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# CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF DICLOFENAC IN HUMAN BIOLOGICAL SAMPLES: A MINI REVIEW

## (Kaedah Kromatografi Untuk Penentuan Diclofenac Dalam Cecair Tubuh Manusia: Satu Ulasan Mini)

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**Abstract**

Diclofenac is a non-steroidal anti-inflammatory drug (NSAID) used to treat pain and inflammatory disorders. Due to a general increase in the usage of pharmaceuticals and incomplete removal of such compounds from through treatment of wastewater, drugs such as diclofenac have been labelled as emerging contaminants of concern. This review covers the chromatographic methods that have been reported in recent literature for the determination of diclofenac in biological fluids of humans. This could be beneficial for researchers interested in the detection and quantification of diclofenac for epidemiological studies to determine the extent of contamination within human populations.

**Keywords**: diclofenac, chromatographic methods, human fluids

**Abstrak**

Diclofenac adalah sejenis ubatan anti-keradangan bukan steroid yang digunakan bagi merawat kesakitan dan gejala keradangan. Peningkatan penggunaan farmaseutikal dan kegagalan penyingkiran sepenuhnya sebatian farmaseutikal dari rawatan sisa buangan mengakibatkan diclofenac dilabel sebagai bahan pencemar baru muncul. Ulasan ini meliputi kaedah kromatografi yang telah dilaporkan dalam penerbitan terkini untuk penentuan kepekatan diclofenac dalam sampel cecair tubuh manusia. Ulasan ini diharap memberi faedah buat penyelidik yang melakukan pengesanan dan pengkuantitian diclofenac untuk kajian epidemiologi bagi penentuan kesan pencemaran ini kepada populasi manusia.

**Kata kunci:** diclofenac, kaedah kromatografi, sampel cecair manusia

## Introduction

Diclofenac, with chemical name 2-[2-(2,6-dichloroanilino)phenyl]acetic acid (Figure 1), is a non-steroidal anti-inflammatory drug (NSAID) used to treat pain and inflammatory disorders. Its mechanism of action involves inhibiting the activity of

cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) [1]. Diclofenac is often formulated as either the sodium or potassium salt, with the difference between these two formulations being the rate of absorption and onset of action. Diclofenac potassium is more water soluble than diclofenac sodium, allowing for quicker absorption and rapid onset of pain relief [2]. Although diclofenac has useful medical applications, it has also been shown to possibly act as an endocrine-disrupting chemical (EDC). In vivo and in vitro studies have described diclofenac's potential as an EDC, with the possibility of affecting estrogen, androgen, glucocorticoid, and thyroid hormone receptors [3, 4].

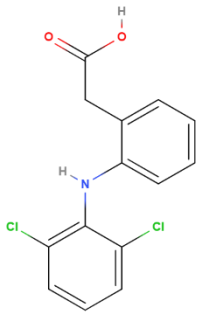


Figure 1. Chemical structure of diclofenac.

The metabolism of diclofenac in humans results in a range of metabolites, including those which are hydroxylated, methoxylated, and acyl glucuronide conjugated. The main metabolites of diclofenac are predominantly 4'-hydroxydiclofenac and 5-hydroxydiclofenac [5]. Most studies in the literature have focused on the toxic effects of diclofenac and its metabolites on avian [6, 7] and marine [8, 9] species. In humans, diclofenac shows acute toxicity. Overdose on NSAIDs such as diclofenac have been reported to result in gastrointestinal, renal, and neurological toxicity [10].

As reviewed by Sathishkumar et al. there is global occurrence of diclofenac contamination in various environmental compartments; thus, diclofenac has been labelled as a contaminant of emerging concern. Diclofenac and other pharmaceuticals can enter water environments through sources such as industrial production sites, wastewater treatment plant effluents, hospitals, and household waste. The presence of diclofenac in water environments can be attributed to a general increase in the usage of pharmaceuticals and incomplete removal of such compounds from wastewater [11]. Studies into the removal of pharmaceuticals from wastewater treatment plants have found that diclofenac and its metabolites are compounds with low removal potential and therefore high detection frequencies in wastewater [12].

Diclofenac and its metabolites have been detected in the drinking water supply of various countries. The unintended exposure of the general population to EDCs such as pharmaceuticals through drinking water supplies is of great concern. In Malaysia, a number of studies have detected diclofenac in drinking water; the maximum concentrations observed were 21.39 ng/L [13], 9.8 ng/L [14], 0.18 ng/L [15], and 0.0821 ng/L [16]. In other Asian countries, such as China, diclofenac has been detected at a maximum level of 3.7 ng/L [17]. At a Japanese drinking water purification plant, the maximum observed level of diclofenac was 16 ng/L [18]. For European countries, diclofenac was detected in surface water and groundwater intended for human consumption in France, with a maximum measured value of 56 ng/L [19]. However, another French study detected comparatively lower concentrations of diclofenac, with a maximum value of 2.5 ng/L being observed [20]. In Spain, a study found that the average concentrations of diclofenac in mineral and tap water were 25 ng/L and 18 ng/L respectively [21].

Although contamination levels of pharmaceuticals are generally lower than the typical dosages that are prescribed for medication, the long-term impacts of such exposure are still not well understood. Therefore, it is of interest to monitor the levels of pharmaceuticals, such as diclofenac, in the general population to determine whether there are long-term impacts. The aim of this review is to give a general overview on the chromatographic methods that have been reported in literature within the past decade for the determination of diclofenac in biological fluids of humans.

## Determination of diclofenac in biological samples

### Sample preparation

Human biological samples are complex matrices which often require preparation before analysis. The aim of

sample preparation is to obtain a sample that is free of interference and will not damage the instrument chosen for carrying out the analysis. Ideally, the sample preparation steps are minimal; and whilst 100% recovery of analytes from the sample is ideal, oftentimes reproducibility of recovery is more important [22]. In terms of determining analytes from biological samples using chromatographic methods, such as liquid chromatography-mass spectrometry (LC-MS), sample preparation is an important aspect; unwanted components that remain in the sample may interfere with the results obtained. Matrix effects are often caused by the presence of co-eluting compounds in the matrix that alter the ionization efficiency of target analytes in the sample. Matrix effects can be observed as either a loss or increase in response, ultimately affecting the accuracy, precision, and sensitivity of a method [23].

One of the most widely used methods for the extraction of analytes from biological samples is protein precipitation. It is considered as one of the fastest and simplest methods for the removal of proteins from biological samples (such as plasma or serum). However, protein precipitation often provides poor selectivity for extraction of target analytes. Another popular method for the extraction of analytes from biological samples is liquid-liquid extraction (LLE), both conventional methods and newer advances such as liquid-liquid microextraction (LLME). Conventional LLE often requires large volumes of organic solvents and is typically a time-consuming process; hence, newer advances – such as LLME – provides solutions to some the issues of LLE by consuming less solvent. Alternatively, solid-phase extraction (SPE) is another established method for the extraction of target analytes from biological samples. Advantages of SPE include high selectivity and lower organic solvent consumption compared to LLE. Additionally, SPE cartridges may also act as a filtration step, preventing suspended solids in the biological sample from clogging the analytical instrumentation. On the other hand, SPE is a time-consuming extraction process, with high running costs as cartridges are often designed for single use. Newer advances in SPE methods to provide solutions to the issue of conventional SPE include solid-phase microextraction (SPME) [24].

**Gas chromatography**

Gas chromatography (GC) is an analytical technique used for the separation of volatile compounds from a mixture which is in the gas phase. As a chromatographic method, GC makes use of a mobile and stationary phase; where the mobile phase is an inert gas, and the stationary phase can either be a solid or a liquid. Upon injection into the GC system, the sample is vaporized and carried through the column by the mobile phase, and compounds are separated based on their interactions with the stationary phase. Benefits of GC include relatively fast run times, good peak separation, and it works in combination with various selective detection methods. However, GC requires the compounds of interest to be thermally stable and sufficiently volatile, which can be an issue [25].

Several GC methods for the determination of diclofenac in human fluids have been developed and reported in literature within the past decade (Table 1). These include methods for serum, plasma, urine, and most recently, whole blood samples. All of the studies from Table 1 made use of capillary columns. For GC, there are two types of columns that can be used: packed columns or capillary columns. A packed column contains a fully-packed stationary phase made up of fine particles. On the other hand, a capillary column contains a stationary phase that that is coated on the inner column wall. Due to the fully-packed stationary phase, packed columns have higher internal pressures than capillary columns. Most packed columns are 2 – 6 m in length with internal diameters of 2 – 4 mm, whilst capillary columns are typically 15 – 100 m in length with internal diameters of 150 – 300 μm. In general, longer columns provide better separation as they have higher theoretical plates per meter. Owing to their internal diameters, packed columns can handle larger sample volumes than capillary columns. However, if too much sample is injected tailing may be observed, resulting in poorer separation. Therefore, capillary columns are often preferred as they provide better resolution, require smaller amounts of sample, and lower pressures compared to packed columns [31].

In terms of the stationary phase, all of the studies in Table 1 used non-polar columns based on

dimethylpolysiloxane. In general, non-polar analytes are more easily separated using a non-polar stationary phase [31]. In terms of the mobile phase, three of the studies used helium [26, 29, 30], whilst one used nitrogen [27]. Helium is often chosen as the inert gas to be used as it is more efficient than nitrogen and is safer than hydrogen. However, helium is more expensive than nitrogen, which may be a point of consideration [32].

In the studies included, the GC system is paired with either a flame ionization detector (FID) or mass spectrometer (MS) for the detection of diclofenac. The study which employed GC-tandem MS (GC-MS/MS) for the analysis diclofenac in whole blood samples reported the lowest limit of quantification (LOQ) of 0.1 µg/L [26]. In comparison, the lowest LOQ reported using GC-FID was 5.0 µg/L, for the analysis of diclofenac in urine samples [30]. Another advantage of GC-MS is its ability to provide structural information, which can be important in cases where there are co-eluting compounds; GC-FID is unable to differentiate compounds with the same retention time. This is especially significant when dealing with human biological samples, as there is often a limited sample amount. With GC-MS, the profiling of multiple EDCs from a single sample is possible even with co-eluting compounds. However, the instrumentation for GC-MS is often much more expensive than GC-FID [33].

**Liquid chromatography**

Liquid chromatography (LC) is an analytical method used for the separation of a mixture of compounds which are soluble in solvent. LC, like GC, makes use of a mobile and stationary phase; the difference lies in the mobile phase being a solvent or solvent mixture, and the stationary phase is a porous solid. The separation of compounds from a mixture relies on the interactions between the compounds of interest with both the mobile and stationary phases. Compounds which have higher affinity for the mobile phase will be eluted more quickly, whereas those with higher affinity for the stationary phase will remain in the column for a longer period of time [33].

Several LC methods for the determination of diclofenac in human fluids have been developed and reported in literature within the past decade (Table 2). Plasma samples have been the focus of these studies, although there are also methods for serum and urine samples reported.

Many of the studies used reversed-phase high-performance liquid chromatography (HPLC); this means that the stationary phase is hydrophobic, whilst the mobile phase is polar. The more hydrophobic the molecules being carried by the mobile phase, the more strongly adsorbed to the stationary phase they are and the longer their retention times [33]. The most common column employed by the studies in Table 2 was the octadecyl silane (ODS) column, which is often referred to as a $C_{18}$ column. One study used an octyl silane column, also known as a $C_8$ column [40]. The difference between a $C_{18}$ and $C_8$ column is the number of alkyl chains which are bonded to the silica. $C_{18}$ columns are more densely packed and hydrophobic in comparison to $C_8$ columns; therefore, the retention times of non-polar compounds will be greater in the $C_{18}$ column [33]. One study used a hydrophilic interaction liquid chromatography (HILIC) system, which is another mode of chromatography used for the analysis of polar and hydrophilic compounds [39]. The advantage of using HILIC over reversed-phase HPLC for the detection of polar pharmaceutical compounds, such as diclofenac, is that the analytes are better retained by the column [41].

Two of the studies used methanol/water for the solvent system [34, 35], whilst the other five studies used acetonitrile/water for the solvent system [36-40]. Historically, the most common organic solvents chosen for reversed-phase LC are methanol, acetonitrile, and tetrahydrofuran; of the three, methanol has the weakest elution strength whilst tetrahydrofuran has the strongest elution strength. However, tetrahydrofuran is rarely used nowadays due to toxicity and safety issues. The choice of acetonitrile as the organic solvent for the mobile phase is often made due to the stronger elution strength compared to methanol; furthermore, acetonitrile is less viscous than methanol resulting in improved column efficiency. Nevertheless, methanol may also be chosen as the organic solvent for the mobile phase as it is less expensive compared to acetonitrile [42].

Table 1. An overview on GC methods for the determination of diclofenac in human fluids within the past decade

| Formulation | Matrix | Sample Treatment | Recovery (%) | Detector | Column | Carrier Gas | Temperature (°C) | LOQ (µg/L) | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| **Diclofenac sodium** | Whole blood | 1. LLE | 92.2 – 105.9 | MS/MS (m/z 277 → 242) | SH-RXI-5MS (30 m × 0.25 mm i.d., 0.25 µm film thickness) | Helium | Start at 60°C. Held for 2 min. Increased to 320°C at 15°C/min. Held for 2 min. | 0.1 | [26] |
| **Diclofenac sodium** | Serum, Urine | 1. Derivatization (ethyl chloroformate)<br>2. LLE | Serum: 99<br>Urine: 97 | FID (310 nm) | DB-1 (30 m x 0.32 mm i.d., with 0.25 µm film thickness) | Nitrogen | Start at 150°C. Held for 3 min. Increased to 280°C at 20°C/min. Held for 5 min. | 1500 | [27] |
| **Diclofenac** | Serum | 1. LLE<br>2. Derivatization (N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% Trimethylchlorosilane (TMCS)) | 70.3 | MS | Agilent J&W HP-5ms UI (30 m x 0.25 µm i.d., with 0.25 µm film thickness) | Not stated | Start at 120°C. Held for 1 min. Increased to 300°C at 15°C/min. | 10 | [28] |
| **Diclofenac sodium** | Plasma | 1. Deproteination (phosphoric acid and acetone)<br>2. LLE<br>3. Derivatization (pentafluoropropionic anhydride (PFPA)) | 89 – 95 | MS (m/z 277) | BP-1 fused silica (15 m x 0.25 µm i.d., with 0.25 µm film thickness) | Helium | Start at 150°C. Held for 4 min. Increased to 180°C at 4°C/min. Held for 0.5 min. Increased to 300°C at 60°C/min. Held for 0.5 min. | 0.25 | [29] |
| **Diclofenac sodium** | Urine | Ultrasound-enhanced air-assisted liquid–liquid microextraction (USE-AALLME) | 74 | FID | BP-20 SGE fused-silica capillary column (30 m x 0.32 mm i.d., with 0.25 µm film thickness) | Helium | Start at 100°C. Increased to 230°C at 30°C/min. Held for 10 min. Increased to 260°C at 30°C/min. Held for 7 min. | 5.0 | [30] |

Two of the studies [39,40] made use of ammonium acetate as buffer solutions to improve peak shape [43]. The study by Klencsár et al. [38] made use of formic acid as an additive; formic acid is often used for MS studies as it provides protons for the MS analysis when carried out in positive mode [44]. It should be noted that diclofenac is a weak acid, with pKa value of 4.15 and logP value of 4.51 [45]. Three of the studies used mobile phases with pH values below 4, which means that the diclofenac is in its non-ionized form and has improved retention on the non-polar stationary phase [35, 37, 40]. Only one study used a mobile phase of pH 6.8, where the diclofenac would be in its ionized form [39]. This choice was made because the mode of chromatography used was a HILIC system, where the retention of analytes is dependent on the electrostatic interactions between the positively charged aminopropyl groups of the stationary phase and the negatively charged carboxylic acid functional groups of NSAIDs such as diclofenac.

In the studies included, the most popular LC technique is high-performance liquid chromatography (HPLC), paired with an ultraviolet (UV) detector. The main advantages of UV detectors are that they are relatively inexpensive and simple to operate [46]. However, specificity may be an issue, as there is no way to confirm that the absorption is not due to possible contaminants that also absorb across the wavelengths being observed. Another detector that can be paired with LC is an MS detector. Much like GC-MS, an advantage of liquid chromatography-mass spectrometry (LC-MS) is its ability to provide structural information, and therefore increase specificity when compared to HPLC-UV. Furthermore, MS detectors are often more sensitive than UV detectors, and lower LOQs can be obtained. However, the instrumentation for LC-MS is often much more expensive than HPLC-UV [33]. Klencsár et al. developed a novel quantification method for the detection of diclofenac using HPLC coupled to tandem inductively coupled plasma-mass spectrometry (ICP-MS), which is another type of MS that uses inductively coupled plasma to ionize the sample [38]. ICP-MS is an analytical technique for the determination of trace level elements in environmental and biological samples. Diclofenac contains chlorine (Cl) atoms, enabling the use of ICP-MS detection.

The study which employed HILIC-MS/MS reported the lowest LOQ of 0.000125 µg/L [39]. In comparison, the lowest LOQ reported using HPLC-UV was 0.0358 µg/L, for the determination of NSAIDs, including diclofenac, in human serum [35]. The lowest LOQ reported using a photodiode array (PDA) as the detector was 0.002 µg/L, for the determination of NSAIDs, including diclofenac, in human plasma and urine [37].

One of the main benefits of using LC over GC for the detection of diclofenac from human biological samples is that the samples are already in liquid form and can be injected into the LC system without the need for vaporization [33]. Furthermore, due to GC requiring volatile samples, temperatures required to run the analysis are often much higher than LC. On the other hand, the speed of elution is often much faster for GC than LC [25].

## Conclusion

Several chromatographic techniques for the determination of diclofenac in human fluids have been reported in literature over the past decade such as hyphenated GC and LC techniques. Generally, the studies employing LC techniques achieved lower LOQs than those using GC. The most popular detector for the detection of diclofenac was either a UV or MS detector. Whilst UV detectors are relatively inexpensive and simple to operate, MS detectors offer improved selectivity and specificity. This is especially important as expected exposure to pharmaceuticals, such as diclofenac, through contamination of drinking water is relatively low. Therefore, the levels of diclofenac present in human biological fluids would also be expected to be at trace levels and require high sensitivity for accurate analysis.

Table 2. An overview on LC methods for the determination of diclofenac in human fluids within the past decade

| Formulation | Matrix | Sample Treatment | Recovery (%) | Detector | Column | Mobile Phase | LOQ (µg/L) | Ref. |
|---|---|---|---|---|---|---|---|---|
| **Diclofenac** | Plasma, Urine | 1. Protein precipitation<br>2. Stir bar sorptive extraction (SBSE) | 69 – 74 | UV (220 nm) | $C_{18}$ (250 x 4.6 mm; 10 µm) | Methanol/0.2% acid acetic/water (70/0.2/29.8, v/v) | 0.65 (Plasma), 0.45 (Urine) | [34] |
| **Diclofenac sodium** | Serum | 1. Protein precipitation | 98 – 99 | UV (230 nm) | Shimadzu Shim-pack CLC-ODS (M) (4.6 mm i.d. x 0.25mm) | Methanol/water pH 3.5 (80:20, v/v) | 0.0358 | [35] |
| **Diclofenac** | Plasma, Serum, Urine | 1. Protein precipitation<br>2. Solid-phase microextraction (SPME) | Plasma: 98 – 99<br>Serum: 97 – 98<br>Urine: 95 – 98 | UV (280 nm) | Eurosphere $C_{18}$ (250 × 4.6 mm) | Acetonitrile/water (50/50, v/v) | 0.14 | [36] |
| **Diclofenac** | Plasma, Urine | 1. Protein precipitation<br>2. Magnetic solid phase extraction (MSPE) | Plasma: 99 – 101<br>Urine: 99 – 100 | Photodiode array (275 nm) | Luna Omega $C_{18}$ (100 mm x 2.1 mm, 1.7 µm) | Acetonitrile and potassium dihydrogen phosphate (10 mM pH 2) in water (50/50, v/v) | 0.002 | [37] |
| **Diclofenac sodium** | Plasma | 1. Protein precipitation | 93 – 96 | ICP-MS/MS (m/z 35 ($Cl^+$) → 37 ($ClH_2^+$) | Pre-concentration: Waters XBridge BEH $C_{18}$ (4.6 x 20 mm; 3.5 µm)<br>Separation: Waters XBridge BEH $C_{18}$ (4.6 x 150 mm; 3.5 µm) | A: 0.1% (v/v) formic acid in MQ water<br>B: 0.1% (v/v) formic acid in acetonitrile | 2 | [38] |
| **Diclofenac** | Plasma | 1. Protein precipitation | 97 – 101 | MS/MS (m/z 294 → 250) | Unison UK-Amino column (50 mm × 3 mm; 3 µm) | A: 10 mM ammonium acetate solution (pH 6.8)<br>B: Acetonitrile | 0.000125 | [39] |
| **Diclofenac potassium** | Plasma | 1. LLE | | UV (230 nm) | Thermo BDS Hypersil C8 (250 x 4.6 mmm; 5 µm) | Acetonitrile and 0.02M ammonium acetate buffer pH 3.5 (53:47, v/v) | 25 | [40] |

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