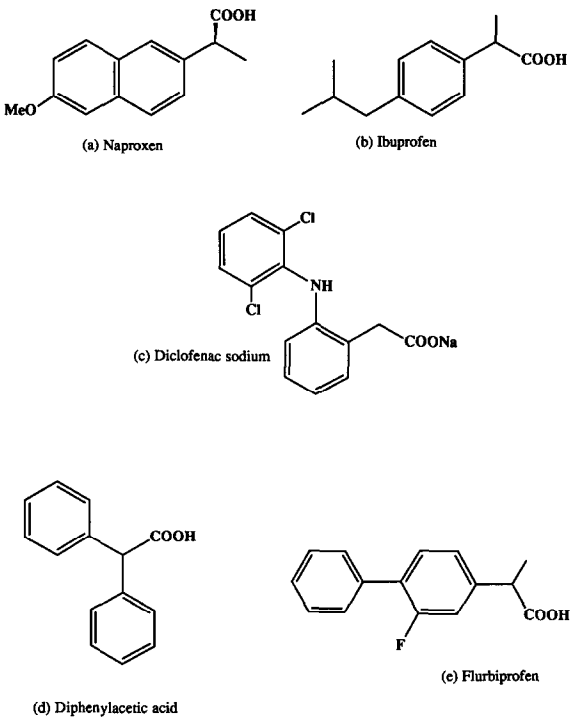

Fig. 1. Structures of (a) naproxen, (b) ibuprofen, (c) diclofenac, (d) diphenylacetic acid and (e) flurbiprofen.

(Fig. 1b), is another member of this group of NSAIDs. The absorption of ibuprofen is rapid, peak serum levels are attained within 2 h after a single oral dose. However, the elimination half-life of ibuprofen is also rapid (typically 2 h) necessitating frequent dosing (to four times daily) to maintain plasma levels within the therapeutic range [3]. A less frequent dosing regimen may be established using a sustained release formulation of ibuprofen. Such a formulation (Brufen Retard) has recently been developed to achieve a once-daily dosing regimen. Diclofenac sodium, 2-[(2,6-dichlorophenyl)amino]benzeneacetic acid monosodium salt (Fig. 1c), is now widely used in the treatment of arthritic conditions. Due predominantly to first-pass metabolism, only about 60% of an oral dose of diclofenac sodium reaches the systemic circulation, and its half-life in man is approximately 1 h [4,5]. Diclofenac sodium is available as a 100-mg polymer matrix slow-release formulation (Voltarol Retard). This formulation releases its total drug content slowly over 8–10 h.

The aim of this study was to determine in symptomatic cases of OA or RA, the plasma and synovial fluid drug levels from serial samples over a minimum period of seven days therapy at conventional dose rates. In order to accomplish this objective, selective high-performance liquid chromatographic (HPLC) analytical procedures for the determination of the three drugs listed above were devised. A number of HPLC assays of varying complexity have been reported for the determination of these drugs in plasma and urine: naproxen [6–8], ibuprofen [9–15] and for diclofenac [16–19]. Other methods reported derivatization techniques [20] and gas chromatography–mass spectrometry [21]. None of these methods report the analysis of synovial fluid.

The assay procedures reported here have included modifications of previously published methods in order to analyze synovial fluid and plasma from patients and hence to achieve some commonality in the assay of NSAIDs in these biological fluids. The use of a single common assay method for the NSAIDs compounds was found to be inappropriate and would have entailed compromise in terms of recovery, reproducibility and accuracy. The levels of naproxen, ibuprofen and diclofenac were determined, using the following analytical procedures, in serial samples of plasma and of synovial fluid from OA and RA patients.

Permission was previously given for these studies in patients from the Ethical Committee of the City Hospital, Nottingham, UK.

EXPERIMENTAL

Materials

Naproxen, diphenylacetic acid (Fig. 1d) and diclofenac were obtained from Sigma (Poole, UK). Ibuprofen and flurbiprofen (Fig. 1e) were obtained from the manufacturer (Boots, Nottingham, UK). Solvents used were HPLC grade (May & Baker, Dagenham, UK). All other materials were of analytical grade or better.

Biological samples

Blank plasma, spun down from expired, citrated, transfusion blood, was stored at −20°C and allowed to defrost at 25°C prior to use. All clinical samples were treated in the same manner.

Apparatus

The HPLC system consisted of a pump and variable-wavelength UV detector (LKB Models 2050 and 2051, respectively, LKB-Produkter, Bromma, Sweden), a Gilson auto-sampling injector fitted with a 20-μl injection loop (Model 231-401, Gilson International, Villiers-le-Bel, France) and a Spectra-Physics data integrator (Model SP4290, Spectra-Physics, San Jose, CA, USA). The analytical column used for the chromatography was a Spherisorb 5-μm ODS 1 column (12.5 cm × 4.6 mm I.D., Hichrom, Reading, UK). A pre-column (2 cm × 2 mm I.D., Uptight C130B, Anachem, Luton, UK), packed with Perisorb RP18 30–40 μm pellicular material, was used to protect the analytical column. The rotary evaporator used was a Gyrovap (VA Howe, London, UK).

Chromatographic system

For the assay of naproxen, samples were eluted with methanol–Sörensen's phosphate buffer (pH 7) (37:63, v/v) at a flow-rate of 1 ml/min. Naproxen and the internal standard, diphenylacetic acid, were detected by their UV absorbances at 260 nm. Ibuprofen was assayed using a mobile phase flow-rate of 1 ml/min at 25°C and the UV detector at 220 nm. The mobile phase was methanol–deionised water (65:35, v/v) adjusted to pH 3.3 with phosphoric acid. Diclofenac was assayed using a mobile phase of methanol–deionised water (63:37, v/v) adjusted to pH 3.3 with phosphoric acid. Mobile phase flow-rate was maintained at 1 ml/min at 25°C and the UV detector at 280 nm.

Preparation of plasma and synovial fluid standards

Plasma or synovial fluid standards (0.5 ml) were prepared by the addition of the appropriate drug solution (50 μl of distilled water–methanol, 60:40, v/v) to blank matrix (obtained from subjects who were not receiving naproxen, ibuprofen or diclofenac) to give a concentration range for each drug as detailed below. Samples were processed and chromatographed as described later. For the preparation of ibuprofen standards the above procedure was employed, but a split calibration curve was employed, consisting of standards from 0.5 to 10 μg/ml and from 10 to 100 μg/ml. This was used to prevent negative bias in low-range concentration values caused by variation in the high-concentration standards. Naproxen concentration range: 5–100 μg/ml; ibuprofen concentration range: 0.5–100 μg/ml; diclofenac concentration range: 10–2000 ng/ml.

Sample preparation

Calibration samples and clinical samples of plasma and synovial fluid were processed in an identical manner.

Naproxen assay. To an aliquot of plasma (0.5 ml) or synovial fluid (0.5 ml) was added internal standard solution (50 μl of 10 mg/ml diphenylacetic acid), and the sample was acidified with 2 *M* hydrochloric acid (200 μl). The sample was then extracted with diethyl ether (5 ml) and tumbled for 10 min on a rotary mixer. The organic layer was separated, transferred to a clean tube and evaporated to dryness using vacuum centrifugation. The dry residue was reconstituted in mobile phase (0.5 ml) and transferred to chromatographic vials for analysis.

Ibuprofen assay. To an aliquot of plasma (0.5 ml) or synovial fluid (0.5 ml) was added internal standard solution (50 μl of 300 μg/ml flurbiprofen), and the sample was acidified with 2 *M* hydrochloric acid (200 μl), vortex-mixed briefly (15 s) and extracted with 5 ml of hexane–diethyl ether (1:1, v/v). The sample was tumbled for 10 min on a rotary mixer at moderate speed and then centrifuged at 10 000 *g* for 5 min to separate the phases. The organic layer was separated, transferred to a clean tube and evaporated to dryness using vacuum centrifugation. The residue was reconstituted in methanol (300 μl) plus deionised water (200 μl) after a 5-min sonication. After a brief vortex-mix (15 s), the reconstituted sample was transferred to a chromatographic vial for subsequent analysis.

Diclofenac assay. To an aliquot of plasma (0.5 ml) or synovial fluid (0.5 ml) was added internal standard solution (50 μl of 24 μg/ml flurbiprofen), and the sample was acidified with 2 *M* hydrochloric acid (200 μl) and extracted with hexane (5 ml). The samples were tumbled for 10 min on a rotary mixer at moderate speed and then centrifuged at 10 000 *g* for 5 min to separate the phases. The organic layer was transferred to a clean tube and evaporated to dryness under vacuum centrifugation. The residue was reconstituted in methanol (150 μl) plus water (100 μl) to give a total reconstituted volume of 250 μl. After a brief vortex-mix (15 s), the reconstituted sample was transferred to a chromatographic vial for subsequent analysis.

Calibration curves comparing peak-area ratios of drug to internal standard *versus* drug concentration were constructed from the results obtained above. Unknown samples were quantified by reference to the linear regression equation derived from the respective standard curve.

Precision, recovery and accuracy

For naproxen, spiked extracts were compared with unextracted standard solutions of naproxen at concentrations representing 5, 60 and 100 μg/ml of sample. Results are based on a minimum of six replicates per concentration. Both intra- and inter-day reproducibility was assessed using the coefficients of variation (C.V.) generated for sets of calibration samples. Absolute recovery as a measure of extraction efficiency was determined according to the mean peak-area values of spiked extracts in comparison with those of standard solutions. Relative recovery was determined from the peak-area ratios (naproxen/internal standard) of extracts with those of standard solutions. The assay accuracy was evaluated by the quantification of drug content as determined from respective regression analyses using the equation:

$$x \text{ (drug concentration)} = y \text{ (peak-area ratio)} - c \text{ (intercept)}/m \text{ (slope)}.$$

For the ibuprofen and diclofenac assays, the above parameters were evaluated in a similar manner to those described above for naproxen.

RESULTS AND DISCUSSION

Representative chromatograms of extracted blank plasma, extracted blank synovial fluid and extracted standards for each of the three assay procedures are shown in Figs. 2–4. The following approximate retention times were observed under the conditions used for each assay procedure: (a) naproxen (8 min), diphenylacetic acid (naproxen internal standard, 5 min); (b) ibuprofen (8 min), flurbiprofen (ibuprofen internal standard,

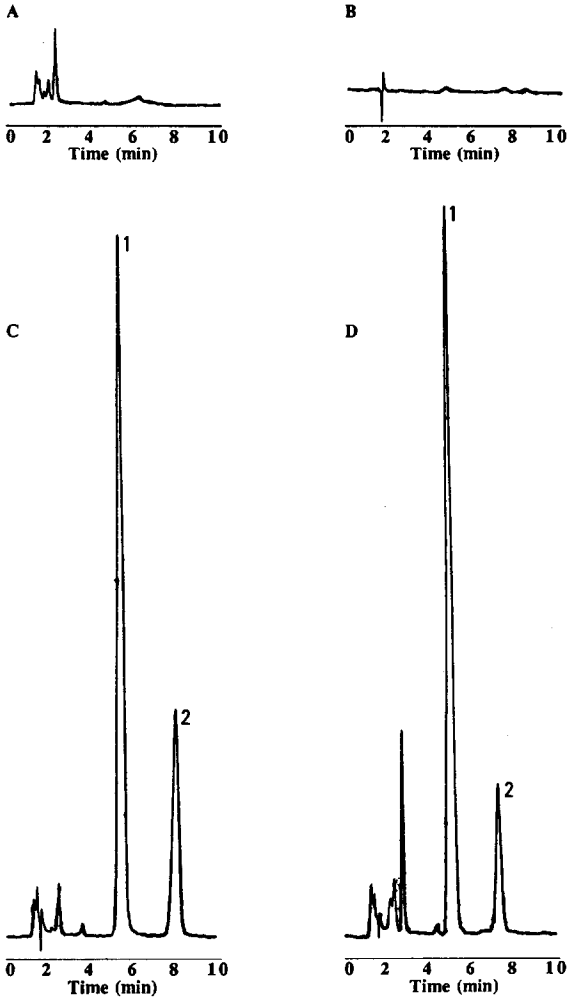

Fig. 2. Representative chromatograms for (A) blank plasma, (B) blank synovial fluid, (C) plasma sample from a patient containing 74.9 μg/ml naproxen and (D) synovial sample from a patient containing 36.2 μg/ml naproxen. Peaks: 1 = desmethylnaproxen (internal standard); 2 = naproxen.

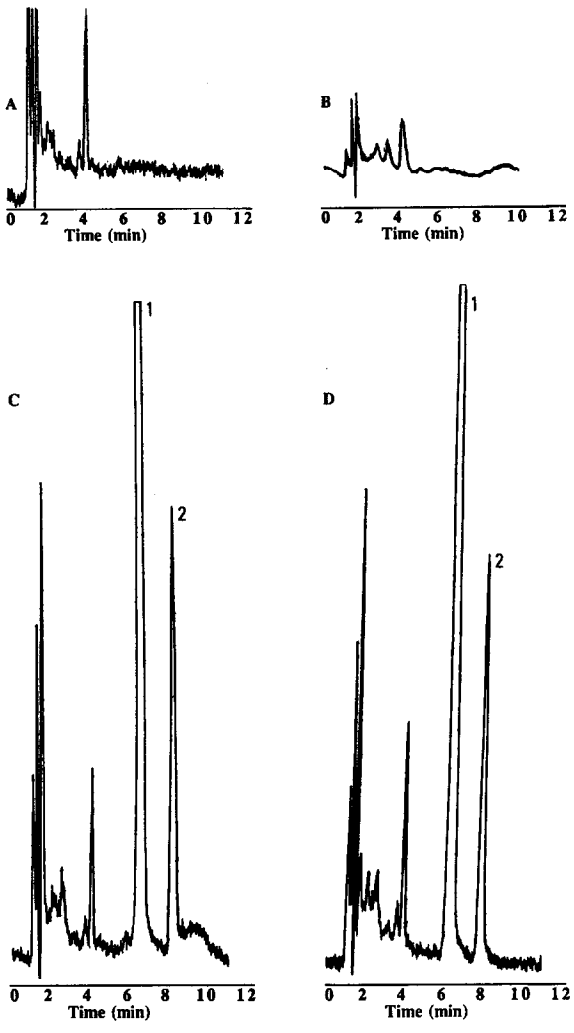

Fig. 3. Representative chromatograms for (A) blank plasma, (B) blank synovial fluid, (C) plasma sample from a patient containing 13.1 μg/ml ibuprofen and (D) synovial sample from a patient containing 8.2 μg/ml ibuprofen. Peaks: 1 = flurbiprofen (internal standard); 2 = ibuprofen.

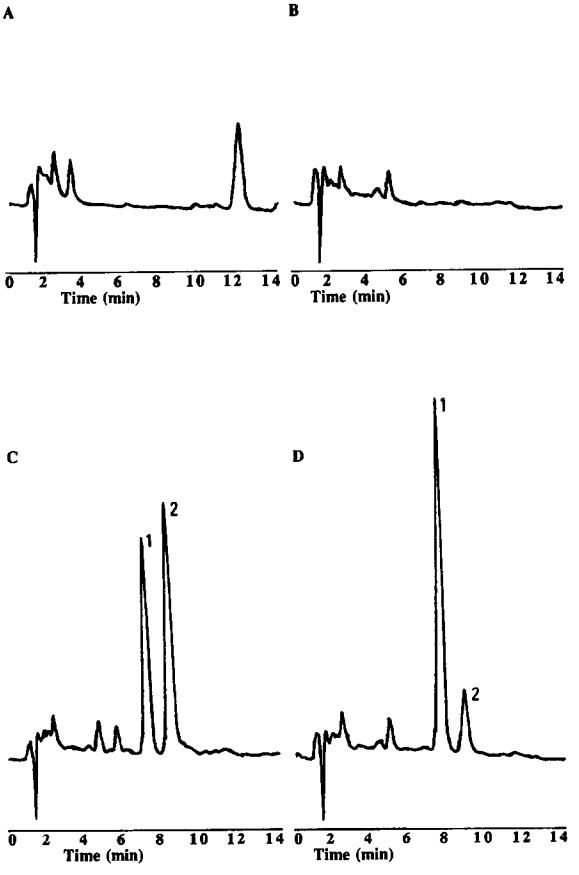

Fig. 4. Representative chromatograms for (A) blank plasma, (B) blank synovial fluid, (C) plasma sample from a patient containing 1628 ng/ml diclofenac and (D) synovial sample from a patient contaning 245 ng/ml diclofenac. Peaks: 1 = flurbiprofen (internal standard); 2 = diclofenac.

6.5 min); (c) diclofenac (7.5 min), flurbiprofen (diclofenac internal standard, 9 min).

No interfering peaks were noticeable in the chromatographic traces of blank plasma or synovial fluid or from those samples obtained from patients who were taking co-administered medication. Plots of peak-area ratios for each of the three assay used were linear over their respective concentration ranges [$r^2 > 0.995$ for naproxen, ibuprofen (low and high range) and dicofenac].

The *intra*-day precision of the three assays was assessed using six replicate trials at two or three different drug concentrations run on the same day (see Table I). For naproxen the C.V. for each assay was less than 3% for concentrations above 5 μg/ml and was still under 10% at the lowest concentration. *Inter*-day precision was similarly calculated using the results from twelve different calibration sets run on different days over a period of approximately one month. Overall, the variation was increased slightly, but was within acceptable limits.

For ibuprofen, reproducibility was assessed in a six replicate trial with three different ibuprofen concentrations (5, 50 and 100 μg/ml) and run on

TABLE I

PRECISION RESULTS FOR THE NAPROXEN, IBUPROFEN AND DICLOFENAC HPLC ASSAYS

Nominal concentration (μg/ml)	Drug/internal standard peak-area ratio (mean ± S.D.)	C.V. (%)
Naproxen		
Intra-day variation		
5	0.068 ± 0.001	1.5
60	0.650 ± 0.015	2.3
100	1.067 ± 0.019	1.8
Inter-day variation		
100	1.065 ± 0.017	1.6
Ibuprofen		
Intra-day variation		
5	0.056 ± 0.003	5.4
50	0.478 ± 0.023	4.8
100	0.861 ± 0.034	3.9
Inter-day variation		
100	0.926 ± 0.035	3.9
Diclofenac		
Intra-day variation		
100 ng/ml	0.096 ± 0.004	4.9
1000 ng/ml	0.973 ± 0.011	1.1
Inter-day variation		
1000 ng/ml	0.888 ± 0.061	6.9

the same day. The results demonstrated excellent reproducibility and precision at 50 and 100 μg/ml, with C.V.s of less than 3%. At 5 μg/ml, precision was still good for the standard solutions with C.V.s less than 5%. *Inter*-day precision was similarly reproducible for the ibuprofen assay procedure.

Reproducibility of the diclofenac assay was assessed in several six-replicate trials using three different diclofenac concentrations (10, 100 and 1000 ng/ml) and assayed on the same day. The results demonstrate excellent reproducibility and precision at diclofenac concentrations between 100 and 1000 ng/ml of plasma, with C.V.s less than 5%. *Inter*-day variation was increased slightly, but was acceptable, at less than 7%.

The recovery of the three compounds was determined by comparing assays of spiked plasma extracts with those of unextracted standard solutions. The recovery of naproxen approached 100% at all concentrations tested and was consistent. The recovery of ibuprofen was found to be 100.5 ± 6% over a range of three concentrations studied. The mean recovery of diclofenac from plasma extracts ranged from 89.5 to 95.1% at concentrations between 100 to 1000 ng/ml but was consistent at each concentration examined. The accuracy of all three assay procedures was determined by comparing the concentration found in spiked samples with the nominal concentration in those samples. The percentage accuracy approached 100% for all three drugs, with values ranging between 97 and 99% depending upon which of the NSAIDs was analyzed.

These assays were then used to determine the plasma and synovial fluid concentrations of the

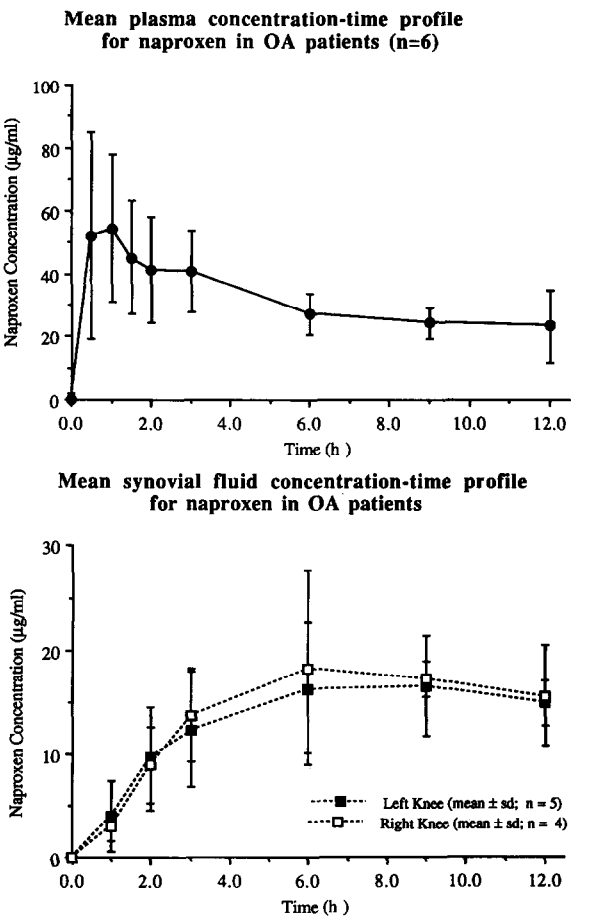

Fig. 5. Mean plasma and synovial fluid concentration–time profiles for naproxen in OA patients.

three drugs in serial samples of these biological fluids which had been withdrawn from patients at various times during pharmacokinetic studies (Fig. 5) [22,23].

Three HPLC assay methods have been developed to determine plasma and synovial fluid levels of naproxen, ibuprofen and diclofenac. These methods have been demonstrated to be sensitive, accurate, precise and reproducible. Furthermore, they are capable of resolving these compounds from endogenous small molecules which may interfere and also from any co-administered compounds. These procedures have been used successfully for the analysis of a large number of plasma and synovial fluid samples obtained during the pharmacokinetic and pharmacodynamic studies of these drugs in patients with OA and RA. These studies have provided valuable information on the use of these NSAIDs in these two matched patient groups.

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