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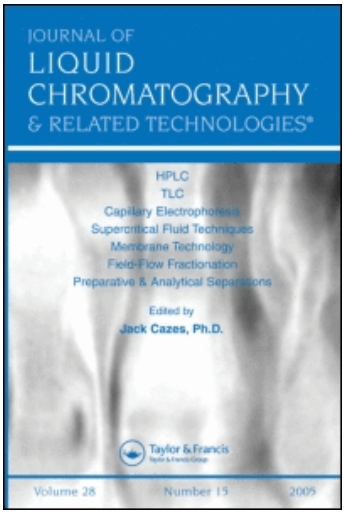

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A Simple Isocratic High-Performance Liquid Chromatographic (HPLC) Determination of Naproxen in Human Plasma using a Microbore Column Technique

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A SIMPLE ISOCRATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC (HPLC) DETERMINATION OF NAPROXEN IN HUMAN PLASMA USING A MICROBORE COLUMN TECHNIQUE

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ABSTRACT

A simple and sensitive HPLC method was developed for the determination of naproxen in human plasma. The assay employs a microbore column packed with a C18 reversed-phase material (5 μm ODS Hypersil) with an isocratic mixture of acetonitrile and 10 mM phosphate buffer, pH 2.5 (40:60, v/v) as the mobile phase. The mobile phase was pumped at a flow rate of 0.5 ml/min. For sample analysis 200 μl of acetonitrile containing internal standard (flurbiprofen) was added to 100 μl of plasma. After centrifugation 10 mM phosphate buffer, pH 7.4 (200 μl) was added to the tube, then vortexed and centrifuged. The supernatant (20 μl) was injected onto the HPLC column. The chromatographic separation was monitored by a fluorescence detector at an emission wavelength of 350 nm with an excitation wavelength of 225 nm. The direct precipitation of plasma protein using acetonitrile gave a good recovery for both naproxen and the internal standard. The detection limit was 0.1 μg/ml for naproxen. The intra- and inter-assay coefficients of variation at different concentrations evaluated were less than 10%.

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INTRODUCTION

Naproxen [(+)-6-methyl-α–naphthalene acetic acid] is a potent non-steroidal anti-inflammatory drug which is widely used in the clinical treatment of rheumatism and osteoarthritis. In man, naproxen undergoes two major biotransformations, viz. demethylation to 6-O-desmethylnaproxen and conjugation with glucuronic acid (1). Therefore the analysis of naproxen in biological samples should be specific and differentiate between the parent drug and its metabolites. A number of methods have been published for the determination of naproxen in human plasma. Gas chromatographic methods have been reported (2-4). However, these methods require a derivatization step for esterification of the carboxylic acid function of naproxen and are consequently tedious and time consuming. Quantitation has been performed by high-performance liquid chromatography (HPLC) involving precipitation of plasma protein followed by injection of the supernatant (5,6) and the direct injection of diluted samples (7). Improvement in the sensitivity of HPLC assays has been reported by incorporation of organic solvent extraction (8-10). Although these methods provide high sensitivity for the quantitation of naproxen, they require a 0.5-1 ml plasma sample and are time consuming since extraction procedures are involved. The present report describes a method for the rapid, simple and sensitive determination of naproxen using an isocratic HPLC system with microbore column and fluorometric detection. This method involves only a direct plasma protein precipitation followed by injection of the supernatant. The applicability of this procedure described is

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demonstrated by the analysis of plasma samples from subjects receiving oral naproxen in a bioavailability study.

MATERIALS AND METHODS

Materials and Reagents

Naproxen and 6-O-desmethylnaproxen were kindly provided by Syntex Australia Limited (Sydney, Australia). Flurbiprofen, used as an internal standard for the analytical procedure, was a gift from Boots Company Limited (Nottingham, UK). HPLC-grade acetonitrile was purchased from May & Baker Limited (Dagenham, England). All reagents used were analytical grade. Water was double glass distilled and MilliQ® filtered. Plasma samples collected from healthy subjects were obtained from a bioavailability study of naproxen. The bioavailability study of naproxen in healthy volunteers was approved by the Otago Area Health Board Ethical Committee.

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Instrumentation

The HPLC system consisted of a Perkin Elmer pump, model 250 (The Perkin Elmer Corporation, Norwalk, CT, USA) equipped with a Waters WISP 712 auto-injector (Waters Associate, Milford, MA, USA). A C18 reversed-phase microbore column (2 mm I.D. x 100 mm) packed with 5 μm ODS Hypersil (Shandon, London, UK) was used. The column efficiency

was over 4,000 plates per 10 cm. The eluate was monitored by a fluorescence detector (Shimadzu, Model RF-350, Shimadzu, Tokyo, Japan). Chromatograms were recorded on a Hitachi D-2500 (Kyoto, Japan) integrator.

Chromatographic Conditions

Analysis of the samples of naproxen was performed using a mobile phase consisting of 40% acetonitrile and 60% Na_2HPO_4 buffer (10 mM, pH 2.5) with a flow rate of 0.5 ml/min. The fluorescence detector was set at an excitation wavelength of 225 nm and an emission wavelength of 350 nm.

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Analytical Procedure and Sample Preparation

Known concentrations of naproxen in plasma were prepared by diluting a stock standard solution (0.1-1 ml) with drug-free plasma (10 to 100 ml). The final naproxen concentrations in the plasma standards were 0, 0.2, 1, 10, 25, 50, 75, 100 and 150 μg/ml naproxen in plasma. The internal standard solution of flurbiprofen of 1.25 mg/ml was prepared in acetonitrile and stored at -20°C until required. The internal standard solution was renewed every week for analysis without significant decomposition under these storage conditions.

Acetonitrile (200 μl) containing 1.25 mg/ml of internal standard (flurbiprofen) was added to 100 μl of plasma in a 1.5 ml plastic Eppendorf® centrifuge tube. The resulting mixture was vortexed for 10 seconds and

centrifuged at 2,000 g for 3 minutes. 10 mM Na_2HPO_4 buffer (pH 7.4) (200 µl) was then added to the tube, the tube vortexed briefly and then centrifuged at 2,000 g for 3 minutes. The clear supernatant was then transferred to a small vial in the auto-injector and 20 µl of this solution was injected onto the HPLC column.

The assay recovery was determined at 1 and 100 µg/ml for naproxen and at 1 µg/ml for the internal standard (flurbiprofen). The peak areas from six extracted plasma samples and six direct injections of the same amount of drug in mobile phase were compared. The assay recovery of each compound was estimated using the following equation:

$$\text{Recovery} = \frac{\text{mean peak area (extracted drug)}}{\text{mean peak area (direct injection)}} \times 100\%$$

A calibration curve was constructed by a plot of the peak area ratio (drug/internal standard) versus plasma naproxen concentration. The specificity of the assay for the naproxen metabolite was assessed by injecting the mixture of its known metabolite, 6-O-desmethylnaproxen, naproxen and the internal standard onto the HPLC column. The overall precision of the assay was evaluated using plasma naproxen standards with three different concentrations of naproxen (i.e. 0.2, 1 and 100 µg/ml) and 4-6 replicates.

RESULTS AND DISCUSSION

It was revealed from the fluorescence emission scan of naproxen and flurbiprofen (internal standard) following excitation at wavelength of 225 that

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the internal standard yielded a much lower fluorescence intensity at the emission wavelength of 350 nm than naproxen. This is the reason for the high concentration of the internal standard solution in acetonitrile (flurbiprofen 1.25 mg/ml) was required.

A mobile phase of 40% acetonitrile and 60% phosphate buffer (10 mM, pH 2.5) gave well resolved, sharp peaks for naproxen and the internal standard (flurbiprofen), with retention times of 2.8 and 5.4 min, respectively (Figure 1a). 6-O-Desmethylnaproxen, the main metabolite of naproxen detected in plasma was also tested for interference with naproxen and the internal standard. Under these chromatographic conditions, the peaks corresponding to 6-O-desmethylnaproxen, naproxen and flurbiprofen were well resolved with retention times of 1.4, 2.8 and 5.5 min, respectively (Figure 1b). These results indicate that the assay is specific and show that the metabolite does not interfere with either the naproxen or flurbiprofen peaks.

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A number of drugs and metabolites (Table 1) have been tested for possible interference with the assay of naproxen. Acetaminophen, aspirin and other commonly used drugs did not interfere with the analytical procedure for naproxen. Ephedrine is the only drug in the list that causes interference with the naproxen assay. Three chromatograms, one obtained from blank plasma, a second from blank plasma spiked with naproxen and a third from plasma taken from a volunteer after ingestion of naproxen are presented in Figure 2. Under these chromatographic conditions, there was also no endogenous interference from plasma.

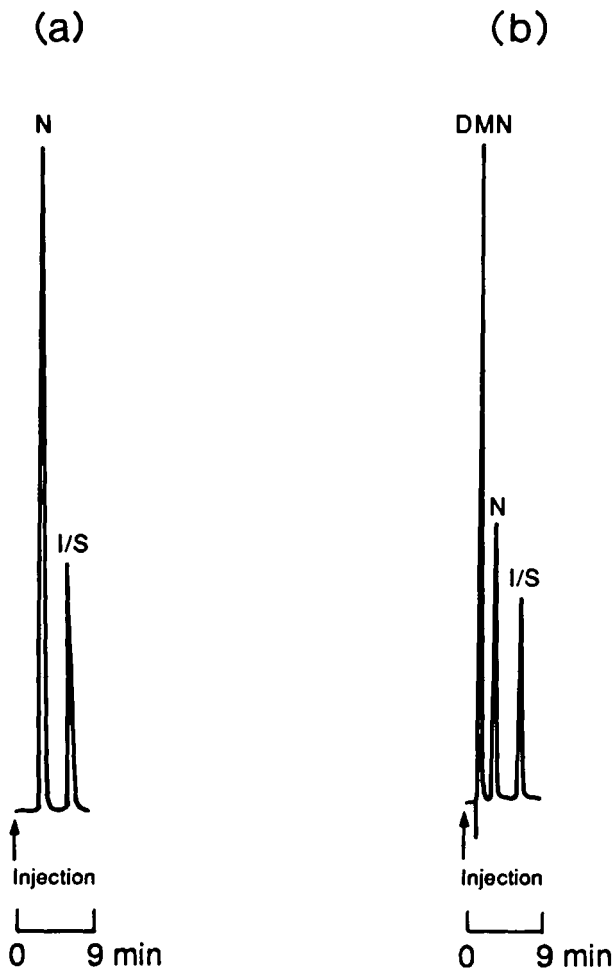

FIGURE 1

Chromatograms of (a): mixture containing 20 μg/ml naproxen (N) and 1,000 μg/ml flurbiprofen, internal standard (I/S) in water and (b): mixture of 5 μg/ml 6-O-desmethylnaproxen (DMN), 15 μg/ml N and 350 μg/ml I/S in water.

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Table 1

Possible Interferences Under Assay Conditions

Drug/Metabolite	Fluorescence at Assay Conditions	Retention* Time (min)
Acetaminophen	No	-
Aspirin	Yes	1.0
Caffeine	No	-
Diazepam	Yes	0.7
Diclofenac	No	-
Ephedrine	Yes	2.6
Indomethacin	No	-
Lidocaine	No	-
Lorazepam	No	-
Metoprolol	No	-
Phenylbutazone	Yes	0.7
Phenytoin	No	-
Pindolol	Yes	1.0
Piroxicam	No	-
Salicylic acid	Yes	1.1
Salicyluric acid	Yes	0.9
Sulphanilamide	Yes	0.8
Sulphinpyrazole	No	-
Theophylline	No	-
Triazolam	Yes	0.7

*Under these chromatographic conditions, the retention times for naproxen and flurbiprofen (the internal standard) were 2.6 and 5.2 min, respectively

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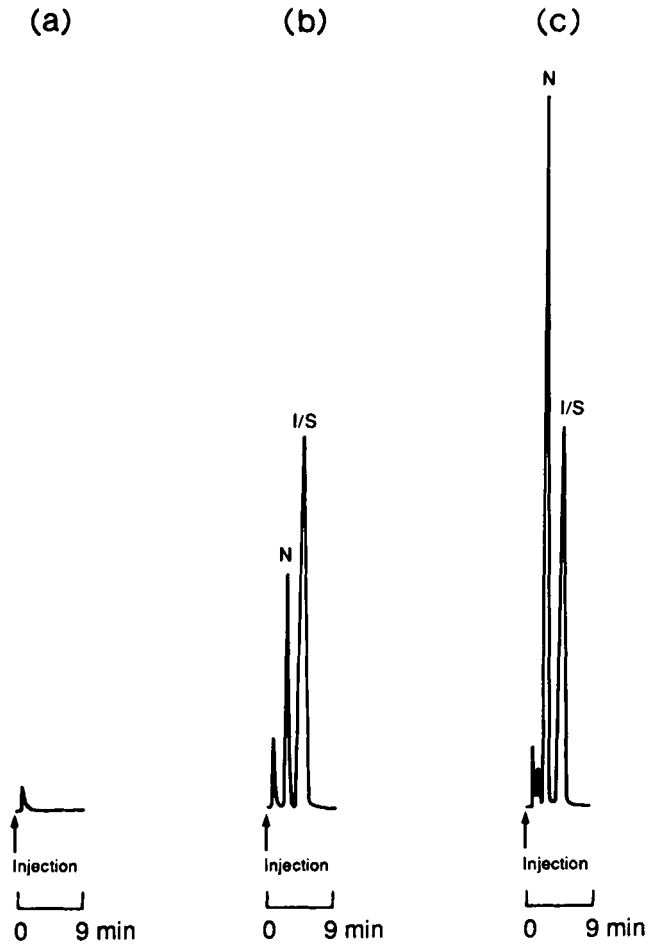

FIGURE 2

Typical chromatograms of human plasma: (a): blank plasma; (b): blank plasma spiked with 10 μg/ml naproxen (N) and the internal standard, flurbiprofen (I/S); (c): plasma with 44.6 μg/ml naproxen taken from a healthy volunteer 14 hours after an oral dose of 500 mg controlled-release naproxen.

The absolute analytical recovery of naproxen from plasma was 97.9 ± 3.9% (S.D.) at 1 μg/ml and 99.1 ± 2.2% at 100 μg/ml of naproxen (n = 6). The recovery of the internal standard, flurbiprofen was also good at the concentration used with a recovery of 96.5 ± 4.3% (n = 6).

The calibration curves were obtained on a number of occasions and were linear over the plasma naproxen concentration range 0.2-150 μg/ml with the determination coefficient (r^2) greater than 0.99. In all cases, the intercepts of the calibration curves were found not to be statistically different from zero and therefore were not included in the calculation.

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Under the assay conditions described above, the day-to-day coefficient of variation in the slope (b) of the calibration curve was 0.9% (n = 4). The sensitivity of the HPLC assay for naproxen was 0.1 μg/ml and was based on a signal-to-noise ratio of 3:1. The coefficient of variation for plasma naproxen concentration at this limit (0.1 μg/ml) was 12.5% (n = 6). This sensitivity appears to be sufficient for the determination of the drug levels usually encountered in the pharmacokinetic study of naproxen.

The reproducibility and accuracy of the naproxen assay in plasma is shown in Table 2. The coefficient of variations were 12%, 4.4% and 0.4% at 0.2, 1.0 and 100 μg/ml, respectively. The intra-assay (within day) coefficients of variation at 0.2 μg/ml and at 100 μg/ml were 5.5% (n = 4) and 1.2% (n = 4), respectively. The inter-assay precision (between days) of the assay for naproxen was reasonable with a coefficient of variation less than 10% at naproxen concentrations of 0.2 μg/ml and 10 μg/ml. The accuracy of

Table 2

Reproducibility and Accuracy of the Naproxen Assay

Naproxen added (μg/ml)	Naproxen concentration found (μg/ml) Mean ± S.D.	Coefficient of variation %	Accuracy* %
0.2	0.20 ± 0.02 (n = 6)	12.0	100.2
1	1.03 ± 0.05 (n = 5)	4.4	103.0
100	100.0 ± 0.4 (n = 4)	0.4	100.0

*Accuracy = (Mean Naproxen concentration found/Naproxen added) x 100

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the assay was greater than 99% for all the evaluated concentrations of naproxen (Table 2).

Repeated determinations of the naproxen concentration in the subject samples were also carried out to assess the accuracy of the analytical method. Random plasma samples from a bioavailability study were selected and analysed for naproxen (4 samples/subject, 15 subjects). The repeated determinations were performed on different days, usually 1-5 days after the original determination. The results indicate a consistency in the naproxen concentration between the original and the repeated determination with an accuracy of 101.0 ± 0.05% (n = 60), range 92.0 to 118%.

The stability of naproxen in plasma samples was studied by an analysis of plasma naproxen concentrations on two occasions using plasma samples

Table 3

Stability of Naproxen in Plasma Samples After Storage at -20°C for 7 weeks

Plasma samples were obtained from two healthy volunteers 12 hours after oral dose of 250 mg Naprosyn®

Sample	n	Naproxen Concentration (μg/ml)[1]	
		Fresh	Stored*
SW	5	35.1 ± 1.0	35.1 ± 0.7
JS	5	28.0 ± 0.7	27.5 ± 0.7

[1]Results given are mean ± S.D.
*No significant difference between the fresh and the stored samples ($p > 0.4$)

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taken from two healthy volunteers after oral administration of naproxen. One of the duplicate samples was analysed on the day the samples were collected whilst the second sample analysis was performed seven weeks after the samples had been stored at -20°C. The results are presented in Table 3. The plasma naproxen concentration was not significantly different between the fresh plasma samples and the stored plasma samples ($p > 0.4$). This indicates that the plasma samples containing naproxen can be stored under these conditions without an appreciable decomposition. The stability of extracted naproxen and the internal standard (flurbiprofen) in the sample mixture was also investigated. Both naproxen and the internal standard were stable in the injection solvent over a period of 24 hours.

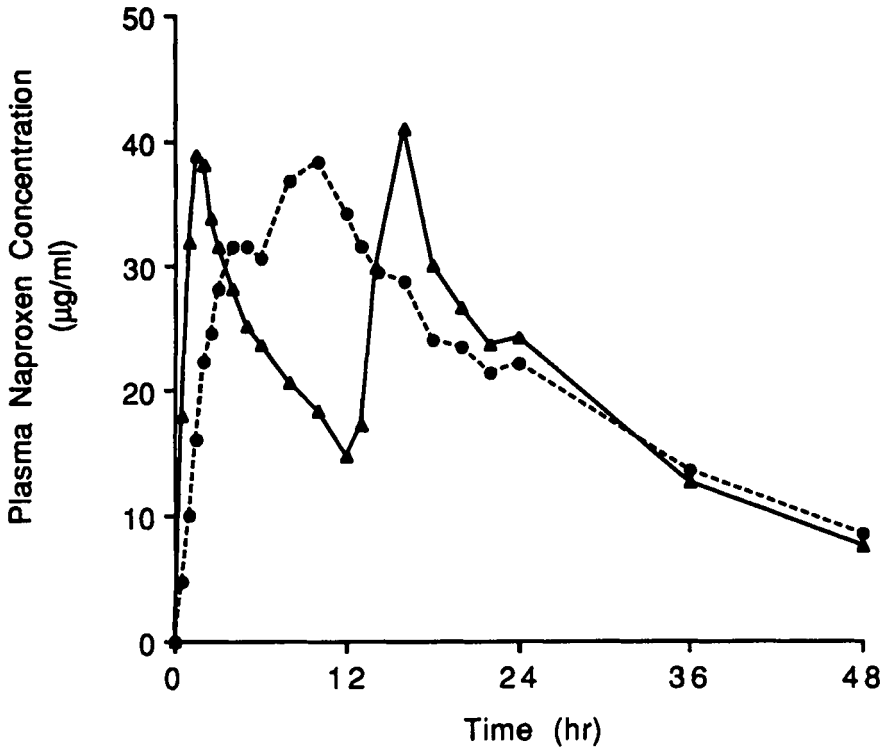

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FIGURE 3

Plasma concentration-time profiles obtained from a representative subject who had taken an oral dose of 250 mg standard naproxen (Δ) or 500 mg controlled-release naproxen (•).

The applicability of the assay described was demonstrated by the analysis of plasma samples from 15 subjects receiving oral doses of naproxen in a bioavailability study. Figure 3 shows the plasma naproxen contentration-time profiles following a single oral dose of either 250 mg standard naproxen or 500 mg controlled-release naproxen in one of the subjects studied. In this

particular study, the plasma samples were collected up to 48 hours after a single dose of naproxen and 72 hours in the other phase of the study after multiple doses of naproxen. None of the samples had a concentration of naproxen below the detection limit of 0.1 μg/ml.

In conclusion, the HPLC assay reported affords a simple, rapid and sensitive method for the determination of naproxen in human plasma suitable for use in clinical studies. The procedure is a direct plasma protein precipitation so is less tedious and rapid. The use of a microbore column provides an additional feature to improve the sensitivity of the HPLC assay.

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