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ARTICLES

Development and Validation of a Bioanalytical Method for the Quantification of Aceclofenac in a Small Volume of Human Serum by RP-HPLC

Fatema Moni[a, b, *], Suriya Sharmin[a], ADA Shahinuzzaman[a], Satyajit Roy Rony[a], Saima Mollick[a], Md. Najem Uddin [a], and Md. Hossain Sohrab[a, **]

[a] *Pharmaceutical Sciences Research Division, BCSIR Laboratories, Dhaka, Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka, 1205 Bangladesh*

[b] *Biomedical and Toxicological Research Institute (BTRI), Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka, 1205 Bangladesh*

**e-mail: moni.fatema@yahoo.com*

***e-mail: mhsohrab@bcsir.gov.bd*

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Abstract—In this study, a simple, accurate, precise, and cost-effective method based on reversed-phase high-performance liquid chromatography with diode array detection for the quantification of aceclofenac in a small volume of human serum (100 μL) was developed and validated according to US-FDA guidelines. Acetanilide was used as an internal standard. The analyte and internal standard were spiked in human serum and extracted using a simple protein precipitation technique. The analysis was carried out on a reversed-phase C_{18} HPLC column (5 μm, 4.6 × 250 mm) using 0.1% formic acid and acetonitrile in a ratio of 40 : 60 (v/v) as the mobile phase with UV detection at 275 nm at a flow rate of 1.2 mL/min. The developed method was found to be sensitive, selective, and linear over the concentration range of 0.8–20 μg/mL with good precision (RSD ≤ 15%) and accuracy (relative error ≤15%). The limit of detection and lower limit of quantification were found to be 0.3 and 0.8 μg/mL, respectively.

Keywords: HPLC-DAD, bioanalytical method, human serum, aceclofenac, acetanilide, validation

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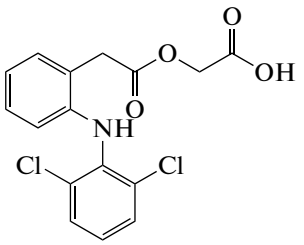
Aceclofenac, a non-steroidal anti-inflammatory drug (NSAID), is a phenylacetic acid derivative (Scheme 1) that exerts its pharmacological activity by an antagonistic effect on cyclooxygenase enzymes, thus inhibiting prostaglandin synthesis [1].



Scheme 1. Chemical structure of aceclofenac.

It is more selective to COX-2 enzymes and hence is called as a preferential COX-2 inhibitor [2]. It is commonly used to treat different painful and inflammatory processes in musculoskeletal disorders like osteoarthritis, rheumatoid arthritis, spondylitis, body pain, low back pain, scapulohumeral periarthritis, extraarticular rheumatism, and dental discomfort [1, 3]. Aceclofenac shows potential anti-inflammatory and analgesic activity, similar efficacy, and improved gastrointestinal tolerance compared to other NSAIDs [1, 2, 4]. The safety and tolerability of aceclofenac make it far superior to other NSAID analogues. As a result, physicians in Asian and European countries frequently recommend it [4]. Aceclofenac is classified as a class II medication by the Biopharmaceutics Class System (BCS), having a log partition coefficient of 2.170. In adults, the daily recommended dose of aceclofenac is 100 mg twice a day. It is well absorbed in the stomach and upper intestine as an unchanged oral delivery form and undergoes first-pass metabolism, necessitating dose adjustment in hepatic impairment. In humans, the mean plasma drug concentration has been reported to be 7–10 μg/mL, achieved in 1–3 h with a mean elimination time ($t_{1/2}$) of around 4 h. It is mainly excreted in urine after metabolizing by the cytochrome P450 enzyme system [5].

Drug development stages can be divided into the discovery of lead molecules after screening from natural or synthetic sources, lead optimization, animal study, and clinical evaluation on human subjects (Phase I to IV). Application of pharmacokinetics

(PK), i.e., the quantification of drugs to monitor the change of drug concentration with time, plays a pivotal role in different stages of drug development process. Over time, poor PK profiles for new drug candidates contributed to the clinical failure and cessation of developments of new molecules as a drug [6]. Thus, quantitative bioanalysis plays an influential role in drug discovery and drug optimization process.

Quantification of drugs in plasma/serum can be done mainly by high-performance liquid chromatography-ultraviolet detection (HPLC-UV) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Each analytical instrument has its own pros and cons. Over the last few decades, LC-MS/MS has become the instrument of choice for its numerous advantages like low detection limits, excellent selectivity, minimal sample requirement, and ability to provide different structural information of broader classes of analytes with different polarities and rapid sample analysis rate. However, analysis using LC-MS/MS requires expert analysts for operation and data integration. Besides, for some instances, the use of LC-MS/MS instruments is limited for bioanalytical method development due to the presence of co-extracted biological matrix components, which causes a matrix-induced effect in ionization efficiencies and ion suppression or enhancement. On the other hand, HPLC coupled with UV, photodiode-array (PDA), or fluorescence detectors offers a cost-effective, simple bioanalytical method and easy handling technique available at different clinical laboratories. However, it has some demerits like poor sensitivity and selectivity compared to LC-MS/MS [7]. The best way to design a method without the need to consult a more experienced analyst or hire a consultant is to take a straightforward approach using a simple analytical technique. So, if adequate sensitivity could be attained, bioanalytical method development using HPLC-UV offers cost-effective and simpler process.

There are several reports in the literature about the quantification of aceclofenac individually or in combination with other drugs in plasma/serum [4, 8–22], including review articles [23]. Techniques used for the quantification of aceclofenac in serum/plasma include HPLC-UV [8–16], narrow-bore HPLC with column switching [17], capillary electrophoresis [18], and LC-tandem mass spectrometry [19–22]. A UV detector is mostly used with HPLC to quantify aceclofenac in serum/plasma. Most of the reported methods were developed using complex sample pretreatment and/or chromatographic separation. Besides, the majority of the reported methods used high volumes of plasma/serum for extraction (≥500 μL).

There are several reasons for bioanalytical method development and validation like the development of new drugs, transfer of methods between laboratories, changes of methodology, reagents, and solvents to be cost-effective, development of a simple method, changes of instruments or analysts, use of a low volume of a biological fluid for analysis, etc. It is a continuous process to develop and validate a bioanalytical method as technological platform and sample processing are upgrading continuously.

Thus, as a part of continuous effort on the development of a simple bioanalytical method, the goal of the investigation was to develop a simple, accurate, cost-effective, and precise method with adequate sensitivity by high-performance liquid chromatography with diode array detection (HPLC-DAD) to quantify aceclofenac in less volume of human serum (100 μL) employing a simple protein precipitation extraction technique. The method is also expected to be suitable for bioavailability, bioequivalence, and pharmacokinetic studies.

EXPERIMENTAL

Materials and reagents. Aceclofenac was kindly gifted by Beximco Pharmaceuticals, Bangladesh. Acetanilide used as the internal standard (**IS**) was purchased from Sigma Aldrich. Methanol and acetonitrile used in the analysis of HPLC grade were purchased from Active Fine Chemicals, Bangladesh. Formic acid with a purity of 85% was also obtained from Active Fine Chemicals, Bangladesh. All other chemicals used were of analytical grade. Deionized water was used throughout the method development and validation process (SUEZ water purification system, UK). Commercially available human serum from human male AB plasma was used as serum matrix for method development and validation purposes obtained from Sigma Aldrich.

Instrumentation and chromatographic conditions. The separation was carried out using a Hitachi Chromaster HPLC system with an Agilent Open Lab software data acquisition system. The HPLC system consisted of an LC pump (Chromaster 5110 pump) with an auto-injection system (Chromaster 5210 autosampler with thermostat), a photodiode array detector (Chromaster 5430 diode array detector), and a column oven (Chromaster 5310 column oven). The system was connected to a personal computer for instrumental operation.

The mobile phase consisted of 0.1% formic acid (pH approx. 3) and acetonitrile in a ratio of 40 : 60. Formic acid was used without considering the assay percent mentioned in the label. Isocratic elution was performed, and the mobile phase was filtered and degassed before use in the analysis.

The separation was performed on a Hitachi Lachrom C_{18} column (5 μm, 250 × 4.6 mm) fitted with a Lachrom C_{18} guard column which was kept at room temperature (25°C) with a flow rate of 1.2 mL/min. The detector response was recorded with a discrete channel at 275 nm. Samples were injected

into the column using an autosampler set at 25°C with an injection volume of 50 μL.

Preparation of stock solutions. A stock solution of aceclofenac with a concentration of 400 μg/mL was prepared in a clear glass volumetric flask by dissolving 20 mg of aceclofenac in 50 mL of methanol. 10 mg of acetanilide was taken in a volumetric flask and dissolved in methanol to prepare the stock solution of acetanilide. These stock solutions were used for preparing calibration standards and quality control (**QC**) samples. Stock solutions were stored at 4°C for further use and were stable for one month.

Preparation of calibration standards and quality control samples. Six different working solutions of aceclofenac were prepared from the above stock solution by appropriate dilutions of accurately taken volumes in methanol to achieve the concentrations of 8, 10, 50, 100, 150, and 200 μg/mL to construct a calibration curve. 10 μL of the working solution of aceclofenac from each dilution was spiked with 90 μL of blank human serum to obtain final concentrations of 0.8, 1.0, 5, 10, 15, and 20 μg/mL which represent the calibration points. Before extraction, 10 μL of acetanilide (IS) was added to the serum sample. A blank sample (serum sample prepared without aceclofenac or internal standard), a zero sample (serum sample prepared with only internal standard), and six non-zero samples covering the expected linearity range, including lower limit of quantification (LLOQ), (serum samples prepared with both aceclofenac and internal standard) were run to construct the calibration curve in serum sample. Ratio of peak areas of aceclofenac to acetanilide against aceclofenac concentration was plotted to construct the calibration curve.

Four quality control samples at different concentrations were also prepared in a similar way, namely, lower limit of quantification (0.8 μg/mL), low (2.0 μg/mL), medium (8 μg/mL), high (16 μg/mL). Quality control samples were prepared from different stock solutions. All the serum samples prepared for the method development and validation were stored at −20°C.

Extraction procedures. A simple protein precipitation technique was employed to extract aceclofenac from serum samples. Serum samples spiked with aceclofenac (100 μL) were prepared in 2-mL Eppendorf tubes. After adding 10 μL of acetanilide (internal standard) to the serum sample, it was vortexed for 30 s to ensure complete mixing of the components. As a protein precipitant, 890 μL of acetonitrile was added to the mixture. Further, the mixture was cyclomixed by a vortexer for 1 min to ensure uniform mixing. The Eppendorf tube was further centrifuged at 12000 rpm for 12 min. Finally, the supernatant was collected, filtered through a 0.22-μm syringe filter, and transferred to an autosampler vial, and 50 μL of the supernatant was directly injected into HPLC.

Method validation. By determining different validation criteria like sensitivity, selectivity, recovery, linearity range, precision, and stability, the proposed method for the quantification of aceclofenac in human serum was validated as per the United States Food and Drug Administration (**US-FDA**) guidelines [24].

Specificity. The specificity of the proposed method was evaluated to determine the degree of interferences from co-eluting substances retained in the sample after extraction like drug metabolites, components co-extracted with the drug from the matrix, impurities, degradants, etc., during chromatographic analysis. It is also expressed as selectivity. The selectivity was investigated at LLOQ level using a blank human serum sample from six batches. Blank serum samples and serum samples processed with analytes at LLOQ level were prepared and extracted as described previously, and the peak areas of these two types of samples were compared. The lack of an interfering peak at the same retention period of the analyte and internal standard ensures the specificity of the proposed method.

Sensitivity. The sensitivity of the developed bioanalytical method was measured in terms of LLOQ. LLOQ is the lowest concentration of analyte used in the calibration curve that can be measured accurately and precisely, while the percent coefficient of variance (CV, %) should be ≤20%. The analyte response should be at least five times the response of blank. The limit of detection (LOD) of a method is the concentration of analyte that can be detected by detector response but not necessarily quantified accurately and precisely. The ratio of response of the analyte to the blank at LOD should be at least three. Five replicate analytical runs were recorded to determine LLOQ.

Recovery. Recovery was determined by spiking human serum samples with aceclofenac at four different QC concentrations (0.8, 2.0, 8, and 16 μg/mL) with five replicate measurements of every concentration. The recovery was calculated by comparing the analyte response extracted from serum to the response of the analyte from post-extraction serum standard sample (i.e., spiking was done after serum extraction) at equivalent concentration. Calculation of recoveries was performed using Eq. (1):

$$\text{Recovery} = (\text{BE}/\text{AE}) \times 100, \quad (1)$$

where BE stands for the response of human serum samples spiked at QC level before extraction, AE stands for the response of human serum samples spiked at QC level after the extraction procedure.

Linearity range. Linearity study is a crucial part of method development because it defines the range of the method within which the results are obtained with accuracy and precision [25]. The calibration standards prepared in human serum were used to test the linearity of the method. The linearity of the proposed method was tested over the concentration range of

0.8–20 μg/mL by plotting the peak ratio of the analyte to the internal standard against the corresponding concentration of the analyte. A six-point calibration curve was constructed using a simple linear regression model to determine the linearity of the developed method (non-zero standard). A blank blood sample was also analyzed to ensure no interfering peaks at the same retention time as the analyte or internal standard. However, this run was not utilized to build the calibration curve. The calibration curve is accepted if the determination coefficient (R^2) is ≥0.99 and the back-calculated concentrations at each point of the calibration curve are within 15% of the nominal value, except for LLOQ, where a 20% variance is acceptable.

Precision and accuracy. The accuracy of an analytical method is the vicinity of measurement results (the value actually found) to the true value (nominal). Precision represents the vicinity of measurement results to each other [26]. The accuracy and precision were measured by five replicate analyses of four QC samples (0.8, 2.0, 8, and 16 μg/mL). The accuracy of the method was determined as the percent of the measured concentration to the nominal concentration and expressed as percent relative error (RE, %). Intra-day precision was assessed by analyzing four QC samples (0.8, 2, 8, and 16 μg/mL) in five replicates within a day, whereas inter-day precision was investigated by analyzing four QC samples on three subsequent days ($n = 5$ per day for each concentration) and expressed as RSD. A standard curve was generated on the same day when samples for accuracy and precision were run and used to measure the concentration of the accuracy and precision sample. For acceptable precision and accuracy, the values of variation (RSD/RE) should be not more than 15%, except for LLOQ, where the value of the deviation was accepted to be up to 20%.

Stability experiments. Analyses are typically not performed immediately after biological samples are collected but rather after they have been processed and stored. So, analyte should be handled properly to ensure stability which is vital to obtaining accurate and precise results from a preclinical and clinical study. As a result, analyte stability must be evaluated during validation of a method over the relevant storage conditions (i.e., the effect of sample storage, sample handling, delay at sample preparation, sample receiving, delay of sample injection during batch run) so that the obtained concentration results adequately imitate those directly after sampling [27]. All relevant situations observed in practice should be included in the stability evaluation.

The stability of aceclofenac was investigated at four different storage conditions: short-term temperature stability, long-term temperature stability, freeze and thaw stability, and post-preparative stability. Four QCs were used for the stability study with five replicate analyses for each concentration. Concentrations obtained after analysis of the stability samples were compared with a freshly prepared sample.

Short-term temperature stability covers the monitoring of analyte stability during shipment, transfer of biological samples from clinical site to bioanalytical site, etc. Spiked serum samples (0.8, 2.0, 8, and 16 μg/mL) were stored at −20°C for 48 h to assess short-term temperature stability. After extraction with acetonitrile as per the extraction method mentioned previously, samples were analyzed by HPLC and compared with the freshly prepared sample. Long-term temperature stability was evaluated after storing the spiked serum sample for one month at −20°C at four different concentrations of QC samples. Freeze and thaw stability covers the analyte stability during a delay in receiving a sample, sample preparation, delay while a large volume of sample is prepared, etc. For the freeze and thaw stability study, QC serum samples were stored at −20°C for 24 h and thawed at room temperature for 24 h. That cycle was repeated three times. Finally, the sample was analyzed after extraction. Post-preparative stability was assessed to monitor the analyte stability throughout the analytical run in HPLC. It may be due to a delay in injection because of malfunctioning of an instrument, large sample volumes, or other causes of a delayed analytical run. In this case, extracted QCs were kept under autosampler conditions (25°C) for 24 h, reanalyzed by HPLC, and compared with a freshly prepared sample.

RESULTS AND DISCUSSION

Method development and optimization. Chromatographic parameters were optimized to obtain a sharp, symmetric, well-resolved peak with maximum intensity and reasonable run time. The mobile phase composition has a significant influence on the separation and selectivity of the method. The composition of the mobile phase was selected based on the ability of the mobile phase to separate the analytes from interfering peaks from a matrix, simplicity of preparation, and easy availability of reagents and solvents. As aceclofenac is weakly acidic in nature, acidic mobile phase additives like 5 mM aqueous ammonium acetate with pH adjusted to approx. 3 using formic acid and 0.1% aqueous formic were tried, while methanol and acetonitrile were used as organic modifiers. The acidic mobile phase will facilitate the retention of aceclofenac in reversed-phase HPLC, which is helpful for the development of a selective method. Acetonitrile was chosen as an organic modifier as practically it was seen that methanol caused elevated pressure in the column and higher background noise than acetonitrile. Finally, the mixture of acetonitrile–0.1% aqueous formic acid with pH 3 (60 : 40) was chosen for better selectivity (capacity to separate aceclofenac and IS from interfering peaks from the biological fluid) along with sharp peak shape, peak intensity, and reproducibility. Besides, there is no need for pH adjustment for

the mobile phase which is tedious. System suitability tests are an essential aspect of liquid chromatographic procedures. System suitability parameters were checked to verify the performance of the chromatographic system for the analysis of intended samples. For this purpose, five consecutive injections were made with the standard solution of aceclofenac at a concentration of 16 μg/mL with the internal standard. Few parameters like peak asymmetry and theoretical plate numbers were calculated and were found to be approx. 1 and ≥2000, respectively, for aceclofenac and the internal standard. Peaks showed symmetry and high resolution. The RSD of the peak area and retention time for aceclofenac and the internal standard were lower than 2%, indicating that the system is appropriate to determine the analyte.

Optimization of extraction method is a crucial step for the determination of drugs in human serum. The major techniques used for this purpose are protein precipitation, liquid–liquid extraction (LLE), and solid phase extraction (SPE). SPE works with most of molecules, but it has a lengthy and intricate technique, low reproducibility, and high cost. LLE is easy and produces clean extracts; however, it has lower drug recovery and is generally inappropriate for thermolabile and hydrophilic compounds [28–31]. As a simple technique was considered, salting out assisted liquid–liquid extraction (SALLE) and protein precipitation techniques were tried. In case of salting out assisted liquid–liquid extraction, it was practically noticed that generated data lacked precision. Though SALLE is a simple technique, its efficiency depends on different parameters like pH, centrifugation rate, time of extraction, salt type, etc. The optimization of several parameters in SALLE to attain the best recovery is time-consuming and laborious [32]. On the other hand, protein precipitation is easier and faster for cleaning plasma/serum samples for chromatographic analysis than other extraction techniques, though it was reported to lead to an increase in column back pressure. Among the protein precipitants, acetonitrile has the best capacity (approx. 96%) to clean up the serum/plasma sample in a ratio of 2 : 1 [33]. However, it was practically observed that using acetonitrile as a protein precipitant in a ratio of 2 : 1 (acetonitrile–serum) with a centrifugation rate of 12000 rpm required rigorous/careful column washing at the end of the day. Otherwise, it caused peak tailing and rise of column back pressure. An experimental design was followed to optimize the ratio of serum to acetonitrile to obtain the best results in response to sample cleanup for better selectivity and the effect of serum sample on rising column back pressure after repeated chromatographic runs. In our study, acetonitrile was used as a protein precipitant in a ratio of 1 : 9 (serum–acetonitrile) with centrifugation rate of 12000 rpm. The described conditions resulted in the precise data acquisition with adequate sensitivity without the rise of column back pressure even after the end of the entire validation process.

Table 1. Extraction recovery of aceclofenac by protein precipitation method from human serum ($n = 5$)

Added concentration, μg/mL	Recovery (mean ± SD), %	RSD, %
0.8	75.4 ± 1.3	1.7
2.0	72.6 ± 1.5	2.2
8	74.0 ± 1.6	2.2
16	76.8 ± 1.0	1.4

Method validation. *Specificity.* Chromatograms obtained from blank serum, blank serum spiked with aceclofenac and/or the internal standard (acetanilide) were compared. No interfering peak from the matrix component was observed at the retention times of aceclofenac or the internal standard, which confirms the specificity of the developed method (Fig. 1). The retention times were 3.4–3.8 min and 8–9 min for the internal standard and aceclofenac, respectively.

Sensitivity. The LLOQ was found to be 0.8 μg/mL. The percent accuracy was found to be 12.5% (as RE), and RSD was found to be 11.1% at LLOQ level, while the ratio of analyte to blank serum sample (zero calibrator) was greater than 10, indicating the acceptance of sensitivity of the proposed method. Representative chromatograms at LLOQ level are shown in Fig. 1. The limit of detection (LOD) was found to be 0.3 μg/mL with the signal to noise ratio >3.

Recovery. The extraction recoveries were performed at four QC concentrations with five replicate analyses of each concentration, which showed 72.6–76.8% recovery of spiked drug; found RSD was <15% (Table 1), which showed the consistent and precise recovery of aceclofenac from human serum. Recovery of the internal standard was consistent and precise.

Linearity, accuracy, and precision. A six-point calibration curve was constructed covering the range of 0.8–20 μg/mL and was found to be linear with the equation of $y = 0.354x + 0.023$ and the determination coefficient (R^2) of 0.997. The back-calculated concentrations at all points of the calibration curve were within ±15% of the theoretical concentrations.

The intra-day precision for four QC samples (0.8, 2.0, 8, and 16 μg/mL) was expressed as the RSD and was found in the range of 4.2 to 11.1%, and inter-day precision was found in the range of 0.3–4.6%. The accuracy was in the range of –4.0 to 12.5% (expressed as RE, %) (Table 2).

Stability. Stability studies of aceclofenac at four tested conditions (post preparative stability, freeze-thaw stability, short-term stability, and long-term stability) are summarized in Table 3. QC samples were prepared with five replicates at each concentration and

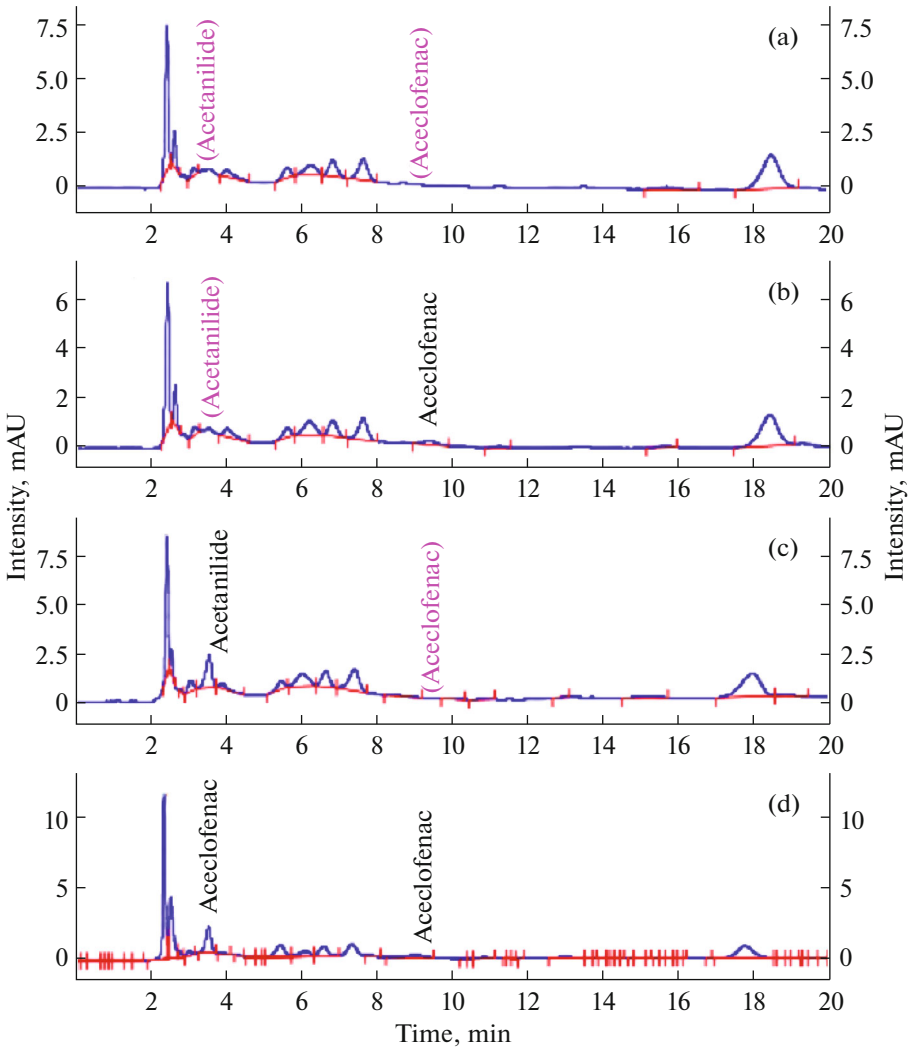


Fig. 1. Representative chromatograms for specificity study of the developed HPLC-DAD method: (a) blank serum, (b) serum spiked with aceclofenac at LLOQ, (c) serum spiked with acetanilide (IS), (d) serum samples spiked with aceclofenac at LLOQ and acetanilide (IS).

processed under the conditions described above. The studied samples were considered to be stable as there was no significant difference between the assay result of the tested sample and the concentration of the freshly prepared sample. It is expected that no stability-related problem would be observed during the analysis of any sample from a clinical laboratory for bioavailability, bioequivalence, and pharmacokinetic study.

CONCLUSIONS

A simple, accurate, precise method using HPLC with DAD detection has been developed and validated to quantify aceclofenac in human serum using a simple protein precipitation technique as an extraction process. The major advantages of the method include simplicity and cost-effectiveness as using a simple instrument, simple mobile phase, less time-consuming and simple extraction method without any drying step and the use of a low volume of serum sample (100 μL) with adequate sensitivity. It is expected that the proposed method could be applied for routine plasma level monitoring of aceclofenac for pharmacokinetic, bioavailability, and bioequivalence study.

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Table 2. Inter- and intra-day precision and accuracy of the developed HPLC method for the determination of aceclofenac in human serum[a]

Intra-day				Inter-day		
added concentration, μg/mL	detected concentration (mean ± SD), μg/mL	precision (RSD), %	accuracy (RE), %	detected concentration (mean ± SD), μg/mL	precision (RSD), %	accuracy (RE), %
0.8	0.90 ± 0.10	11.1	12.5	0.88 ± 0.04	3.8	10.0
2.0	1.92 ± 0.08	4.2	–4.0	1.930 ± 0.017	4.6	–3.5
8	7.7 ± 0.7	8.6	–4.0	7.81 ± 0.14	0.3	–2.4
16	16.2 ± 0.9	5.6	1.0	16.10 ± 0.08	3.6	0.6

[a] $n = 5$ for intra-day and $n = 15$ for inter-day measurements.

Table 3. Results of stability study of aceclofenac in human serum by the proposed HPLC method at different QC concentrations ($n = 5$)

Declared concentration, μg/mL	Condition	Measured concentration (average ± SD), μg/mL	RSD, %
0.8	Post-preparative stability (autosampler) after 24 h	0.800 ± 0.004	0.5
	Freeze–thaw stability after three cycles	0.79 ± 0.04	5.1
	Long-term stability	0.78 ± 0.02	2.6
	Short-term stability	0.780 ± 0.016	2.1
2.0	Post-preparative stability (autosampler) after 24 h	1.87 ± 0.03	1.6
	Freeze–thaw stability after three cycles	2.10 ± 0.13	6.2
	Long-term stability	2.04 ± 0.03	1.5
	Short-term stability	1.90 ± 0.03	1.6
8	Post-preparative stability (autosampler) after 24 h	7.99 ± 0.04	0.5
	Freeze–thaw stability after three cycles	8.27 ± 0.10	1.2
	Long-term stability	8.22 ± 0.29	3.5
	Short-term stability	8.35 ± 0.15	1.8
16	Post-preparative stability (autosampler) after 24 h	15.91 ± 0.11	0.7
	Freeze–thaw stability after three cycles	16.22 ± 0.09	0.6
	Long-term stability	15.95 ± 0.14	0.9
	Short-term stability	15.79 ± 0.07	0.5

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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