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## Fully validated diclofenac HPLC assay

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### ABSTRACT

A simple, sensitive high-performance liquid chromatography (HPLC) assay for diclofenac measurement in human plasma was fully validated and diclofenac stability was studied. After one-step extraction of 1 ml plasma with 5.0 ml of tert. butyl methyl ether and reconstitution in mobile phase, naproxen (internal standard, IS) and diclofenac eluted at 3.9 and 8.3 minutes, respectively, on a Nova-Pak C<sub>18</sub> 4- $\mu$ m cartridge at room temperature (RT), and were detected using a 996 photodiode array detector set at 276 nm. The mobile phase, 0.2% glacial acetic acid (pH = 3.0) and acetonitrile (51:49, v/v), was delivered at 2.0 ml/min. Calibration curves were linear in the range 0.02-1.92  $\mu$ g/ml, and intra- and inter- run coefficients of variation were  $\leq$  5.3 % and  $\leq$  10.1 %, respectively. Extraction recovery and intra- and inter-run bias were = 86 %,  $\leq$  12%, and  $\leq$  10 %, respectively. Diclofenac was stable in plasma for 5 hours at RT ( $\geq$  97 %), 7 weeks at -20°C (= 93 %), and after 3 cycles of freeze at -20°C and thaw at RT ( $\geq$  91 %). In extracted samples, diclofenac was stable for 16 hours at RT ( $\geq$  97 %) and 48 hours at -20°C ( $\geq$  99 %). Stock solution of diclofenac (1 mg in methanol) was stable for 24 hours at RT (104 %) and 7 weeks at -20°C (91 %). The assay was successfully used to determine plasma diclofenac level after the ingestion of a therapeutic dose. The data indicate that the described assay is suitable for therapeutic drug monitoring and bioequivalence studies in humans.

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### KEYWORDS

Diclofenac;  
Naproxen;  
HPLC;  
Stability;  
Validation.

### INTRODUCTION

Diclofenac sodium, 2-[(2, 6-dichlorophenyl) amino] -benzeneacetic acid monosodium salt (CAS number: 15307-79-6), belongs to the non-steroidal anti-inflammatory drug group with analgesic and antipyretic activities<sup>[1,2]</sup>. Its oral bioavailability is about 50% and it circulates 99% bound to plasma proteins<sup>[2]</sup>. It has 2 main inactive metabolites, an aromatic hydroxylated metabolite and a conjugated metabolite<sup>[3]</sup>.

Several methods for the determination of diclofenac

in pharmaceuticals<sup>[4-7]</sup> and biological fluids have been reported<sup>[1,8-22]</sup>, including high performance liquid chromatography (HPLC)<sup>[1,8-16]</sup>, gas chromatography (GC)<sup>[17-21]</sup>, and gas chromatography coupled with mass spectrophotometry (GC-MS)<sup>[22]</sup>. However, they were not validated for clinical use. Moreover, little information is available on the stability of diclofenac<sup>[15]</sup>.

The aims of the current study were to 1) establish a simple, fully validated diclofenac HPLC assay in human plasma to be used in bioequivalence studies and therapeutic level monitoring, and 2) determine the sta-

bility of diclofenac under various clinical laboratory conditions.

## EXPERIMENTAL

### Apparatus

The liquid chromatograph consisted of Waters Alliance 2690 Separations Module, a 4- $\mu\text{m}$  (particle-size), 8 $\times$ 100 mm Nova-Pak C<sub>18</sub> radial compression cartridge in conjunction with an RCM-100 radial compression module, a Nova-Pak C<sub>18</sub> 4- $\mu\text{m}$  insert in conjunction with Guard Pak pre-column module, and Waters 996 photodiode array detector (Water Associates, Milford, MA, USA) set at 276 nm. Data were collected with a Pentium III computer using Millennium<sup>32</sup> Chromatography Manager Software (Water Associates, Milford, MA, USA).

### Chemicals and reagents

Diclofenac (Figure 1-a), and the internal standard (IS) naproxen (CAS number; 22204-53-1) (Figure 1-b) were analytical grade and obtained from USP (Rockville, MD, USA). Acetonitrile and methanol (HPLC grade) were purchased from Fisher Scientific (Fairlawn, NJ, USA). Tert. butyl methyl ether was supplied by Fluka Chemika AG (Buchs, Switzerland). Glacial acetic acid was purchased from BDH chemicals Ltd (Poole, England). Water for HPLC was prepared by reverse osmosis and further purified by passing through a Milli-Q System obtained from Millipore Co. (Bedford, MA, USA).

### Chromatographic conditions

The mobile phase consisted of 0.2% glacial acetic acid (pH = 3.0) and acetonitrile (51:49, v/v) and was delivered at a flow rate of 2.0 ml/min at room temperature. The mobile phase was filtered through a 0.22 $\mu\text{m}$  size membrane filter (Millipore Co., Bedford, MA, USA) and degassed before use. The autosampler was programmed to inject 100  $\mu\text{l}$  into the chromatograph with a run time of 10 minutes.

### Preparation of stock and working solutions

10 mg diclofenac sodium was dissolved in 10 ml methanol to produce a stock solution of 1 mg/ml. The stock solution was used for stability studies and to pre-

pare a 20 $\mu\text{g/ml}$  working solution in plasma. The working solution was used within one week.

10 mg naproxen was dissolved in 10 ml methanol to produce a stock solution of 1 mg/ml. An IS working solution of 5.0 $\mu\text{g/ml}$  was prepared weekly in mobile phase.

### Calibration standard/Quality control samples

Calibration standards were prepared by mixing nine different volumes of diclofenac working solutions in blank human plasma to produce final concentrations of blank, zero (blank plasma spiked with IS only), 0.02, 0.04, 0.05, 0.08, 0.12, 0.24, 0.48, 0.96, and 1.92 $\mu\text{g/ml}$ . Quality control (QC) samples were prepared by mixing four different volumes of diclofenac working solution in blank human plasma to produce final concentrations of 0.02, 0.06, 0.90, and 1.80  $\mu\text{g/ml}$ . Samples were vortexed for 20 seconds, then aliquots of 1 ml of calibration standards QC samples were transferred into Teflon-lined, screw-capped, borosilicate glass culture tubes and stored at -20 $^{\circ}\text{C}$ .

### Sample preparation

Aliquots of 1 ml of calibration standard, QC, or volunteer samples were allowed to equilibrate to room temperature. To each tube, 150 $\mu\text{l}$  of the 5.0 $\mu\text{g/ml}$  IS working solution was added and vortexed for 10 seconds. After the addition of 5.0 ml of tert.butyl methyl ether, samples were vortexed again for 1 minute and centrifuged for 5 minutes at 6000 rpm at room temperature. The organic layer was carefully collected, dried under a gentle stream of nitrogen at 40 $^{\circ}\text{C}$ , and the residue was reconstituted in 250 $\mu\text{l}$  mobile phase. After centrifugation at 3500 rpm for 2 minutes, the superna-

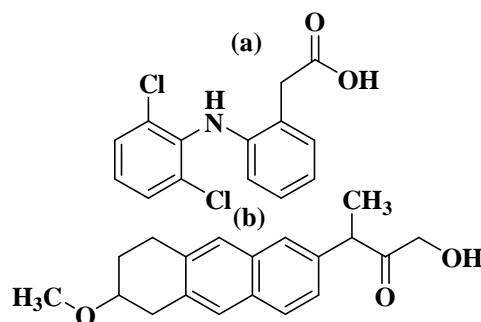


Figure 1: (a) Structure of diclofenac (C<sub>22</sub>-H<sub>24</sub>-Cl-N<sub>5</sub>-O<sub>2</sub>). (b) Structure of naproxen, the internal standard (C<sub>23</sub>-H<sub>29</sub>-Cl-F-N<sub>3</sub>-O<sub>4</sub>)

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TABLE 1: Specificity of diclofenac assay

Drug name	Retention time
Diclofenac	8.3
Naproxen (IS)	3.9
Aspirine	1.8
Acetaminophen	1.4
Ranitidine	1.7
Nicotinic Acid*	1.5
Ascorbic Acid*	1.2
Caffeine*	1.4
Ibuprofen	2.5
Omeprazole	5.6

1 mg/ml solutions in methanol (or water\*) were diluted in mobile phase to 10 µg/ml and 100 µl were injected.

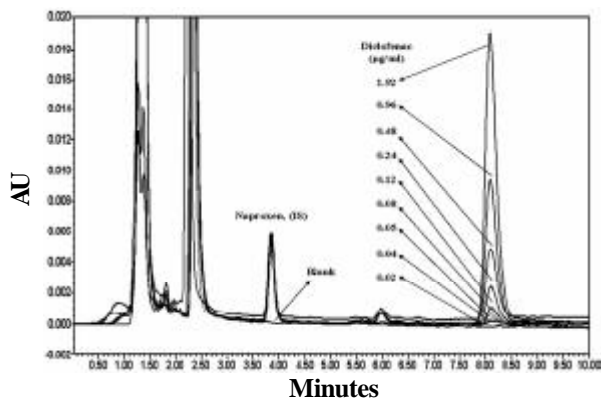


Figure 2: Overlay of calibration curve chromatograms of diclofenac spiked with IS, internal standard (naproxen)

tant was transferred into the auto-sampler vials and 100µl were injected. The run time was 10 minutes.

### Stability studies

**Stability of diclofenac in plasma:** Adequate number of aliquots of two QC samples (0.06, and 1.80 µg/ml) were prepared. Five aliquots of each QC sample were extracted and immediately analyzed (baseline), five aliquots were left for 2 or 5 hours at room temperature before analysis (counter stability), five aliquots were extracted, stored at room temperature for 16 hours or at -20°C for 48 hours, and then analyzed (auto-sampler stability), and five aliquots were stored at -20°C for 7 weeks before analysis (long term freezer stability). Finally, fifteen aliquots of each QC sample were stored at -20°C for 24 hours. They were then left to completely thaw unassisted at room temperature before being returned to -20°C for another 24 hours. The cycle was repeated three times (freeze-thaw stability). **Stock solutions stability:** Five aliquots of diclofenac or

the IS stock solution were diluted to 10 µg/ml in mobile phase and analyzed at baseline, after storage for 24 hours at room temperature, or after storage at -20°C for 7 weeks.

## RESULTS

### Optimization of chromatographic conditions

In order to improve specificity and minimize interference from plasma or solvent system that may occur at lower wavelengths, we optimized the absorbance wavelength based on photodiode array extracted spectra. We performed the analysis at 276 nm. A mobile phase composed of acetonitrile and glacial acetic acid (pH = 3.0) was employed to achieve separation of diclofenac from IS and minimize background absorbance. A satisfactory resolution of the peaks of interest was obtained. Under the described conditions, the IS and diclofenac were well resolved within a run time of 10 minutes, and their retention time were 3.9 and 8.3 minutes, respectively.

### Assay validation method

The procedures used for validation were as described in US food and drug administration (FDA) bioanalytical method validation guidance<sup>[23]</sup>.

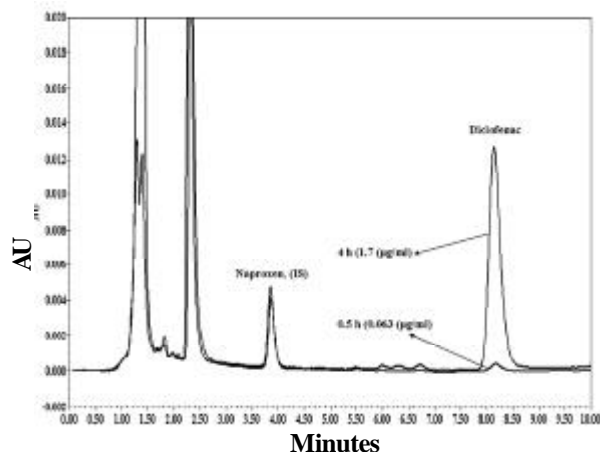
### Specificity

To evaluate specificity, we screened eight frequently used medications (dissolved in methanol: water, 50:50, v/v) and six different batches of human plasma. All batches of blank plasma were free from interfering components. None of eight commonly used drugs co-eluted with diclofenac or the IS (TABLE 1).

### Linearity

Linearity was determined in the range of 0.02 - 1.92 µg/ml using eight calibration curves. The data were analyzed by linear regression using the formula:  $\text{Conc.} = a + b (\text{PHR})$ , where Conc. is the concentration of diclofenac, a is the intercept, b is the slope, and PHR is the peak height of diclofenac divided by the peak height of the IS. The concentrations of the calibration standards of the eight calibration curves were back-calculated using the individual regression lines. Linearity studies (n=8) showed mean (SD) for  $R^2$  of 0.999 (0.0002)

a slope of 0.5581 (0.004), and an intercept of 0.0111 (0.0023). Figure 2 depicts an overlay of chromatograms of a representative standard curve. Figure 3 shows an overlay of two chromatograms of plasma samples collected from a healthy volunteer 0.5 and 4 hours after the oral administration of a single 50 mg diclofenac sodium tablet. The reported  $C_{max}$  of diclofenac sodium level after the oral administration of a therapeutic dosage of 50 mg is  $1.5\mu\text{g/ml}$ <sup>[24]</sup>, indicating that the described



**Figure 3:** Overlay of two chromatograms of plasma samples of a volunteer 0.5 and 4 hours after the oral administration of a single 50 mg diclofenac tablet

**TABLE 2:** Extraction recovery of diclofenac and naproxen

Nominal concentration ( $\mu\text{g/ml}$ )	Plasma		Mobile Phase		** Recovery (%)
	*Mean peak height	SD	*Mean peak height	SD	
Diclofenac 0.06	466	15.9	545	22.1	86
0.90	8382	198.7	9355	459.2	90
1.80	14650	365.8	16035	753.7	91
Naproxen 0.75	4997	605.2	5856	14.2	85

\*Mean peak height of 5 replicates. \*\*Mean peak height of spiked plasma sample divided by mean peak height of spiked mobile phase sample  $\times 100$ . SD, Standard Deviation

**TABLE 3:** Intra-run and inter-run accuracy and precision of diclofenac assay

Nominal concentration ( $\mu\text{g/ml}$ )	Intra-run (n=10)				Inter-run (n=20)			
	Mean measured concentration ( $\mu\text{g/ml}$ )	SD	Precision (CV*, %)	** Bias (%)	Mean Measured Concentration ( $\mu\text{g/ml}$ )	SD	Precision (CV*, %)	** Bias (%)
0.02	0.0225	0.0012	5.3	12	0.0208	0.0019	9.4	4
0.06	0.0589	0.0030	5.2	2	0.0564	0.0051	9.1	6
0.90	0.9291	0.0195	2.1	3	0.8727	0.0808	9.3	3
1.80	1.7086	0.0509	3.0	5	1.6213	0.1638	10.1	10

\*Coefficient of variation (CV) = Standard Deviation (SD) divided by mean measured concentration  $\times 100$ . \*\*Bias = 1 minus mean measured concentration divided by nominal concentration  $\times 100$ .

method is suitable for therapeutic drug monitoring.

### Limit of detection

The limit of detection (LOD) was defined as three times the baseline noise. LOD of diclofenac was set at  $0.01\mu\text{g/ml}$ .

### Recovery

The extraction recovery of diclofenac and the IS was determined by dividing mean peak heights of five replicates of three quality control samples (0.06, 0.90, and  $1.\mu\text{g/ml}$ ) and five replicates of the IS ( $0.75\mu\text{g/ml}$ ) that were prepared in plasma (as described under sample preparation above), by mean peak heights of five replicates of equivalent concentrations prepared in the mobile phase. The results of the extraction recovery studies of diclofenac and the IS are presented in TABLE 2. Recovery was = 86% (mean 89%) for diclofenac and 85% for the IS.

### Precision and bias

Precision was calculated as coefficient of variation (standard deviation divided by mean measured concentration  $\times 100$ ) and inaccuracy (bias) as the absolute value of (1 minus mean measured concentration divided by nominal concentration)  $\times 100$ . The intra-run and inter-run precision and bias were determined by analyzing four QC samples: 0.02, 0.06, 0.90, and  $1.80\mu\text{g/ml}$  over three different days (TABLE 3). Intra-run precision and bias ( $n = 10$ ) ranged from 2.1% to 5.3% and from 5% to 12%, respectively. The inter-run precision and bias ( $n = 20$ ) ranged from 9.1% to 10.1% and from 4% to 10%, respectively.

### Stability

The stability of diclofenac in plasma, in processed samples, and under usual storage conditions was in-



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TABLE 4: Stability of diclofenac in plasma samples and stock solution

Nominal concentration (µg/ml)	Stability (%)										
	*Plasma Samples									**Stock solution	
	Unextracted			Extracted		Freeze-thaw			24 h	7 wks	
	2 h	5 h	7 wks	16 h	48 h	One	Two	Three	24 h	7 wks	
	RT	RT	-20°C	RT	-20°C	cycle	cycles	cycles	RT	-20°C	
0.06	97	97	93	100	99	100	100	91	100	91	
1.80	99	100	100	97	100	108	100	100			

Stability (%) = mean measured concentration (n=5) at the indicated time divided by mean measured concentration (n=5) at baseline x 100. \*Spiked plasma samples were extracted with tert. butyl methyl ether and analyzed immediately (baseline, data not shown), after 2 or 5 hours at room temperature (2 h RT and 5 h RT), after 7 weeks at -20°C (7 wks -20°C), or after 1 to 3 cycles of freezing at -20°C and thawing at room temperature (freeze-thaw); or extracted and analyzed after 16 hours at room temperature (16 h RT) or 48 hours at -20°C (48 h -20°C). \*\* Diclofenac, 1 mg/ml in methanol.

investigated. The results of diclofenac stability studies are presented in TABLE 4. The data indicate that: 1) diclofenac in plasma is stable for at least 5 hours at room temperature and 7 weeks at -20°C, 2) in extracted samples, diclofenac is stable for at least 16 hours at room temperature and 48 hours at -20°C, 3) diclofenac in plasma is stable after at least three cycles of freeze at -20°C and thaw at room temperature and 4) diclofenac in methanol (1 mg/ml) is stable for at least 24 hours at room temperature and 7 weeks at -20°C. The IS in methanol (1 mg/ml) was also stable under the same conditions (108 % and 97 %, respectively).

### Robustness

The robustness of a method is a measure of its capacity to remain unaffected by small variations in method conditions. It provides an indication of the reliability of the method during normal applications. The robustness of the proposed method was evaluated by slightly altering the strength of the glacial acetic acid, pH, and amount of acetonitrile in mobile phase. No significant effects were observed. Further, the chromatographic resolution and peak responses were stable over about 850 injections of processed plasma samples using one cartridge.

### DISCUSSION

We describe a rapid, simple, accurate, and precise HPLC assay for the determination of therapeutic levels of diclofenac in human plasma. The advantages of the current assay include: (1) one-step liquid-liquid extraction rather than two steps organic extraction<sup>[1]</sup> or solid-phase extraction<sup>[8,9]</sup>, (2) a run time of 10 minutes compared to 40 minutes<sup>[1]</sup>, and (3) mean extraction recov-

ery for diclofenac of 89 % compared to 50 %<sup>[1]</sup>. Other previously reported assays were not developed to measure diclofenac level in biological fluids<sup>[4-7]</sup>, had relatively high limit of detection of  $\geq 25$  ng/ml<sup>[7,14-16]</sup>, or required derivatization<sup>[17-19]</sup>.

Limited information has been previously reported on the stability of diclofenac under various conditions observed in the clinical laboratory<sup>[15]</sup>. The comprehensive stability experiments that are reported here extend the known limits of stability of the drug in plasma to 5 hours at room temperature, 7 weeks at -20°C, and three freeze-thaw cycles.

We have also successfully used the assay to determine diclofenac level in a healthy volunteer after the oral administration of a single 50 mg diclofenac sodium tablet.

The performance characteristics of the assay together with the information on the stability of diclofenac under various conditions encountered in the clinical laboratory, indicate that the assay is suitable for use in therapeutic drug monitoring and bioequivalence studies.

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