Journal of Chromatography, 616 (1993) 283–290
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6855

High-performance liquid chromatographic determination of diclofenac in human plasma using automated column switching

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(First received January 12th, 1993; revised manuscript received March 15th, 1993)

ABSTRACT

A validated high-performance liquid chromatographic procedure employing ultraviolet detection for the analysis of diclofenac in human plasma is reported. The method is rapid and, coupled with column switching, leads to a sensitive, accurate and reproducible assay. The retention times of diclofenac and the internal standard (4′-methoxydiclofenac, CGP-4287) are 6.4 and 7.6 min, respectively. The peak height *versus* plasma concentration is linear over the range 5.0–2000 ng/ml with a detection limit below 2.5 ng/ml. The mean absolute recovery of diclofenac using the described assay is 96.5% (n = 24). The inter- and intra-day accuracy and precision are within 8.3% of the actual values for all concentrations investigated. Furthermore, this procedure is applied to assess the pharmacokinetics of a single 75-mg oral dose of diclofenac sodium.

INTRODUCTION

Diclofenac sodium (Voltaren, 2-[(2,6-dichlorophenyl)amino]benzeneacetic acid monosodium salt, Fig. 1) is a non-steroidal anti-inflammatory agent used in the treatment of rheumatoid arthritis and severe osteoarthritis [1]. Several approaches exist for the determination of diclofenac in biological fluids, including thin-layer chromatography (TLC) [2], gas chromatography (GC) [3–7] and high-performance liquid chromatography (HPLC) [8–15]. TLC lacks the sensitivity required for the analysis of diclofenac in clinical samples and GC methods, although sensitive and selective, require extensive sample work-up, including derivatization. HPLC methods exist for the quantification of diclofenac using UV [9–14], fluorescence [8,9] and electrochemical [15] detection. Unfortunately, endogenous plasma constituents usually limit the sensitivity of diclofenac by HPLC. However, the use of column switching provides a means to enhance the selectivity of diclofenac, thereby allowing a detection limit of 2.5 ng/ml to be obtained without derivatization.

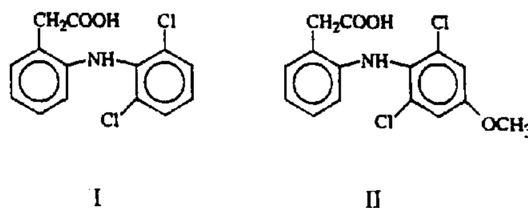

Fig. 1. Chemical structures of diclofenac (I) and internal standard (II).

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The method reported herein for the determination of diclofenac in human plasma is validated over the range 5.0–2000 ng/ml. This concentration range is predicted to be suitable for following the pharmacokinetics of a single 75-mg oral dose of Voltaren in humans. Furthermore, various stability parameters were evaluated including freeze–thaw cycles, plasma storage at –20°C and in-process stability.

EXPERIMENTAL

Materials

Diclofenac sodium and the internal standard (I.S., CGP-4287) were obtained from Ciba-Geigy (Ardsley, NY, USA). Phosphoric acid, 85% purity, was purchased from Fisher Scientific (Montreal, Canada). HPLC-grade acetonitrile, hexane and isopropanol were purchased from Caledon (Georgetown, Canada). Glass-distilled methanol and sodium acetate were purchased from BDH (Ville St. Pierre, Canada). The water was deionized Type 1, reagent grade (Millipore, Ville St. Laurent, Canada). All reagents were used without further purification.

Instrumentation

The chromatographic system consisted of two Waters Model 590 pumps, a WISP 710B autosampler, a Lambda Max Model 481 UV detector and a Waters automated switching valve (Waters Assoc. Milford, MA, USA). The system also included two columns: a Nucleosil C_{18}, particle size 10 μm (3.5 cm × 4.6 mm I.D., prepared inhouse) for sample clean-up, and a Nucleosil C_{18} column, particle size 10 μm (15 cm × 4.6 mm I.D., prepared in-house) for the analytical separation. The columns were maintained at ambient temperature. The UV detector was set at 280 nm (0.05 a.u.f.s.) to monitor the analytes. The mobile phase consisted of 22 m*M* sodium acetate, pH 7.1–acetonitrile–methanol (52:23:25, v/v/v). Both pumps supplied the mobile phase at a flowrate of 1.5 ml/min. Under these conditions, the retention times for diclofenac and the I.S. were 6.4 and 7.6 min, respectively.

Column-switching routine

The chromatographic separation of diclofenac and I.S. from plasma constituents is achieved isocratically using the column-switching configuration depicted in Fig. 2. With the switch-valve in position 1, the sample is injected onto the cleanup column, which is vented to waste. At 2 min post injection, the valve switches to position 2, which sends the eluent from the clean-up column onto the analytical column. At 4 min post injection, the valve returns to position 1. This

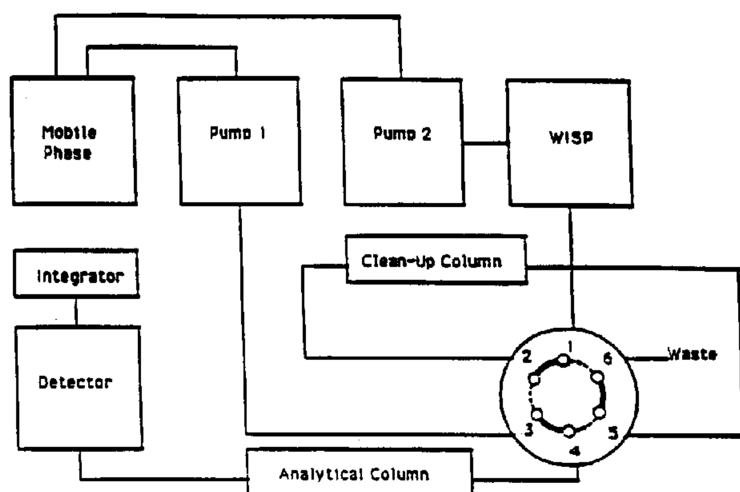

Fig. 2. Column-switching system. Position 1 is indicated by the solid lines within the switch valve, while position 2 is indicated by the broken lines.

"heart-cut" contains diclofenac and internal standard without most of the plasma constituents.

The column-switching routine is determined by connecting the clean-up column directly to the UV detector and injecting a reference solution of diclofenac and I.S. Once the retention times for diclofenac and I.S. have been established, the switch times can be determined. The first switch time is typically 30–45 s prior to the elution of diclofenac, while the second switch time is 30–45 s following the elution of the internal standard.

Biological samples

Blood samples were collected from healthy male volunteers after receiving a single 75-mg oral dose of Voltaren. Blood was drawn into evacuated EDTA collection tubes (Becton Dickinson vacutainer systems, Rutherford, NJ, USA), centrifuged immediately, and the plasma was stored at −20°C until analyzed.

Preparation of standards

Stock solutions of diclofenac were prepared at 1.00 mg/ml in methanol. Appropriate dilutions of the stock solutions were made with deionized water to prepare plasma standards of diclofenac at concentrations of 5.0, 10.0, 20.0, 100, 250, 500, 1000 and 2000 ng/ml. Quality control (QC) samples spiked in plasma were prepared in pools of 30.0 ml at final diclofenac concentrations of 25.0, 800 and 1600 ng/ml. Individual aliquots of 1.00 ml were stored in 100 mm × 16 mm screw-cap glass culture tubes and stored at −20°C until analyzed. A stock I.S. solution was prepared at 1.00 mg/ml in methanol and diluted to 1.5 μg/ml with deionized water. All stock solution were stable for at least one month when stored at −20°C.

Sample preparation

Aliquots of plasma (1.00 ml) were added to 100 mm × 16 mm screw-cap glass culture tubes. Plasma samples were treated with 200 μl of 1.5 μg/ml I.S. in deionized water (working internal standard), then acidified by the addition of 4.0 ml of 2.0 *M* phosphoric acid. The analytes were extracted with 6.0 ml of hexane–isopropanol (9:1, v/v) on a reciprocating shaker at low speed (150 ± 20 oscillations/min) for 15 min. It is imperative to shake at low speed or else an emulsion will form. After centrifugation for 10 min at *ca.* 1500 *g*, the organic layer was transferred into a clean 100 mm × 16 mm disposable borosilicate tube and evaporated to dryness at 37°C under a gentle stream of nitrogen. The residue was reconstituted in 250 μl of mobile phase, and 100 μl were injected into the liquid chromatograph under the previously stated conditions. The reconstituted samples were stable at room temperature for at least 20 h.

Data acquisition

The peak heights of diclofenac and internal standard were measured with a Spectra-Physics Model 4270 integrator and down-loaded to a Chrom-Station (Spectra-Physics, Mountain View, CA, USA). The chromatographic data were automatically processed for the peak-height ratios of diclofenac to I.S. and fitted to a weighted ($1/c$) linear regression.

RESULTS AND DISCUSSION

Chromatography

Typical chromatograms obtained from extracted plasma samples are illustrated in Fig. 3A–C. Fig. 3A shows a representative chromatogram of a processed plasma blank. This chromatogram indicates that no endogenous compounds exist at the retention time of diclofenac or the internal standard. Fig. 3B is a chromatogram amplified to the same degree as the blank exhibiting the limit of quantification (LOQ, 5.0 ng/ml). Fig. 3C is a plasma sample obtained from a subject 3.0 h after a single 75-mg oral dose of Voltaren. The retention times of diclofenac and the internal standard were 6.4 and 7.6 min, respectively. The overall chromatographic run time was 9.5 min.

Linearity and quantification limit

A linear response in peak-height ratio of diclofenac to I.S. over the range 5.0–2000 ng/ml was observed with a minimum signal-to-noise ratio of

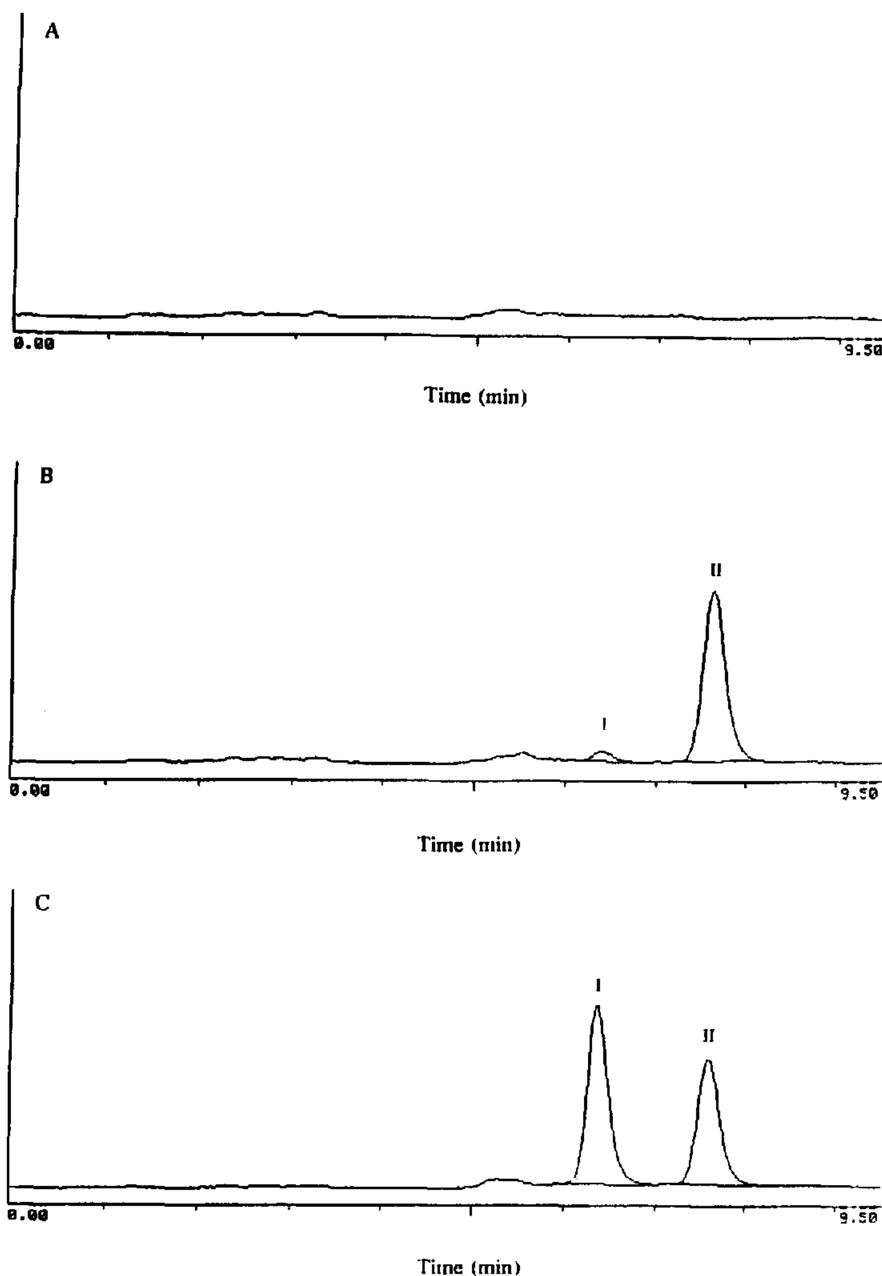

Fig. 3. Chromatograms of samples prepared according to the described procedure. (A) Plasma blank, attenuation 2; (B) plasma spiked at 5.0 ng/ml with diclofenac, attenuation 2; (C) plasma of a subject 3.0 h after a 75-mg oral dose of Voltaren, attenuation 4. Peaks: I = diclofenac; II = I.S.

5:1. The correlation coefficient from the standard curves used to validate this method were 0.9985 or better.

Recovery

The absolute recovery of diclofenac was evaluated by comparing the concentrations found in

plasma samples spiked with known amounts of each analyte to the concentrations found in solution (adjusted for concentration effects in the extraction). Spiked human plasma at four concentrations (5.0, 250, 1000 and 2000 ng/ml in replicates of six) were extracted as described previously except the I.S. was not added. The absolute peak heights from the extracted samples were compared to unextracted standard solutions prepared in the mobile phase. Similarly, the recovery of the I.S. was determined at the final recommended concentration. These results are provided in Table I.

TABLE I

RECOVERY OF DICLOFENAC AND INTERNAL STANDARD FROM HUMAN PLASMA (n = 6)

Drug	Concentration (ng/ml)	Recovery (%)	R.S.D. (%)
Diclofenac	5.0	101.0	3.8
	250	99.9	1.4
	1000	92.0	6.4
	2000	92.9	1.8
Internal standard	1200	97.5	5.3

TABLE II

TWELVE-WEEK STABILITY OF DICLOFENAC IN HUMAN PLASMA AT −20°C

Nominal concentration (ng/ml)	n	Mean found concentration (ng/ml)	R.S.D. (%)	R.E. (%)	Comments
25.0	6	26.8	9.2	7.1	Stable
800	6	777	5.8	−2.9	Stable
1600	5	1637	3.1	2.3	Stable

TABLE III

FREEZE–THAW STABILITY OF DICLOFENAC IN HUMAN PLASMA

Cycle No.	Found concentration (ng/ml)		
	25.0 ng/ml	800 ng/ml	1600 ng/ml
0[a]	27.4	793	1583
	26.9	786	1578
1	25.7	840	1581
	25.6	788	1567
2	23.8	827	1540
	25.0	791	1571

[a] Cycle 0 indicates the concentration from freshly spiked human plasma.

TABLE IV

IN-PROCESS STABILITY OF DICLOFENAC IN HUMAN PLASMA

Set No.	Time (h)	Found concentration (ng/ml)		
		25.0 ng/ml	800 ng/ml	600 ng/ml
1	0	27.3	801	1457
		25.2	829	1446
2	1	23.7	741	1577
		28.6	791	1584
3	2	23.0	819	1532
		24.6	854	1627
4	3	25.8	777	1585
		27.3	785	1572
5	4	26.0	767	1575
		24.4	782	1530

TABLE V

INTER-DAY PRECISION AND ACCURACY OF DICLOFENAC IN HUMAN PLASMA

Nominal concentration (ng/ml)	n	Concentration found (ng/ml)		R.S.D. (%)	R.E. (%)
		Mean	S.D.		
Standard					
5.0	7	5.2	0.38	7.3	3.0
10.0	7	10.1	0.53	5.2	1.0
20.0	7	19.9	0.92	4.6	−0.5
100	7	96.6	6.72	7.0	−3.4
250	7	245.9	11.46	4.7	−1.7
500	7	514.9	33.16	6.4	3.0
1000	6	997.1	48.63	4.9	−0.3
2000	7	1869.6	36.99	2.0	−6.5
Quality control					
25.0	14	23.1	1.35	5.8	−7.7
800	14	745.6	24.89	3.3	−6.8
1600	13	1467.2	104.67	7.1	−8.3

TABLE VI

INTRA-DAY PRECISION AND ACCURACY OF DICLOFENAC IN HUMAN PLASMA

Nominal concentration (ng/ml)	n	Concentration found (ng/ml)		R.S.D. (%)	R.E. (%)
		Mean	S.D.		
Std 5.0	6	5.1	0.21	4.1	2.0
QC 25.0	6	24.3	1.56	6.4	−2.8
QC 800	6	757.9	17.35	2.3	−5.3
QC 1600	6	1522.8	42.37	2.8	−4.8

Selectivity

Human plasma was collected from ten healthy donors and screened for interference at the retention times of diclofenac and the internal standard. No significant interference had been observed in drug-free plasma samples. Also, the following over-the-counter (OTC) drugs were tested for possible interference: caffeine, ibuprofen, aspirin, nicotine, acetaminophen, theophylline, phenylpropanolamine and dextromethorphan. These OTC drugs did not interfere with the analysis of diclofenac.

Stability

The stability of diclofenac under experimental conditions in stock solutions and frozen human plasma was investigated. The stock solutions of diclofenac and I.S. prepared in methanol were stable for at least one month at −20°C (difference ≤5%).

The reanalysis of plasma QC samples after storage at −20°C over a twelve-week period against a freshly prepared calibration curve showed that diclofenac was stable under the above condition (difference ≤15%, Table II).

The influence of two freeze–thaw cycles at each QC concentration level was examined in duplicate (Table III). Since no appreciable differences were observed between cycles (difference ≤15% with no trend), it was concluded that two freeze–thaw cycles can be tolerated.

The stability of the reconstituted residue, *i.e.* extracted diclofenac from plasma which has been dissolved in the mobile phase, was examined. Extracted QC samples were chromatographed immediately after preparation and also after 20 h in the autosampler. Virtually no changes had occurred (<3%) in the concentration of diclofenac after 20 h.

Lastly, the stability of diclofenac was investigated during the extraction proces (Table IV). After the addition of internal standard and buffer to five sets of QC samples, one set was extracted immediately, while the other sets were extracted 1, 2, 3 and 4 h later. Since no significant differences were observed among samples extracted immediately compared to those left at room temperature over the specified time periods (difference ≤15% with no trend), it was concluded that diclofenac and its I.S. were stable during the sample processing described herein.

Precision and accuracy

The inter-day precision and accuracy were assessed by the repeated analysis of plasma specimens containing different concentrations of diclofenac (Table V). The precision was based on the calculation of the relative standard deviation (R.S.D.). The accuracy was based on the calculation of the relative error (R.E.) of the mean found concentration as compared to the actual concentration. Two samples at each QC concentration (low, medium and high), together with a calibration curve, were run as a single batch. At spiked QC plasma concentrations of 25.0, 800 and 1600 ng/ml for diclofenac, the method yields an R.S.D. of 5.8, 3.3 and 7.1%, respectively. The R.E. obtained from the calibration curve ranged from −6.5 to 3.0% of the nominal concentrations for diclofenac.

The intra-day precision and accuracy were determined by the evaluation of a typical production run. Plasma samples spiked with diclofenac at concentrations of 5.0, 25.0, 800 and 1600 ng/ml were evaluated. The R.S.D. for samples analyzed were within 6.4% and the R.E. ranged from −5.3 to 2.0% of the nominal concentrations. These results are presented in Table VI.

Application

Plasma samples were obtained prior to dosing and at thirteen subsequent time points following a 75-mg oral dose of Voltaren. Following collection, the plasma samples were stored at −20°C until analyzed.

Estimates of the maximum concentration (C_{max}), time to reach C_{max} (t_{max}) and half-life ($t_{1/2}$) were 1400 ng/ml, 2.6 h and 0.9 h, respectively. All samples were analyzed by the method presented here. A typical plasma concentration–time profile of Voltaren is depicted in Fig. 4.

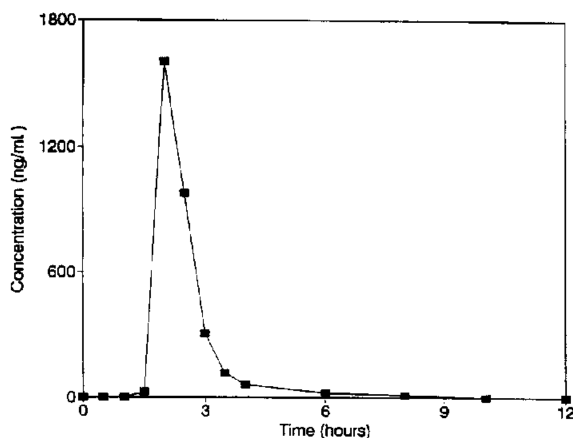

Fig. 4. Representative concentration–time profile of a subject following a single 75-mg oral dose of Voltaren.

CONCLUSION

The extraction process was modified slightly from that originally reported by Chan *et al.* [14]. Attempts to reproduce some reported diclofenac assays proved unsuccessful with respect to sensitivity and/or precision [10,11,14]. This was attributed primarily to the endogenous peaks resulting from the acidic extraction. Attempts to eliminate the interfering endogenous peaks by utilizing different acids (hydrochloric, sulfuric and perchloric), extraction solvents (ethyl acetate, diethyl ether and dichloromethane) and back-extractions were unsuccessful. Consequently, column switching was investigated and proved to be an acceptable alternative in determining diclofenac in human plasma.

The described method for the analysis of diclofenac in human plasma is selective, sensitive and robust. The intra- and inter-assay precision of the method was at or below 7.3%, while the accuracy of the method was within 8.3% even at the LOQ. Furthermore, the method requires a simple sample preparation, resulting in *ca.* 90 samples being processed daily. This assay has been used to monitor plasma levels in clinical trials generating over 1000 samples. More than 1000 plasma samples have been injected on a single analytical column with more than 500 injections being made on the clean-up column with minimal loss of chromatographic integrity.

Moreover, this procedure allows the quantification of diclofenac in human plasma for at least 12 h following a single 75-mg oral dose of Voltaren and permits the complete characterization of the resulting plasma profile.

ACKNOWLEDGEMENT

The author would like to thank Janet Miller for her help in preparing this manuscript.

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