EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 5
Issue: 6
217-221
Year: 2018

# VALIDATED UPLC-PDA METHOD FOR SIMULTANEOUS DETERMINATION OF SOME ANTI-INFLAMMATORY AND ANALGESIC DRUGS

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**Article Received on 26/03/2018** **Article Revised on 16/04/2018** **Article Accepted on 07/05/2018**

## ABSTRACT
In the presented work identification and quantification of some anti-inflammatory and analgesic drugs, namely aceclofenac, diclofenac, paracetamol and para-aminophenol were carried out by validated UPLC-PDA method. The chromatographic separation was achieved on Acquity UPLC BEH C18 (100.0 x 2.1 mm, 1.7 µm) column using isocratic mobile phase consisting of acetonitrile-2mM ammonium acetate (50:50, v/v) at a flow rate of 0.25 mL/min. The PDA detection was carried out at 275 nm for all the compounds. The elution of aceclofenac, diclofenac, paracetamol and para-aminophenol was occurred at 2.75, 3.80, 0.70 and 1.70 min, respectively. The calibration curves were linear over the concentration range of 1-1000 ng/mL for the compounds. The developed method was validated according to ICH guidelines. The method was applied in the identification and quantitative determination of these compounds during routine quality control analysis, in stability studies and in pharmacokinetic study.

**KEYWORDS:** UPLC-UV, Aceclofenac, Paracetamol, Diclofenac, Para-aminophenol, Validation.

## INTRODUCTION
In the era of developed and modified chromatographic techniques, the UPLC-PDA is the simplest, most reliable, easy handling and worldwide used technique in the various stages of drug development. It is simple, sensitive and fast as compared to HPLC and has been thoroughly applied in pharmaceutical development particularly in the identification and quantitative analysis of drug products. The technique is particularly very useful when analysis is carryout simultaneously for several compounds with less time. Aceclofenac (ACF), is chemically [[[2-[(2, 6-Dichlorophenyl) amino] phenyl] acetyl] oxy] acetic acid, is a non-steroidal anti-inflammatory drug (NSAIDS). It is used for the relief of pain and inflammation in several types of arthritis. Diclofenac (DCF), is chemically [2-[(2, 6-Dichlorophenyl] amino] phenyl] acetic acid, also used as anti-inflammatory and analgesic drug and is a major metabolite and/or degradation product of aceclofenac. Paracetamol (PCM) is chemically N-(4-hydroxy phenyl) acetamide and is used as analgesic and antipyretic drug in the treatment of pain and fever. Para-aminophenol is a major metabolite and/or degradation product of paracetamol.[1] The combination tablets containing 100 mg of aceclofenac and 500 mg of paracetamol have been used for acute painful condition in osteoarthritis and rheumatoid arthritis.

The literature survey revealed that aceclofenac in presence of its degradation product, diclofenac was reported by spectrophotometry, densitometry, and/or HPLC.[2-5] The determination of paracetamol in presence of its degradation product, para-aminophenol, was reported by spectrophotometry[6,7] and HPLC.[8-10] Simultaneous determination of aceclofenac and paracetamol alongwith their unknown degradation products was reported by HPLC.[11,12] Simultaneous determination of aceclofenac and paracetamol with demonstrating its separation from their major degradation products, diclofenac and para-aminophenol has been reported recently in our previously published work by UPLC/Q-TOF-MS.[13] But this method was found sensitive and suitable for structural identification of the compounds. Method was not suitable for routine quality control analysis because it was not separating all the compounds in a single chromatogram. The single chromatogram from tablet sample of all the compounds makes the method complicated when the analysis is carried out by UPLC-MS/MS technique due to the different mass (MS/MS) quantification parameters for every compound. Therefore the compounds were quantified by selecting their individual MS/MS parameters. In our previous investigation these drugs were also analyzed by HPLC-UV method. But this method consumed about 10 min for total chromatographic separation time for all the compounds with 2-Dimensional peak analysis.[14] Hence, in the

present work an UPLC-PDA method was developed and validated for simultaneous determination of aceclofenac, diclofenac, paracetamol and para-aminophenol in tablets. All the compounds were separated within the total chromatographic run time of 3 min with 3-Dimentional peak purity. The method was applied in the identification and quantitative determination of these compounds during routine quality control analysis, in stability studies and in pharmacokinetic studies.

## EXPERIMENTAL

### Chemicals and Reagents

Pure samples of aceclofenac and paracetamol were kindly supplied by Moraceae Pharmaceuticals Ltd. (Uttarakhand, India). Diclofenac was supplied by Arti Drugs Ltd. (Mumbai, India). Para-aminophenol was supplied by Hema Pharmaceuticals Ltd. (Gujarat, India). Tablet formulations, Aceclo Plus (Aristo Pharmaceuticals Ltd., Mumbai, India) containing 100 mg of aceclofenac and 500 mg of paracetamol were obtained commercially. HPLC grade water, acetonitrile, methanol, potassium dihydrogen phosphate were purchased from Fluka analytical, Sigma-Aldrich Corporation, St. Louis, MO, USA. All other reagents used were of HPLC grade.

### UPLC-PDA Conditions

UPLC was performed with a Waters Acquity UPLC system (Waters Corporation, MA, USA) equipped with a binary solvent manager, an auto-sampler, column manager. Chromatographic separation was performed on a Waters Acquity UPLC BEH C18 (100.0 x 2.1 mm, 1.7 mm) column. The mobile phase for UPLC analysis consisted of acetonitrile–2mM ammonium acetate (50:50, v/v) which was filtered through 0.45 mm membrane filter and degassed by sonication. For isocratic elution, the flow rate of the mobile phase was kept at 0.25 mL/min and 10 µL of sample solution was injected in each run. The total chromatographic run time was 4.0 min. The PDA detection was carried out at 275 nm for all the compounds.

### Preparation of Standard Solutions

50 mg each of aceclofenac, diclofenac, paracetamol, and para-aminophenol were weighed accurately and transferred to 50 mL volumetric flasks separately. The powders were then dissolved with approximately 25 mL of methanol and ultrasonicated for 10 min. The final volume was made up with methanol. The solutions were further diluted with methanol: water (50:50, v/v) to give a series of standard solutions containing required concentrations for each compound.

### Preparation of sample solutions

Twenty tablets were weighed accurately and powdered. Powder equivalent to 50 mg of aceclofenac and 250 mg of paracetamol was taken and transferred to a 50 mL volumetric flask. The powder was dissolved with approximately 25 mL of methanol and ultrasonicated for 10 min. The final volume was made up with methanol. This solution was filtered through a 0.45 mm nylon membrane filter to remove all the excipients. The resultant filtrate was further diluted with methanol: water (50:50, v/v) to give a sample solution containing 100 ng/mL of aceclofenac and 500 ng/mL of paracetamol.

### Validation of the Method

The developed method was validated according to ICH validation guidelines. [15] The validation parameters addressed were linearity and range, limit of detection and quantitation, precision, accuracy, robustness, and specificity.

### Linearity, Range, LOD, and LOQ

Different standard concentrations each compound in the range of 1-1000 ng/mL was prepared separately in methanol: water (50:50, v/v). The solutions were filtered through a 0.20 mm nylon syringe filter and injected in to the UPLC-PDA system for analysis. Average peak area at each concentration level was subjected to linear regression analysis with the least squares method. Linearity was described by slope, intercept, and correlation coefficient obtained from regression equations. Standard stock solutions were diluted appropriately to obtain concentrations for the estimation of the limit of detection (LOD) and the limit of quantitation (LOQ) based on signal to noise (S/N) ratio of 3:1 and 10:1, respectively.

### Precision

The intra-day precision was assessed by performing six analyses using tablet sample solution containing 100 ng/mL of aceclofenac and 500 ng/mL of paracetamol, spiked with 100 ng/mL each of diclofenac and para-aminophenol. Similarly inter-day precision was assessed by performing replicate analysis using same concentration of all the analytes for three consecutive days under the same experimental conditions. The intermediate precision of the method was also evaluated by a different analyst in the same laboratory under the same experimental conditions. The mean of percentage recoveries and the RSD (%) was calculated.

### Accuracy

The accuracy of the method was determined by standard addition technique. Three different levels (50, 100, and 150%) of standards were added to pre-analyzed tablet sample in six replicates and the mixtures were re-analyzed by the proposed method. The percentage recoveries of all the compounds at each level and each replicate were determined. The mean of percentage recoveries and the RSD (%) was calculated.

### Robustness

Robustness is the ability of the method to remain unaffected by small and deliberate variations in the method parameters and provides an indication of its reliability for routine analysis. The robustness was determined by analyzing the sample solution containing 100 ng/mL of aceclofenac and 500 ng/mL of paracetamol, spiked with 100 ng/mL of diclofenac and

para-aminophenol under a variety of conditions of the method parameters, such as flow rate, mobile phase composition, column temperature, and injection volume. The mean of percentage recoveries and the RSD (%) was calculated from six replicates.

**Specificity**

Specificity is the ability of the method to measure the analyte response in the presence of sample components or matrix such as excipients, potential impurities and degradation products. The samples were chromatographed to determine the extent to which mobile phase components and excipients could contribute to the interference with the analytes. Specificity of the developed method was also assessed by performing forced degradation studies. According to ICHQ1A (R2), stress testing of the drug substance can help in the identification of degradation products and the intrinsic stability of the molecule and to investigate the stability-indicating power of the analytical methods. [16] The stress conditions employed for the degradation study includes acid hydrolysis (1 N HCl), alkali hydrolysis (1 N NaOH), oxidation (3% $H_2O_2$), and light (carried out as per ICH Q1B). For acid, alkali hydrolysis, and oxidation, the study period was 1 h whereas for light study it was 24 h. All stress conditions employed for forced degradation studies were carried out at room temperature (25ºC).

**Analysis of Marketed Tablets**

The procedure for analysis of marketed tablets was similar as described in preparation of sample solution. The amount of aceclofenac and paracetamol in tablets was determined by calibration equations obtained from respective calibration plot.

## RESULTS AND DISCUSSION

**Optimization of UPLC-PDA Conditions**

Using isocratic mobile phase composition of acetonitrile–2mM ammonium acetate buffer (50:50, v/v) at a flow rate of 0.25 mL/min gave good peak shapes with short separation times. The retention time was found to be 0.75 min for aceclofenac, 3.80 min for diclofenac, 0.70 min for paracetamol, and 1.70 min for para-aminophenol with the total chromatographic run time of 4 min. UPLC-PDA chromatogram obtained from mixed standards (1ng/mL each) of all four compounds is shown in Figure **1**.

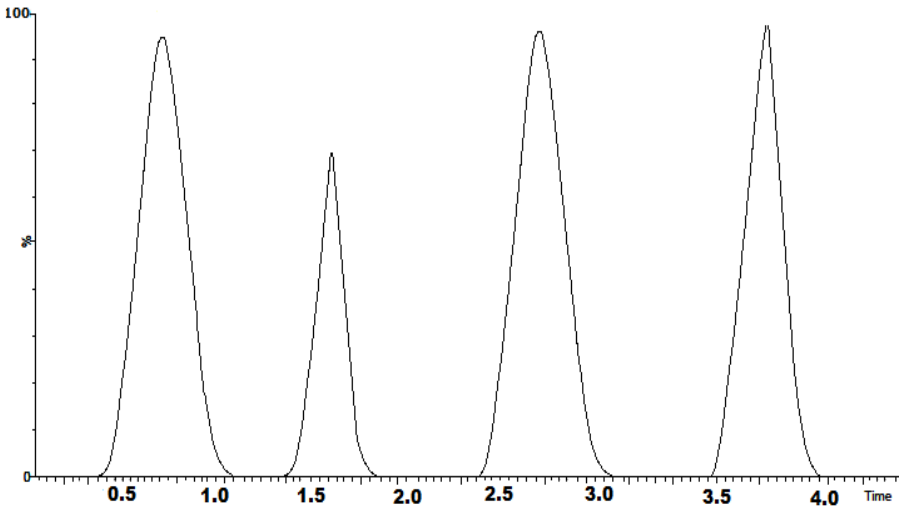


**Figure 1: UPLC-PDA chromatogram obtained from mixed standards (1ng/mL each) of Aceclofenac ($R_t$ 2.75 min), Diclofenac ($R_t$ 3.80 min), Paracetamol ($R_t$ 0.70 min) and Para-minophenol ($R_t$ 1.70 min).**

**Linearity and Range**

The linear calibration plot was obtained over the concentration range of 1-1000 ng/mL for all the compounds with correlation coefficient was more than 0.999. The results exhibited that an excellent correlation existed between the peak area and concentration ranges as stated for all the compounds. The linearity data and results of LOD and LOQ are presented in Table **1**.

**Table 1: Results obtained from Linearity, LOD, and LOQ.**

| Parameters | ACF | DCF | PCM | PAP |
|---|---|---|---|---|
| Linear range (ng/mL) | 1-1000 | 1-1000 | 1-1000 | 1-1000 |
| Slope | 11.512 | 11.132 | 10.545 | 8.220 |
| Intercept | 245.55 | 235.25 | 130.34 | 127.35 |
| Correlation coefficient[a] | 0.9997 | 0.9998 | 0.9998 | 0.9997 |
| LOD (ng/mL) | 0.1 | 0.1 | 0.1 | 0.1 |
| LOQ (ng/mL) | 1 | 1 | 1 | 1 |

[a]Mean of six replicates (n = 6)

**Precision**

The low values of RSD less than 2% were obtained for all the compounds by evaluation of intraday, interday, and different analysts precision suggested an excellent precision of the method. The results of precision are presented in Table **2**.

**Table 2: Results obtained from Precision.**

| Analyte | ACF | | DCF | | PCM | | PAP | |
|---|---|---|---|---|---|---|---|---|
| | Recovery (%)[a] | RSD (%) | Recovery (%)[a] | RSD (%) | Recovery (%)[a] | RSD (%) | Recovery (%)[a] | RSD (%) |
| Intraday | 98.78 | 1.75 | 99.52 | 1.58 | 98.25 | 1.33 | 98.95 | 1.72 |
| Interday | 99.45 | 1.85 | 98.75 | 1.32 | 99.44 | 1.35 | 99.92 | 1.64 |
| Different analyst | 99.72 | 1.77 | 99.78 | 1.57 | 99.80 | 1.80 | 99.84 | 1.92 |

[a]Mean of six replicates (n = 6)

**Accuracy**

After spiking 50, 100, and 150% levels of standards to pre-analyzed tablet sample, the recoveries of all the compounds was found to be in the range 98-101% with RSD less than 2%, indicating that accuracy of the method was adequate.

**Robustness**

The method was found to be robust with respect to variability in chromatographic conditions. The retention times and peak area of each compound did not change significantly when mobile phase composition, flow rate, injection volume, and column temperature were deliberately modified.

**Specificity**

The comparison of the chromatograms of the blank and sample solutions indicated that no interferences were detected from mobile phase components and excipients of the formulation. Degradation was observed when the aceclofenac and paracetamol individual standard solutions were subjected to acidic and alkaline hydrolysis as seen from the significant drop in assay values and appearance of degradation peaks in the chromatograms. After acidic and alkaline hydrolysis, aceclofenac was degraded into diclofenac with the appearance of peak and paracetamol was degraded into para-aminophenol with the appearance of intense peaks in their chromatogram. This was further confirmed by co-injection of reference standard solution of diclofenac and para-aminophenol. The obtained chromatograms were found similar with that of reference standard of each compound, indicating that there was no co-elution of unknown degradation peak at the retention times of respective compounds. No degradation was observed when the drugs were subjected to oxidative and photolytic stress conditions. The hydrolytic degradation mechanism of aceclofenac and paracetamol is presented in Figure **2** and Figure **3**, respectively.

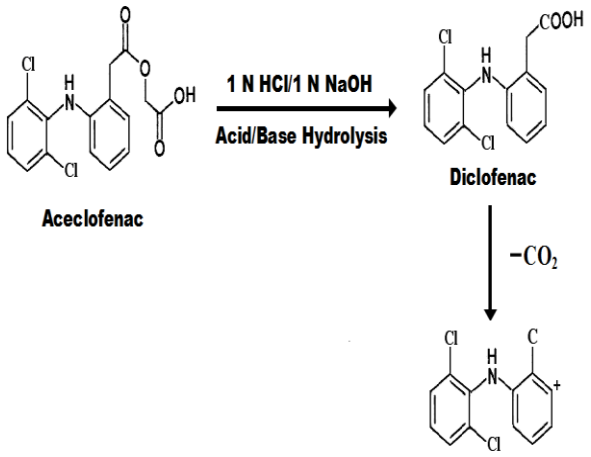


**Figure 2: Proposed degradation mechanism of Aceclofenac.**

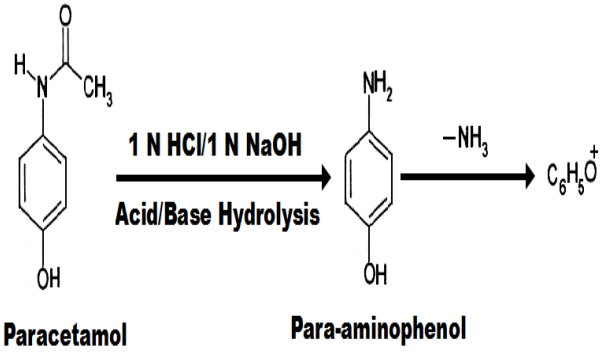


**Figure 3: Proposed degradation mechanism of Paracetamol.**

**Analysis of Marketed Tablets**

The validated method was applied to the determination of aceclofenac and paracetamol in commercially available tablets containing 100 mg of aceclofenac and 500 mg of paracetamol. The aceclofenac content from tablets was 97.95–98.98% with RSD 1.65% and paracetamol content was 98.85–99.98% with RSD 1.77%. The low values of RSD indicated that method was suitable for routine analysis of aceclofenac and paracetamol in tablets without any interference from excipients.

**CONCLUSION**

UPLC-PDA method has been developed and validated

for rapid qualitative and quantitative analysis of aceclofenac, diclofenac, paracetamol and para-aminophenol in tablets. The advantages of our developed method are simple, cost effective, all compounds are separated in single chromatographic run time (4 min), and high sensitivity (LOQ: 1.0 ng/mL for all the compounds). Hence, it is suggested that the method should be applied for routine quality control analysis and during stability studies of all four compounds in their bulk drugs and in tablet formulation. It can also be applied for pharmacokinetic study of these compounds in human plasma.

**ACKNOWLEDGMENTS**

The authors are grateful to Moraceae Pharmaceuticals Ltd., Uttarakhand, India, for providing pure samples of aceclofenac and paracetamol. The authors are also grateful to Arti Drugs Ltd., Mumbai, India, for providing pure sample of diclofenac, and Hema Pharmaceuticals Ltd., Gujarat, India, for providing pure sample of para-aminophenol. The authors are also thankful to Dean and In-charge of Instrumentation Facilities, Faculty of Pharmacy, Hamdard University, New Delhi, India, for providing opportunities to work on UPLC-PDA system.

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