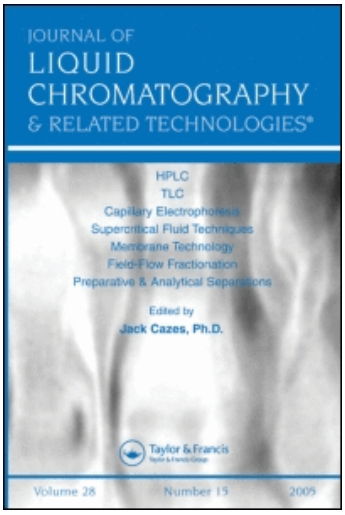

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A Rapid High Performance Liquid Chromatographic Assay for the Measurement of Diclofenac in Human Plasma

Jennifer L. Mason[a]; Gregory J. Hobbs[a]
[a] University Department of Anaesthesia Queens Medical Centre, Nottingham, United Kingdom

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A RAPID HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THE MEASUREMENT OF DICLOFENAC IN HUMAN PLASMA

JENNIFER L. MASON AND GREGORY J. HOBBS
University Department of Anaesthesia
Queens Medical Centre
Clifton Boulevard
Nottingham NG7 2UH, United Kingdom

ABSTRACT

A high performance liquid chromatographic method is described for the determination of diclofenac in human plasma, using naproxen as the internal standard. This method is simple, rapid and cost-effective. Zinc sulphate (5%) and methanol were used for extraction. Prepared samples were analysed on a nucleosil C18 column using a 35:65 acetonitrile-water phosphate buffered mobile phase (pH 2.8) and ultraviolet detection at 280 nm. The assay was linear in the range 30 ng/ml to 2 μg/ml, with recovery of extraction ranging from 82 to 97% and a detection limit of 30 ng/ml.

INTRODUCTION

Diclofenac sodium ([o-(2,6-dichloroanilino)-phenyl] acetate) is a non-steroidal anti-inflammatory drug prescribed commonly for acute and chronic musculo-skeletal pain, including arthritis, and more recently for the

treatment of post-operative pain. Diclofenac is 99% bound to serum protein, particularly albumin. Only 60% of oral diclofenac reaches the systemic circulation, mainly due to first pass metabolism, and the elimination half-life is approximately 1.3 ± 0.3 h [1].

The pharmacokinetics of diclofenac sodium are well documented, with maximum plasma concentrations (C_{max}) and time to C_{max} (T_{max}) depending upon the formulation and dose investigated, eg Voltaren-Retard (R) 100 mg 654 ± 329 ng/ml after 6.4 h, sucralfate-covered tablets 50 mg 773 ± 80 ng/ml after approximately 1 h, Diclogesic 100 mg 536 ± 63 ng/ml after 4.1 ± 0.9 h, Voltaren 75 mg 1400 ng/ml after 2.6 h [2-5].

High Performance Liquid Chromatography (HPLC) is the preferred method of analysis for the measurement of diclofenac, with preparation of samples being more rapid than Gas Chromatography (GC) [6-7]. Although Schumacher *et al* described a method using Thin Layer Chromatography (TLC), this lacks sensitivity and Battista *et al* commented on the absence of useful colour reagents for detection on TLC plates [8-9]. Many of the HPLC methods reported for the measurement of diclofenac involve evaporation and reconstitution as part of the sample preparation [5,10-19]. The method described here requires no evaporation stage and therefore allows a more rapid turnover of samples. Other methods not involving evaporation have been described [20-23]. One of these [20] not only has a limit of detection that is higher than this procedure (400 compared to 30 ng/ml) but also involves protein precipitation using perchloric acid which

attacks diclofenac and is therefore best avoided [23]. The method described by Wiese and Hermansson [21] involves a post-column UV photoreaction transformation of diclofenac into a fluorescent derivative, and that by Brunner and Luders [22] involves automation. Moncrieff [23] also described fluorimetric detection of diclofenac, using heat (85°C) to denature the samples. Linearity was reported from 40 ng/ml (compared to 30 ng/ml for this method) and baseline separation was not achieved for the diclofenac peak at 5.92 minutes.

The procedure described here is a simple, rapid and relatively inexpensive method for the measurement of diclofenac, eg extraction is liquid-liquid and does not require the use of solid-phase cartridges. It provides baseline separation of diclofenac with no inherent background interference at the retention times of interest in blank plasma samples.

MATERIALS

Zinc sulphate heptahydrate (ACS grade), diclofenac sodium and naproxen ((s)-6-methoxy-(-methyl-2-naphthaleneacetic acid)) were purchased from Sigma Chemical Company (Poole, UK). Sodium lauryl sulphate (AR grade), sodium dihydrogen orthophosphate (AR grade), acetonitrile (HPLC grade), orthophosphoric acid (AR grade) and methanol (HPLC grade) were purchased from Fisons Scientific Equipment (Loughborough, UK). Water used for analytical applications was purified using a Purite Select Analyst HP system (Thame, UK).

Apparatus

The HPLC system consisted of an SP8800 pump, SP8780 autosampler, SpectraChrom 100 variable wavelength detector and SP4400 Chromjet integrator (Thermo Separation Products, Stone, UK) and a C_{18} Nucleosil, 25 cm x 4.6 mm I.D., 5 μm particle size, reversed-phase column (Jones Chromatography Ltd, Hengoed, UK).

Chromatographic Conditions

The mobile phase was comprised of 1 mM sodium lauryl sulphate, 10 mM sodium dihydrogen orthophosphate and acetonitrile-water (35:65 v/v). Orthophosphoric acid was used to adjust the pH to 2.8. A 0.45 μm Gelman Sciences membrane was used to filter the mobile phase before use. The flow rate of the pump was 1.5 ml/min and the temperature ambient (range 27-30°C). The UV detector was set at a wavelength of 280 nm and 0.01 a.u.f.s.. Naproxen was used as the internal standard.

METHODS

Calibration

Fresh stock solutions of diclofenac and naproxen (1 mg/ml in water and 50% acetonitrile mobile phase respectively) were made on a weekly basis and stored at 4°C until use. Freshly made calibration standards were

prepared on a daily basis when required in the following concentrations, diclofenac 30 ng/ml to 2 μg/ml and naproxen 1 μg/ml.

Sample Extraction and Preparation

Blood samples were collected in 5 ml lithium heparin tubes and centrifuged at 2000g, 27°C for 10 min. The plasma was decanted off and either used immediately or stored at -70°C until use. Disposable polypropylene test tubes were used for all subsequent work.

Naproxen (1 μg/ml) was used to spike 1 ml of plasma. After the addition of 100 μl of 5% zinc sulphate (w/v in water) the sample was vortex mixed for 2 min. Methanol (3 ml) was added before vortex mixing for a further 2 min. Aqueous buffer (0.44 ml) was then added. This buffer consisted of 100 mM sodium dihydrogen orthophosphate and 10 mM sodium lauryl sulphate. Orthophosphoric acid was used to adjust the pH to 2.8 before the solution was filtered with a Gelman Sciences 0.45 μm filter. The sample was then finally vortex mixed for a further 1 min. The sample was centrifuged at 2000g, 27°C for 10 min and the supernatant decanted off, 100 μl of which was injected onto the HPLC column.

Extraction Efficiency

Three different concentrations of diclofenac (0.1, 1 or 2 μg/ml) and 1 μg/ml naproxen (n=6 for each concentration) was used to spike drug-free

plasma which was then taken through the extraction procedure. The extraction efficiency was then determined by comparing the results to a series of non-extracted aqueous standards.

Reproducibility

Aliquots of plasma (1 ml) were spiked with either 0.05, 0.2 or 1 μg/ml diclofenac (n=10 for each concentration) and naproxen 1 μg/ml and analysed during one working day in order to determine intra-assay reproducibility. Inter-assay reproducibility was investigated using pooled plasma (diclofenac 1 μg/ml and naproxen 1 μg/ml) as a quality control. This was analysed over an eight week period, being stored at -70°C until use with a single 1 ml aliquot used per sample run.

Light Sensitivity

To determine whether the storage conditions and/or the length of time before analysis was critical, twenty 1 ml aliquots of drug-free plasma were dispensed into colourless test tubes and spiked with diclofenac 1 μg/ml and naproxen 1 μg/ml. Ten of these were kept under darkened conditions while the other ten were left under normal laboratory conditions (ambient temperature, in daylight but out of direct sunlight) for between 15 min and 6 h.

Stability

Sample stability at ambient temperature was investigated over a 20 h period. The extract from pooled plasma, which had been spiked to a final concentration of either 0.05, 0.2 or 1 μg/ml diclofenac and 1 μg/ml naproxen, was analysed every hour for 20 h using an autosampler.

Measurement of Diclofenac Following 50 mg Oral Dose

After taking a baseline blood sample, a male volunteer received oral diclofenac 50 mg. Blood samples (5 ml) were collected via an intravenous cannula at 15, 30, 45, 60, 70, 80, 90, 105, 120, 150, 180, 240, 300, 360 min after administration. The blood samples were centrifuged and analysed immediately.

RESULTS

Measurement of Diclofenac

Figure 1 shows chromatograms of peaks for diclofenac (II) and naproxen (I) from a) aqueous standard, diclofenac 1 μg/ml and naproxen 1 μg/ml, b) plasma blank, c) plasma sample spiked with diclofenac 1 μg/ml and naproxen 1 μg/ml and d) plasma 80 min after oral administration of diclofenac 50 mg plus naproxen 1 μg/ml. Retention times for naproxen and diclofenac were approximately 5.8 and 10.6 min respectively.

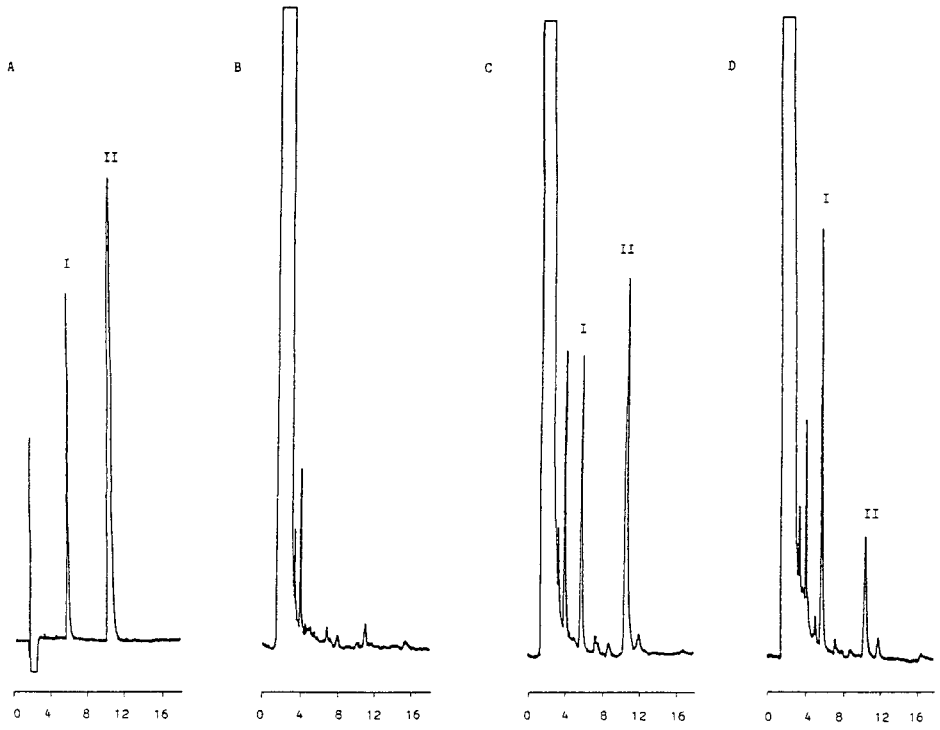

FIGURE 1. Typical chromatograms of the recovery of diclofenac (II) and naproxen (I) from aqueous standard diclofenac 1 μg/ml naproxen 1 μg/ml (A), plasma blank (B), plasma spiked with diclofenac 1 μg/ml naproxen 1 μg/ml (C) and plasma 80 minutes after an oral dose of 50 mg diclofenac (D).

Calibration

Figure 2 shows the linear correlation obtained between diclofenac concentration and peak-height ratio over the range 30 ng/ml to 2 μg/ml (n=6 for each of the 8 concentration points). The regression equation was $y = 1.32x - 0.014$ and the correlation coefficient 0.999. The standard

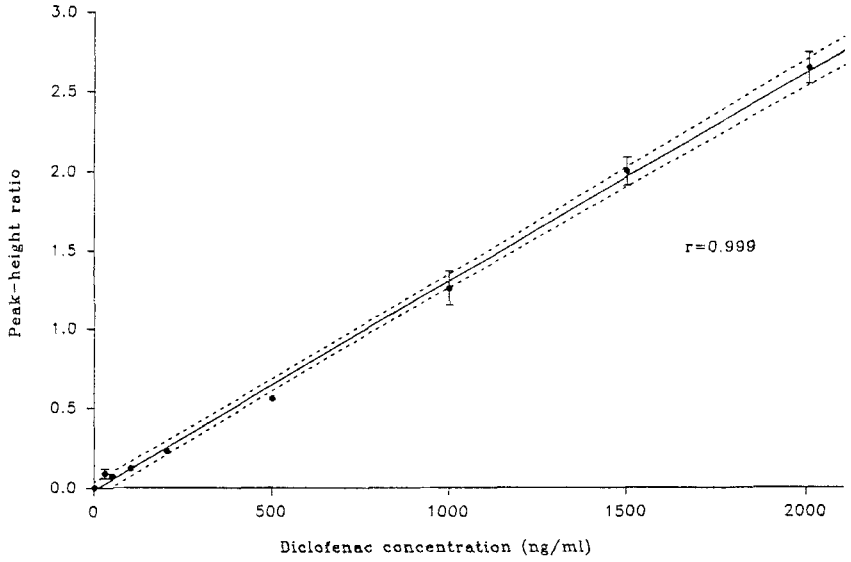

FIGURE 2. Calibration curve of diclofenac in human plasma.

TABLE 1. Recovery of Extraction of Diclofenac and Naproxen (Internal Standard)

			Naproxen 1μg/ml	
Diclofenac concentration (μg/ml)	Recovery (mean ± SD) (%)	CV (%)	Recovery (mean ± SD) (%)	CV (%)
0.1	97.2 ± 6.78	7.00	89.7 ± 1.08	1.20
1.0	83.7 ± 0.98	1.17	89.7 ± 1.75	1.95
2.0	82.0 ± 1.06	1.29	91.7 ± 2.57	2.80

n=6 for each concentration
CV=coefficient of variation

deviation for the slope and intercept were 0.021 and 0.023 respectively. The limit of detection was 30 ng/ml (signal-to-noise ratio of 3:1).

Extraction Efficiency

The recovery of extraction of diclofenac from human plasma was between 82 and 97% over the range of concentrations 0.1 to 2.0 μg/ml (Table 1). The coefficient of variation was 7% or less for all three concentration levels of diclofenac studied.

Reproducibility

Intra-assay reproducibility for the three concentrations of diclofenac investigated is shown in Table 2 (n=10 for each concentration). The coefficient of variation for intra-assay reproducibility ranged from 2.4 to 11.1%. Inter-assay reproducibility, determined by analysis of the quality control plasma samples over an 8 week period, had a coefficient of variation of 4.4%.

Light Sensitivity

Using the statistical package Minitab release 10 for Windows, neither the length of time before analysis (over the range 15 min to 6 h) nor exposure to light had a statistically significant effect upon the amount of diclofenac measured in spiked plasma samples ($p = 0.214$ and 0.063 respectively).

TABLE 2. Diclofenac Reproducibility and Stability Over a 20 Hour Period

Diclofenac concentation (ng/ml)	Intra-assay reproducibility (n=10) (peak-height ratio drug/IS)			Diclofenac concentration of pooled extract (ng/ml)	
	mean	SD	CV (%)	mean	SD
50	0.09	0.01	11.1	56	5.7
200	0.22	0.01	4.5	177	10.1
1000	1.25	0.03	2.4	989	8.0

CV=coefficient of variation

Stability

The diclofenac content of pooled plasma was stable over the 20 h period investigated. Table 2 shows the mean and standard deviations for the three concentrations investigated.

Measurement of Diclofenac Following 50 mg Oral Dose

Plasma levels of diclofenac 50 mg after oral administration are shown in Figure 3. Using the procedure described, plasma levels of diclofenac were still measurable 6 hours after oral administration, ie approximately 4 to 5 elimination half-lives.

DISCUSSION

Using the described procedure, concentrations as low as 30 ng/ml of diclofenac (signal-to-noise ratio of 3:1) can be measured, with no inherent background interference in blank plasma samples. The calibration curve was

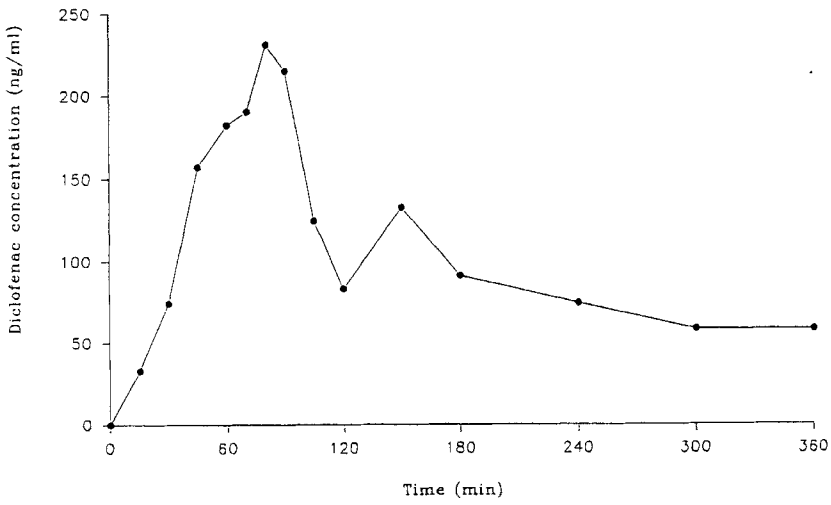

FIGURE 3. Plasma levels of diclofenac in a volunteer after a 50 mg oral dose.

linear between 30 ng/ml and 2 μg/ml (correlation coefficient 0.999). Recovery of diclofenac from human plasma was good for a variety of concentrations, eg for 0.1 and 2.0 μg/ml recovery was 97 and 82% respectively. This procedure was reproducible, both in terms of within-day and day-to-day reproducibility. Coefficient of variation for intra- and inter-assay reproducibility were 2.4-11.1 and 4.4% respectively.

Diclofenac (in plasma) was not effected by exposure to light, over the six hour period investigated. Once taken through the extraction procedure, diclofenac (in extract) was stable over twenty hours. Consequently a batch of samples could be prepared and extracted simultaneously and then analysed overnight using an autosampler.

Our procedure has been used to analyse blood taken from a volunteer following an oral dose of diclofenac 50 mg up to six hours post-administration and could be used for clinical research.

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