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# UPLC-MS/MS Determination of Aceclofenac and Diclofenac in Bulk, Dosage forms and in At-line Monitoring of Aceclofenac Synthesis

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***Authors' contributions***

*This work was carried out in collaboration among all authors. Authors RIEB and HMEA designed the study and analyzed data. Author HMEA edited the paper and funded the study. Authors EFE and FF carried out the experiments, analyzed data, and wrote first draft. All authors read and approved the final manuscript.*

***Original Research Article***

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## ABSTRACT

**Aim:** The derivatization product of diclofenac (DCL), aceclofenac (ACL), is a non-steroidal anti-inflammatory drug (NSAID) which causes faster and extended action with reduced gastrointestinal (GI) inflammation. The detection of DCL in ACL bulk and pharmaceutical products indicates incomplete synthesis and hydrolysis.
In this article we have developed a UPLC-MS/MS method for analysis of ACL and DCL. The method was designed as an at-line monitoring tool for process analytical technology (PAT) application to ACL synthesis. The method was also applied for analysis of ACL and DCL in bulk and tablets.
**Methodology:** Isocratic elution was performed on a UPLC C18 column (2.1 x 50 mm, 1.7 µm) using a mobile phase consisting of acetonitrile, water and formic acid (80:20:0.5,

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*v/v/v*). Flow rate was 0.2 mL/min and total run time was 1 min. Auto-sampler temperature was maintained at 5ºC to prevent any further degradation of ACL. Electrospray positive ionization (ESI +Ve) in multiple-reaction monitoring mode (MRM) was used for the simultaneous determination of ACL and DCL. Monitoring was performed at $[M+H]^+$ 354.23: 250.09 and 296.13:250.1 *m/z*; respectively. The method was validated according to ICH guidelines Q2(R1).

**Results:** The linearity range was 20 – 3000 ng/mL for both drugs. The developed method was accurate and precise (RSD<2%) for the determination of ACL and DCL in single solution (99.65±1.33 and 100.37±1.02 for ACL and DCL; respectively) and laboratory prepared mixtures (101.01±1.07 and 100.45±1.54 for ACL and DCL; respectively). The method was applied to Bristaflam® and Cataflam® tablets and the recovery was 100.95±0.18 and 99.15±0.62; respectively. The average recovery from reaction mixture was101.21±0.06 and 98.89±0.64 for ACL and DCL; respectively.

**Conclusion:** The proposed UPLC-MS/MS method is valid for at-line monitoring of ACL and DCL during PAT application to ACL synthesis and drug determination in bulk and tablets.

*Keywords: Aceclofenac;diclofenac; synthetic impurities; UPLC- MS/MS; process analytical technology.*

## 1. INTRODUCTION

ACL, 2-[2-[2-[(2,6-dichlorophenyl)amino]phenyl]acetyl] oxyacetic acid, is a non-steroidal anti-inflammatory drug (NSAID) with reduced GI complications. ACL is rapidly absorbed with 100% bioavailability in human. It is metabolized to 4′-hydroxy ACL, DCL, and 4′-hydroxy DCL in rat, monkey and human [1].

DCL,2-(2-(2,6-dichlorophenylamino) phenyl) acetic acid, is also a NSAID. The anti-inflammatory and analgesic effects of DCL are due to the inhibition of prostaglandin (PG) synthesis. The PG inhibition is caused by suppressing leukocyte migration as well as cylcooxygenases (COX-1 and COX-2). The antipyretic action of DCL is due to its effect on the hypothalamus leading to peripheral dilatation and increased cutaneous blood flow resulting in heat dissipation. DCL is absorbed in 2-3 hours and its bioavailability is 50% in human. Patients (20%) who receive DCL experience side effects ranging from GI inflammation, gastric ulcers to ulcerative bleeding and perforations. Other side effects include fluid retention and edema [1].

DCL which is used as a precursor for the synthesis of ACL may be detected in ACL [2–6]. In addition, DCL is a degradation product [7] and a metabolite of ACL [8]. For the determination of ACL, spectrophotometric [9,10], chromatographic [8,11–17], electrochemical [18] and densitometric thin layer chromatography (TLC) [19] methods have been applied. Spectrophotometric [20–23], chromatographic [24–26], TLC [27], electrochemical [28–32] and capillary zone electrophoretic [33] methods have been applied for the determination of DCL in pharmaceutical preparations and biological fluids.

The simultaneous determination of ACL and DCL in pharmaceutical matrix has been reported by spectrophotometry with compromised sensitivity [7,34]. Chromatographic determination of ACL and DCL mixture with quadruple - time of flight tandem mass detector (Q-TOF) has been described in literature with excellent sensitivity. The Linearity range for

the determination of DCL was 0.01–1 ng/mL, while that for ACL was 1–1000 ng/mL [12]. The method was validated in presence of up to 0.2% DCL in ACL [12]. The described method is not valid if the DCL impurity exceeds 0.2%. Thus it cannot be applied in monitoring ACL synthesis or in extensive degradation of ACL where the DCL level might exceed 0.2% [12]. This is due to the possible interferences that might occur between ACL and DCL determination due to structural similarity. The described method [12] has relied on Q-TOF detection which is less common and more expensive type of MS/MS detector hindering the wide spread application of the method in laboratories. Due to the previous limitations the described method [12] could not be used for PAT application in synthesis of ACL from DCL. US Food and Drug administration (FDA) has defined Process Analytical Technology (PAT) as "a mechanism to design, analyze, and control pharmaceutical manufacturing processes through the measurement of Critical Process Parameters (CPP) which affect Critical Quality Attributes (CQA)" [35].

In this paper, a UPLC-MS/MS method for simultaneous determination of ACL and DCL in bulk and tablets was developed. The method was validated as an at-line monitoring method for the application of PAT to the synthesis of ACL from DCL.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals and Reagents

ACL and DCL salt were obtained from National Organization for Drug Control & Research; Egypt. The purity levels were 99.98 and 99.99 % for ACL and DCL; respectively. HPLC grade methanol, acetonitrile, TFA and formic acid were all obtained from Sigma Aldrich; Germany. De-ionized water (DI) was produced in house by Milli-Q system; USA. Cataflam® tablets, Novartis; Egypt and Bristaflam® tablets, Bristol Myers Squibb; Egypt were obtained from public pharmacies.

### 2.2 Instrumentation

Acquity UPLC-MS/MS system equipped with a UPLC BEH C-18 column (2.1 x 50 mm, 1.7 µm), electro spray ionization (ESI) probe and a triple quadruple tandem mass detector (TQD) was used for the assay (Waters; USA). The system included a vacuum degasser, quaternary mobile phase pump, thermostated auto-sampler and column oven compartment. MassLynx 4.2 software was used for controlling the instrument and data acquisition.

### 2.3 Standard Solutions

#### 2.3.1 Solutions As

50 mg of ACL and DCL were separately weighed and transferred into two 250 mL volumetric flasks then dissolved in methanol and water; respectively to give a concentration of 0.2 mg/mL each.

#### 2.3.2 Solutions Bs

I – Five mL of solution ACL and DCL (solution A) were separately transferred into two 100 mL volumetric flask and completed to volume with water (final concentration10 µg/mL; solutions BI).

II- One mL of solution A for ACL and 5 mL of solution A for DCL were transferred into 100 mL volumetric flasks and completed to volume with water (solutions BII) equivalent to 2 µg/mL of ACL and 10 µg/mL DCL.

III- Five mL of solution A for ACL and 1 mL of solution A for DCL were transferred into 100 mL volumetric flasks and completed to volume with water (solution BIII) equivalent to 10 µg/mL of ACL and 2 µg/mL DCL.

## 2.4 MS/MS Tuning Conditions

A mixture of the two drugs each at a concentration of 100 ng/mL in mobile phase was used as the tuning mixture. ESI +Ve was used and conditions were optimized regarding capillary, cone and collision voltages. Cone gas flow was adjusted at 50 L/hr. Desolvation gas flow was 900 L/hr. Collision gas flow was set at 0.1 mL/min and source temperature was 120ºC to get the best sensitivity of the target ions.

## 2.5 Chromatographic Conditions

UPLC BEH C18 column (2.1 x 50 mm, 1.7 µm) was used as stationary phase at ambient temperature (20 – 25ºC). Isocratic elution was performed with mobile phase of acetonitrile, water and formic acid in ratio (80:20:0.5, *v/v/v*). Flow rate was 0.2 mL/min and total run time was 1 min. Auto-sampler temperature was 5ºC.

## 2.6 Method Validation

The method was validated according to ICH guide lines Q2 (R1) (Validation of Analytical Procedures: Text and Methodology) [36].

### 2.6.1 Linearity

Different volumes (0.1 – 15 mL) from solutions BI of ACL and DCL were separately transferred into two series of 50 mL volumetric flasks and completed to volume with the mobile phase (20 – 3000 ng/mL for ACL and DCL). Three replicates were prepared from the calibration curve series for each drug. Area under the peak (AUP) was recorded and data was used for calibration curve plot. Best-fit calibration curves of peak area against concentration were drawn and calibration equations were calculated.

### 2.6.2 Accuracy

*2.6.2.1 Accuracy in single solution*

The accuracy of the assay was tested by quality control (QC) samples of known concentrations. Three replicates of the QC samples (6 different concentrations, 100 – 2600 ng/mL) were injected into UPLC system and analyzed. AUP was recorded and applied to regression equations to calculate concentrations.

*2.6.2.2 Accuracy in lab prepared mixtures*

Different volumes of solution BII (1.5 – 12.5 mL) and B III (1.5 – 13 mL) were separately transferred into two series of 50 mL volumetric flasks and completed with mobile phase. This

was done to prepare a series of concentration ratio 1 ACL: 5 DCL (60 – 500 ng/mL ACL and 300 – 2500 DCL) and another series of concentration 5 ACL: 1DCL (300 –2600 ACL and 60 – 520 ng/mL DCL); respectively. Samples were injected into the system and analyzed. AUP was recorded and applied to regression equations to calculate concentrations.

### 2.6.3 Precision

Three replicates of QC samples of different concentrations of single solutions and laboratory prepared mixtures were prepared and analyzed as described above. Precision was calculated based on CV% of the analytical response (peak area). The procedures were repeated on three successive days. Inter and intraday precision were calculated.

### 2.6.4 System carryover test

A QC sample of the highest concentration of the calibration curve (3000 ng/mL) of each drug was injected into the UPLC system and analyzed. Afterwards, a blank sample (mobile phase) was injected. The MRM chromatogram was examined for the two drugs to see if traces of the analytes were carried over by the system for subsequent analysis.

### 2.6.5 LOD and LOQ

LOD was calculated mathematically using the equation LOD = SD x 3.3/ Slope. LOQ was calculated based on the visual evaluation method. Six samples at LOQ were analyzed and the accuracy of the analysis was determined.

### 2.6.6 Specificity and matrix effect

*2.6.6.1 Specificity in bulk samples*

To ensure absence of sample cross-talk; pure ACL and DCL were injected into the system. Each was monitored using MRM representing its *m/z* transition and the MRM of the other drug transition.

*2.6.6.2 Specificity in tablet samples*

The specificity was determined on tablet extracts for ACL and DCL. Tablets' extracts were injected into the UPLC system and detection was done using total ion chromatogram (TIC) monitoring mode where the MS and MS/MS transitions of both drugs were included. The generated TIC peaks were MS/MS analyzed to check for interference from other molecular ions of matrix.

*2.6.6.3 Specificity in hydrolysis reaction matrix samples*

Two mixtures of ACL, DCL and TFA were heated at 60ºC and 100ºC. The mixtures were cooled down and 10 µL were transferred into a 100 mL volumetric flask and completed to volume with the mobile phase after cooling. Ten µL were injected and monitored in TIC and MRM modes.

## 2.7 Application to Pharmaceutical Preparations

Twenty Bristaflam® tablets (ACL) were weighed and grinded. Weight equivalent to 50 mg was taken and extracted with 200 mL of methanol on four portions. The extract was filtered through a membrane filter, transferred into a 250 mL volumetric flask and volume was completed with methanol to give a final concentration of 0.2 mg/mL. Five mL of the extract was transferred into a 100 mL volumetric flask and the volume was completed with water. Different volumes (2, 3 and 5 mL) were taken and transferred into 50 mL volumetric flask and completed to mark with mobile phase. Prepared samples equivalent to 400, 600 and 1000 ng/mL were injected into the system and analyzed.

Twenty Cataflam® tablets (DCL) were weighed and grinded. Weight equivalent to 50 mg was taken and extracted with 200 mL of water on four portions. The extract was filtered through a membrane filter, transferred into a 250 mL volumetric flask and volume was completed with water to give a final concentration of 0.2 mg/mL. Five mL of the extract was transferred into a 100 mL volumetric flask and the volume was completed with water. Different volumes (2, 3 and 5 mL) were taken and transferred into 50 mL volumetric flask and completed to mark with mobile phase. Prepared samples equivalent to 400, 600 and 1000 ng/mL were injected into the system and analyzed.

## 2.8 Quantitative Application to simulated hydrolysis Reaction Mixture

Five mL of solution As of ACL and DCL were separately transferred into two 100 mL flasks, 1 mL of TFA was added. The contents of the flasks were completed with water. Different volumes (2, 3 and 5 mL) were taken and transferred into 50 mL volumetric flasks and completed to the mark with the mobile phase. Prepared samples equivalent to 400, 600 and 1000 ng/mL were injected into the system and analyzed.

# 3. RESULTS AND DISCUSSION

## 3.1 Tuning Conditions

Tuning conditions were optimized using each drug at a concentration of 100 ng/mL. Optimum conditions are displayed in Table 1.

**Table 1. MS/MS ESI+Ve ionization tuning conditions of a 100 ng/mL mixture of ACL and DCL**

| Drug | Parent ion | Daughter ion | Collision voltage (V) |
|---|---|---|---|
| ACL | 354.24 | 250.09 | 20 |
| DCL | 296.13 | 250.10 | 25 |

At quadruple MS1; The molecular ion of ACL (354 g/mol) was observed at 354 *m/z*$[M+H]^+$. The molecular ion of DCL (297 *m/z*) was observed at 296 *m/z* $[M+H]^+$. Cone gas flow was adjusted as 50 (L/hr). Desolvation gas flow was 900 (L/hr) and source temperature was 120ºC to get the best sensitivity of the target ions. Capillary voltage was adjusted as 3 KV. The cone voltage was varied until the best sensitivity of the ACL and DCL molecular ions were obtained. The best sensitivity was observed at a cone voltage of 20 V for both drugs.

At quadruple MS2; Argon as a collision gas at a flow rate of 0.1 mL/min was used to fragment the molecular ions produced at MS1. Varying collision energies was applied to obtain molecular ion fragments of highest sensitivity and resolution.

For the MS/MS fragments of ACL; a cone voltage of 20 V was suitable for the determination of ACL fragments 215, 250 and 278 *m/z* at collision energy of 20 V. For the MS/MS fragments of DCL; a cone voltage of 20 V was suitable for the determination of DCL fragments 215, 250.1 and 278 *m/z* at collision energy of 25 V (Figs. 1, 2 and Table 1).

Proposed fragmentation patterns created by Chemdraw Ultra 8.0 software for ACL and DCL are displayed in Figs. 3 and 4.

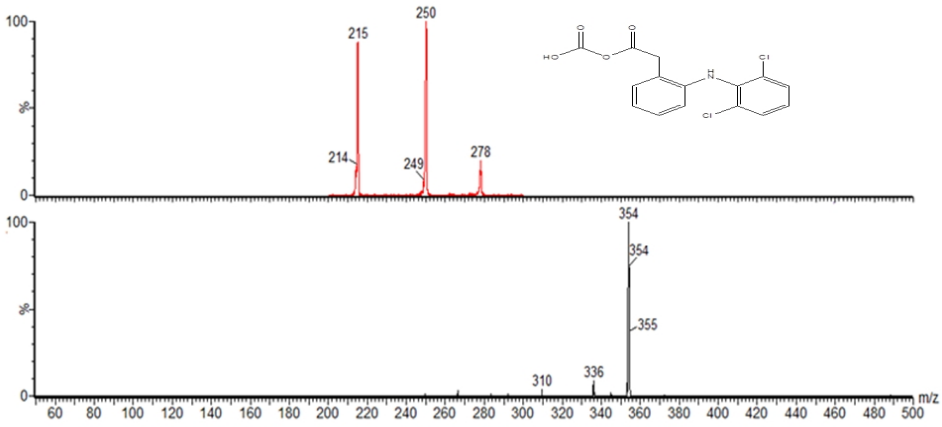



**Fig. 1. Fragmentation of ACL molecular ions (354.23 *m/z*) under capillary voltage 3 kV, cone voltage 20 V and 20 V collision energy. Figure displaying the parent ion of ACL (354 *m/z*) and the molecular ion fragments of 278, 250 and 215 *m/z*.**

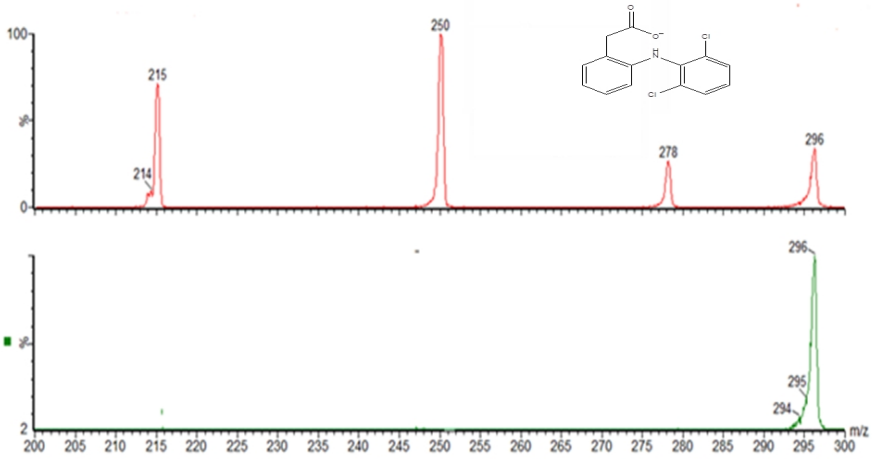
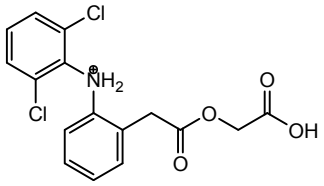
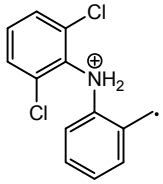



**Fig. 2. Fragmentation of DCL molecular ion (296.13 *m/z*) under capillary voltage 3 kV, cone voltage 20 V and 25 V collision energy. Figure displaying the parent ion of DCL (296 *m/z*) and the molecular ion fragments of 278, 250 and 215 *m/z*.**

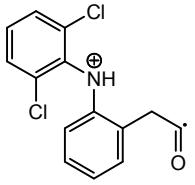


$C_{16}H_{14}Cl_2NO_4^{2+}$
Exact Mass: 354.03
Mol. Wt.: 355.19
m/e: 354.03 (100.0%), 356.03 (64.8%), 355.03 (17.8%), 357.03 (11.3%), 358.02 (10.2%), 359.03 (1.9%), 358.03 (1.5%), 356.04 (1.5%)
C, 54.10; H, 3.97; Cl, 19.96; N, 3.94; O, 18.02



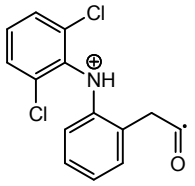
$C_{13}H_{11}Cl_2N^{\bullet+}$
Exact Mass: 251.03
Mol. Wt.: 252.14
m/e: 251.03 (100.0%), 253.02 (63.9%), 252.03 (14.2%), 255.02 (10.2%), 254.03 (9.1%), 256.02 (1.5%)
C, 61.93; H, 4.40; Cl, 28.12; N, 5.56

A



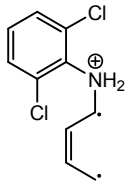
$C_{14}H_{10}Cl_2NO^{\bullet}$
Exact Mass: 278.01
Mol. Wt.: 279.14
m/e: 278.01 (100.0%), 280.01 (64.0%), 279.02 (15.3%), 282.01 (10.3%), 281.01 (9.9%), 283.01 (1.6%), 280.02 (1.3%)
C, 60.24; H, 3.61; Cl, 25.40; N, 5.02; O, 5.73

B



$C_{14}H_{10}Cl_2NO^{\bullet}$
Exact Mass: 278.01
Mol. Wt.: 279.14
m/e: 278.01 (100.0%), 280.01 (64.0%), 279.02 (15.3%), 282.01 (10.3%), 281.01 (9.9%), 283.01 (1.6%), 280.02 (1.3%)
C, 60.24; H, 3.61; Cl, 25.40; N, 5.02; O, 5.73

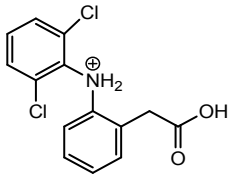
C



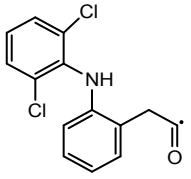
$C_{10}H_{10}Cl_2N^{\bullet\bullet+}$
Exact Mass: 214.02
Mol. Wt.: 215.1
m/e: 214.02 (100.0%), 216.02 (64.0%), 215.02 (11.2%), 218.01 (10.2%), 217.02 (7.0%), 219.02 (1.1%)
C, 55.84; H, 4.69; Cl, 32.96; N, 6.51

D

**Fig. 3. Proposed molecular ions of ESI +Ve MS-MS fragmentation of ACL (354 *m/z*); Figure created by Chemdraw Ultra 8.0 displaying fragments of ACL molecular ions fragments at 250 *m/z* (A) 278 *m/z* (Band C) and 215 (D)**

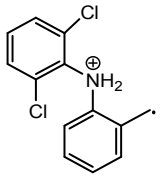


$C_{14}H_{12}Cl_2NO_2^+$
Exact Mass: 296.02
Mol. Wt.: 297.16
m/e: 296.02 (100.0%), 298.02 (64.0%), 297.03 (15.4%), 300.02 (10.3%), 299.02 (9.9%), 301.02 (1.6%), 298.03 (1.5%)
C, 56.59; H, 4.07; Cl, 23.86; N, 4.71; O, 10.77



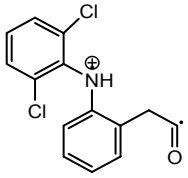
$C_{14}H_{10}Cl_2NO^{\bullet}$
Exact Mass: 278.01
Mol. Wt.: 279.14
m/e: 278.01 (100.0%), 280.01 (64.0%), 279.02 (15.3%), 282.01 (10.3%), 281.01 (9.9%), 283.01 (1.6%), 280.02 (1.3%)
C, 60.24; H, 3.61; Cl, 25.40; N, 5.02; O, 5.73

A



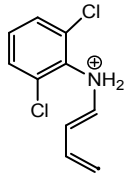
$C_{13}H_{11}Cl_2N^{\bullet}$
Exact Mass: 251.03
Mol. Wt.: 252.14
m/e: 251.03 (100.0%), 253.02 (63.9%), 252.03 (14.2%), 255.02 (10.2%), 254.03 (9.1%), 256.02 (1.5%)
C, 61.93; H, 4.40; Cl, 28.12; N, 5.56

B



$C_{14}H_{10}Cl_2NO^{\bullet\bullet+}$
Exact Mass: 278.01
Mol. Wt.: 279.14
m/e: 278.01 (100.0%), 280.01 (64.0%), 279.02 (15.3%), 282.01 (10.3%), 281.01 (9.9%), 283.01 (1.6%), 280.02 (1.3%)
C, 60.24; H, 3.61; Cl, 25.40; N, 5.02; O, 5.73

C



$C_{10}H_9Cl_2N^{\bullet}$
Exact Mass: 213.01
Mol. Wt.: 214.09
m/e: 213.01 (100.0%), 215.01 (64.0%), 214.01 (11.2%), 217.01 (10.6%), 216.01 (7.2%), 218.01 (1.1%)
C, 56.10; H, 4.24; Cl, 33.12; N, 6.54

D

**Fig. 4. Proposed molecular ions of ESI +Ve MS-MS fragmentation of DCL (296 *m/z*); Figure created by Chem-draw Ultra 8.0 displaying fragments of ACL molecular ions at 250 *m/z* (B) 215 (D) and 278 *m/z* (A and C)**

The Chemdraw mass analysis results were close to the practically obtained results by MS/MS tuning Figure 3 displays the molecular ion of ACL (354 *m/z*) and its possible

fragments where A was 251 *m/z*, B and C were 278 *m/z* and D was 215 *m/z*. Fig. 4 displays the molecular ion of DCL (296 *m/z*) and its possible fragments where A and C were 278 *m/z*, B was 251 *m/z* and D was 215 *m/z*.

Small peaks were observed in the ACL spectrum (Fig. 1) at 310 and 336 *m/z*. These peaks may be resulting from system contaminants, drug substance impurities or unexpected slight fragmentation of ACL at MS1.

Shouldered spectral peaks appeared in DCL spectrum (Fig. 2). These shoulders may be representing fragments of close *m/z* values to the major detected signals (215, 250 and 278 *m/z*). However these fragments are of minor relative abundance. This interpretation supports the Chemdraw software interpretation (Fig. 4).

MRM monitoring mode was used for detection and quantitation of the analytes. This is to increase specificity of the analytical method. By using MRM mode the extra detected peaks will not affect the method performance.

## 3.2 Chromatographic conditions

UPLC BEH C18 column, 2.1 x 50 mm, 1.7 µm was used as stationary phase. Isocratic elution was performed with mobile phase of acetonitrile, water and formic acid (80:20:0.5, *v/v/v*). Flow rate was 0.2 mL/min and total run time was 1 min. The Auto-sampler temperature was maintained at 5ºC to guard against further degradation of ACL and the injection volume was 10 µL.

Formic acid was added to the mobile phase as 0.5% to enhance positive ionization and improve sensitivity. MRM mode was used for the separation of ACL and DCL. MS/MS channels 354.23: 250.1 and 296.13: 250.09 for ACL and DCL; respectively were found to show the least noise and best calibration fit. Retention times were 0.70 and 0.78 min for ACL and DCL; respectively (Fig. 5).

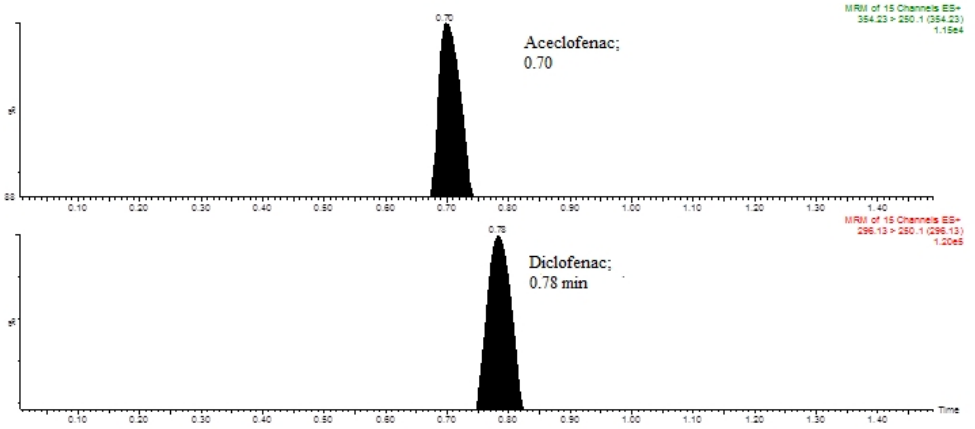



**Fig. 5. Chromatographic elution of ACL and DCL; using mobile phase of acetonitrile, water, formic acid (80:20:0.5; *v/v/v*) and a UPLC C-18 column. Figure displays the MRM chromatogram of ACL at 0.70 min and DCL at 0.78 min.**

## 3.3 Method Validation

### 3.3.1 Linearity

A calibration curve was established covering the dynamic range 20 – 3000 ng/mL of ACL and DCL. The method was linear and the regression parameters are shown in Fig. 6. The maximum deviation of data points from the nominal concentration was 97 – 103% for ACL and 97.4 – 102% for DCL.

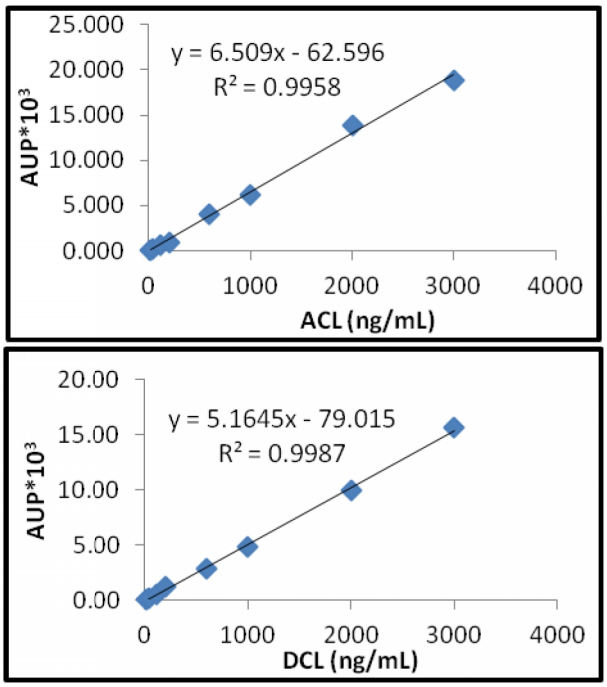



**Fig. 6. Calibration curves of ACL and DCL determination (20 – 3000 ng/mL). Maximum CV% of all determinations was 2.00 and 1.89 for ACL and DCL; respectively.**

### 3.3.2 Accuracy

To calculate accuracy in single solution; Replicates of QC samples of known concentration ranging from 100 – 2600 ng/mL were prepared from solutions BI and injected into the UPLC system. The analytical response (peak area) was used for calculating drug concentration using the corresponding regression equation. Accuracy percentages were calculated. The method was found accurate for both drugs with average recovery of 99.65±1.33 and 100.37±1.02 for ACL and DCL; respectively (Table 2).

To calculate accuracy in laboratory prepared mixture; three replicates of two laboratory prepared mixtures (1ACL:5DCL and 5 ACL: 1 DCL) of varying concentrations of both drugs were analyzed. The recovery was calculated based on the regression equations. As indicated in Table 3; the average recovery was 101.01±1.07 and 100.45±1.54.

**Table 2. Accuracy of the UPLC-MS/MS method for the determination of ACL and DCL determination in single drug solution**

| Concentration (ng/mL) | ACL | | | DCL | | |
|---|---|---|---|---|---|---|
| | Calculated conc. (ng/mL) | Accuracy % | CV% | Calculated conc. (ng/mL) | Accuracy % | CV% |
| 100 | 102.00 | 102.00 | 1.23 | 98.88 | 98.88 | 1.23 |
| 400 | 400.01 | 100.00 | 1.37 | 399.28 | 99.82 | 0.64 |
| 800 | 799.12 | 99.89 | 1.33 | 809.58 | 101.20 | 2.02 |
| 1200 | 1183.42 | 98.62 | 0.73 | 1205.20 | 100.43 | 2.19 |
| 1600 | 1572.88 | 98.31 | 0.27 | 1628.01 | 101.75 | 1.49 |
| 2600 | 2576.45 | 99.09 | 1.22 | 2603.12 | 100.12 | 1.64 |
| Average accuracy % | | 99.65 | | | 100.37 | |
| SD | | 1.33 | | | 1.02 | |
| RSD | | 1.34 | | | 1.02 | |

**The British pharmacopoeia stated the purity limit of ACL as 99 – 101 % [37].*

**Table 3. Accuracy of the UPLC-MS/MS method for the determination of ACL and DCL in laboratory prepared mixtures**

| | ACL | | | | DCL | | | |
|---|---|---|---|---|---|---|---|---|
| | Concentration (ng/mL) | AUP | Calculated (ng/mL) | Accuracy % | Concentration (ng/mL) | AUP | Calculated (ng/mL) | Accuracy % |
| Mixture 1 (1 ACL: 5DCL) | 60 | 330.49 | 60.39 | 100.65 | 300 | 1442.82 | 294.67 | 8.22 |
| | 80 | 455.34 | 79.57 | 99.47 | 400 | 1959.08 | 394.64 | 98.66 |
| | 160 | 998.38 | 163.00 | 101.88 | 800 | 4049.08 | 799.32 | 99.92 |
| | 300 | 1923.14 | 305.08 | 101.69 | 1500 | 7531.12 | 1473.55 | 98.24 |
| | 500 | 3246.28 | 508.35 | 101.67 | 2500 | 13005.14 | 2533.48 | 101.34 |
| | | **Accuracy %** | | 101.07 | | | | 99.28 |
| | | **SD** | | 1.02 | | | | 1.35 |
| Mixture 2 (5ACL: 1DCL) | 300 | 1889.89 | 299.97 | 99.99 | 60 | 234.71 | 60.75 | 101.24 |
| | 800 | 5107.28 | 794.27 | 99.28 | 160 | 758.69 | 162.21 | 101.38 |
| | 1200 | 7900.16 | 1223.35 | 101.95 | 240 | 1182.71 | 244.31 | 101.79 |
| | 1600 | 10509.79 | 1624.27 | 101.52 | 320 | 1604.69 | 326.02 | 101.88 |
| | 2600 | 17194.25 | 2651.23 | 101.97 | 520 | 2654.70 | 529.33 | 101.79 |
| | **Accuracy %** | | | 100.94 | | | | 101.62 |
| | **SD** | | | 1.23 | | | | 0.29 |
| **Overall accuracy** | **Accuracy%** | | | 101.01 | | | | 100.45 |
| | **SD** | | | 1.07 | | | | 1.54 |

**Table 4. Inter and intraday precision of UPLC-MS/MS determination of ACL and DCL in single drug solution**

| Concentration (ng/mL) | ACL | | | | DCL | | | |
|---|---|---|---|---|---|---|---|---|
| | CV% | | Accuracy % | | CV% | | Accuracy % | |
| | Intraday | Inter-day | Intraday | Inter-day | Intraday | Inter-day | Intraday | Inter-day |
| 100.00 | 0.18 | 0.27 | 102.00 | 101.09 | 1.10 | 2.00 | 98.88 | 98.82 |
| 400.00 | 1.37 | 1.03 | 100.00 | 100.83 | 0.64 | 0.40 | 99.82 | 99.43 |
| 800.00 | 1.33 | 0.53 | 99.89 | 100.77 | 2.02 | 1.97 | 101.20 | 101.62 |
| 1200.00 | 0.73 | 0.77 | 98.62 | 98.30 | 2.19 | 2.06 | 100.43 | 101.55 |
| 1600.00 | 0.27 | 0.40 | 98.31 | 98.20 | 1.49 | 1.75 | 101.75 | 102.34 |
| 2600.00 | 1.22 | 0.21 | 99.09 | 99.64 | 1.64 | 2.20 | 100.12 | 99.73 |

** Analysis was performed on triplicate analysis intraday and inter-day analysis in 3 successive days.*

**Table 5. Inter and intraday precision of UPLC-MS/MS determination of ACL and DCL in laboratory prepared mixtures**

| | ACL | | | DCL | | |
|---|---|---|---|---|---|---|
| | Concentration (ng/mL) | CV% | | Concentration (ng/mL) | CV% | |
| | | Intraday | Inter-day | | Intraday | Inter-day |
| mixture 1 (1 ACL: 5DCL) | 60 | 1.00 | 1.20 | 300 | 0.92 | 0.23 |
| | 80 | 0.96 | 1.27 | 400 | 1.61 | 1.99 |
| | 160 | 0.26 | 0.75 | 800 | 0.96 | 0.61 |
| | 300 | 1.59 | 0.14 | 1500 | 0.24 | 0.48 |
| | 500 | 0.29 | 0.97 | 2500 | 0.67 | 0.97 |
| Mixture 2 (5 ACL: 1 DCL) | 300 | 0.56 | 0.76 | 60 | 1.74 | 0.98 |
| | 800 | 0.36 | 0.54 | 160 | 0.97 | 0.77 |
| | 1200 | 0.11 | 0.27 | 240 | 1.41 | 0.77 |
| | 1600 | 0.95 | 0.47 | 320 | 0.37 | 0.58 |
| | 2600 | 0.57 | 0.19 | 520 | 0.64 | 0.36 |

** Analysis was performed on triplicate analysis intraday and inter-day analysis in 3 successive days.*

### 3.3.3 Precision

Precision was calculated based on three replicates of different concentrations of single solution and the laboratory prepared mixtures. CV% of the analytical response was calculated. The procedure was repeated on three consecutive days The CV% was within limit and indicated acceptable intraday and inter-day precision of the assay (Tables 4 and 5).

### 3.3.4 System carry-over

No traces of ACL and DCL appeared when a blank sample (mobile phase) was analyzed after ACL and DCL samples of highest concentration on calibration curve (3000 ng/mL). The negative result indicated proper elution and absence of system carryover for ACL and DCL by the developed method

### 3.3.5 LOD and LOQ

The LOD was calculated (3.3*SD/Slope) to be 0.52 and 0.45 ng/mL for ACL and DCL; respectively. The LOQ was 10 ng/mL for ACL and 20 ng/mL for DCL. The average recovery of six determinations at LOQ was 108.23±0.92 and 92.32±1.23 for ACL and DCL; respectively.

### 3.3.6 Specificity

In the analysis of ACL and DCL samples only spectral peaks of the tested analyte appeared with no appearance of the other analyte spectral peaks indicating no interference (analyte cross talk). In the tablet mixture sample, only spectral peaks corresponding to ACL or DCL appeared indicating no interference from tablet matrix (Fig. 7). Similar results were obtained in reaction mixture.

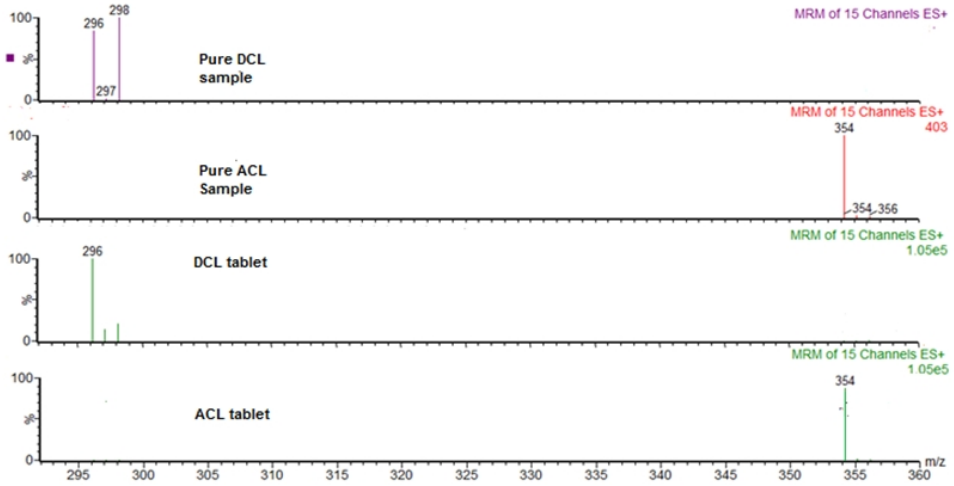



**Fig. 7. Specificity of the UPLC-MS/MS method for the analysis of ACL and DCL in tablets. The figure displays the MS analysis of the TIC for pure DCL, pure ACL, DCL and ACL tablets mixture; respectively. In the total ion chromatogram (TIC) of tablets only MS fragments corresponding to active constituent (DCL and ACL) are present indicating that there is no interference from tablets' additives**

## 3.4 Application to Tablets

Injected samples representing ACL and DCL tablets showed recovery of 100.95±0.18 and 99.15±0.62; respectively, indicating applicability of the method to tablets and absence of interference with tablet ingredients (Table 6).

**Table 6. Application of the LCMSMS for the determination to ACL and DCL in tablets**

| Theoretical concentration (ng/mL) | ACL | | DCL | |
|---|---|---|---|---|
| | Calculated concentration (ng/mL) | Recovery % | Calculated concentration (ng/mL) | Recovery % |
| 400 | 403.31 | 100.83 | 397.92 | 99.48 |
| 600 | 606.96 | 101.16 | 597.16 | 99.53 |
| 1000 | 1008.68 | 100.87 | 984.28 | 98.43 |
| **Average recovery %** | | 100.95 | | 99.15 |
| SD | | 0.18 | | 0.62 |
| RSD | | 0.18 | | 0.63 |

## 3.5 Optimization for at-line Monitoring of ACL Synthesis during PAT Application

In order to design and develop an at-line monitoring method for the application of PAT to the ACL synthesis from DCL several steps were followed. Steps included study and understanding of the ACL synthesis methods [2 – 4] as well as defining the critical point that is most likely to affect the flow of the process and the product final quality. Assigning of the parameters that can be adjusted to control the process (process controls) was done. The at-line monitoring method (process measurement system) was designed based on the gathered information from understanding the synthesis process, the synthesis critical point and its process controls.

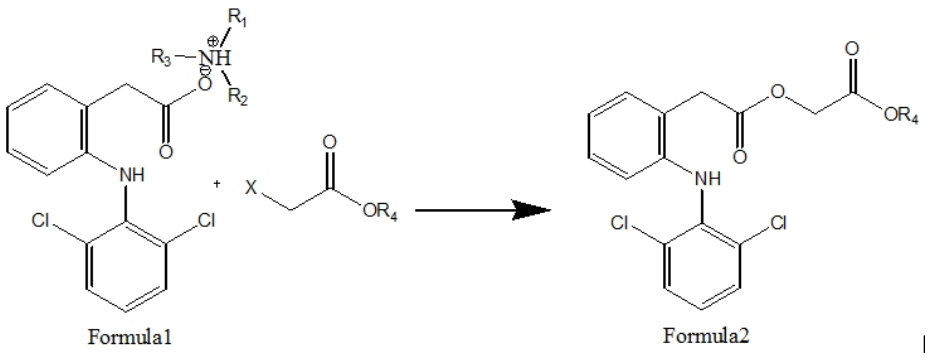
### 3.5.1 Process understanding (Interpretation of the synthesis process of ACL from DCL)

Three synthesis schemes for ACL from DCL were investigated for process understanding as described later [2–4]. The reactions were interpreted thoroughly for the determination of the CPP and other parameters of the PAT. All processes involved alkylation of DCL or its salt with halo ester of acetic acid to give a protected from of ACL (formula 2). The reaction intermediate (formula 2) is then acid hydrolyzed to ACL.

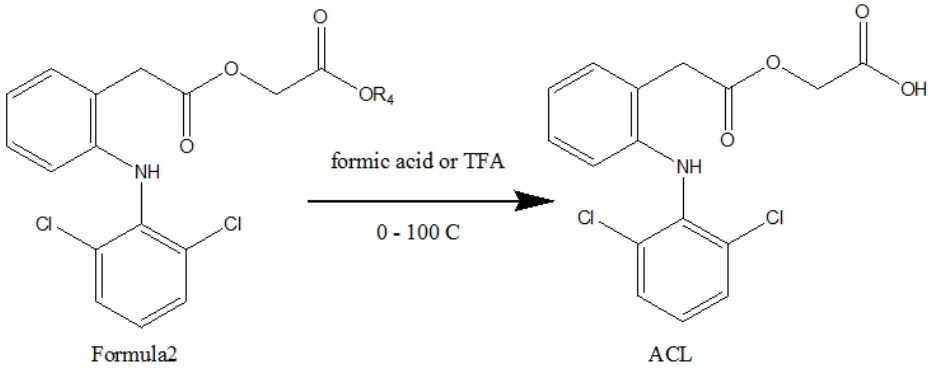
### Synthesis process 1 [2]

**Step 1:** Compounds of formula (I), is prepared by reacting DCL with triethylamine, diisopropylamine or ammonia in a solvent at a temperature of from 20 – 60ºC.

**Step 2:** The product is directly reacted with an appropriate α -haloacetic acid ester (e.g tertiary butyl bromoacetate) to form acetates (formula 2) which are de-protected to give ACL.

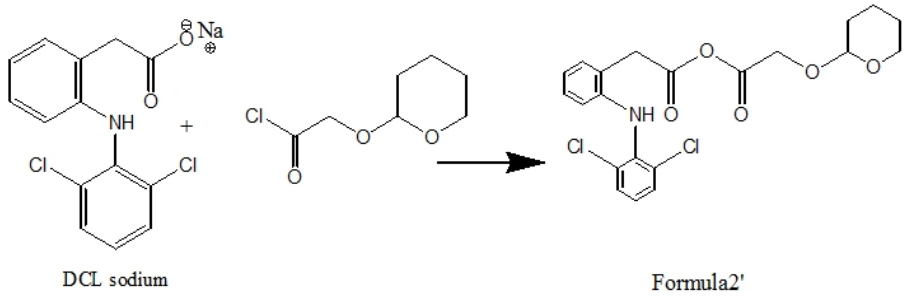



**Step 3:** For the conversion of formula 2 into ACL, de-protection step is done with formic acid or TFA at mild conditions (0 – 100ºC). The process is as 1 pot reaction where all steps are present in one medium.

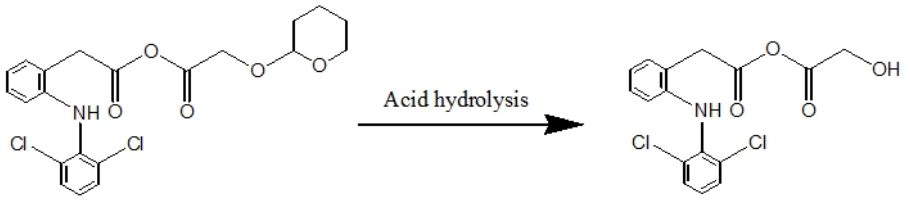



## Synthesis process 2 [3]

**Step 1:** reaction of DCL sodium salt with tetrahydropyranyl/furanyl chloroacetate to give product formula 2'



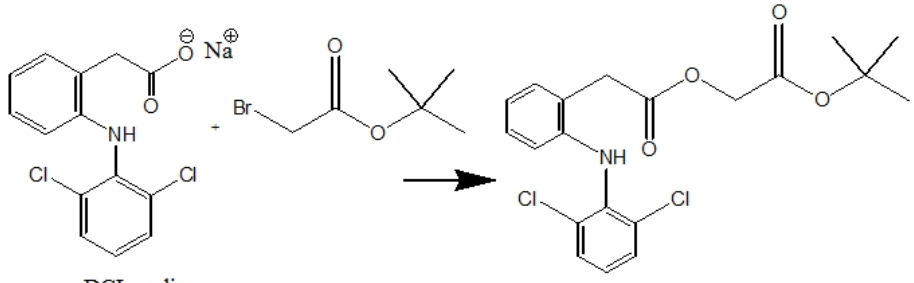
**Step 2:** Acid hydrolysis of formula 2' into ACL




Formula2' ACL

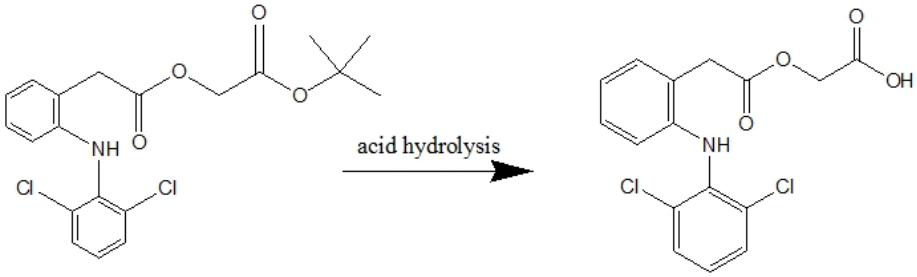
## Synthesis process 3 [4]

**Step 1:** reaction of DCL sodium salt with tertiary butyl bromoacetate to give product formula 2"




DCL sodium Formula2"

**Step 2:** Acid hydrolysis of formula 2" into ACL




Formula2" ACL

The acid hydrolysis step should only hydrolyze formula 2 to ACL [2–4]. Further undesired hydrolysis due to harsh conditions (heat and time) might cause degradation of ACL back to DCL. Direct hydrolysis of formula 2 into DCL might also occur. In both unplanned scenarios of ACL further degradation, ACL yield will decrease and the DCL impurity will increase leading to defective and subsequently rejected product. The undesired further hydrolysis can be avoided by continuous at-line monitoring of ACL and DCL levels. The monitoring data will help optimizing the reaction conditions for better results. The optimization of hydrolysis temperature and duration will reduce DCL impurity in final product.

### 3.5.2 Determination of CPP

CPP was suggested as the acid hydrolysis step which produces ACL from the intermediates (formula 2, 2' or formula 2''). Further acid hydrolysis may cause acid degradation of ACL back to DCL. Continuous at- line monitoring of ACL and DCL will give the required information about the reaction flow.

### 3.5.3 Design of a process measurement system

The UPLC-MS/MS method was designed to suit the continuous at-line monitoring of ACL and DCL. The sample size was small (10µL) in order not to affect the reaction yield. The analysis time was short (1 min.) to allow continuous monitoringand fast decision on condition adjustment.

### 3.5.4 Setting of process controls that provide adjustments

The acid hydrolysis can result in conversion of ACL to DCL. The optimization of the acid hydrolysis conditions can affect the final reaction yield. Process control points are suggested to be the duration and temperature of acid hydrolysis.

The application of the developed UPLC-MS/MS method fulfils the at-line monitoring required for the ACL synthesis procedures. The adoption of at-line monitoring in synthesis process will improve the conventional synthesis protocol. The developed UPLC-MS/MS method will help making the right decisions about optimization of process controls. This improvement will increase the ACL yield and decrease the DCL impurity [35].

### 3.5.5 Application to simulated synthesis reaction matrix

Injected samples representing ACL and DCL with TFA showed recovery of 101.21±0.06 and 98.89±0.64; respectively, indicating applicability of the method to determination of ACL and DCL in reaction mixture. These results indicate the suitability of the developed UPLC-MS/MS for at-line monitoring of ACL synthesis process (Table 7).

**Table 7. Application of the UPLC-MS/MS method for the determination of ACL and DCL in simulated reaction mixture**

| Theoretical concentration ng/mL | ACL | | DCL | |
|---|---|---|---|---|
| | Calculated concentration ng/mL | Recovery % | Calculated concentration ng/mL | Recovery % |
| 400 | 404.56 | 101.14 | 397.92 | 99.48 |
| 600 | 607.53 | 101.26 | 593.84 | 98.97 |
| 1000 | 1012.23 | 101.22 | 982.28 | 98.23 |
| **Average recovery %** | | 101.21 | | 98.89 |
| SD | | 0.06 | | 0.64 |
| RSD | | 0.06 | | 0.64 |

## 4. CONCLUSION

The developed method offers the fastest determination of DCL in ACL while maintaining accuracy; precision and Linearity. The method is feasible as an at-line monitoring method for PAT application to the ACL synthesis. The developed method is valid for ACL and DCL determination in bulk and pharmaceutical products.

## CONSENT AND ETHICAL APPROVAL

Not applicable.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## REFERENCES


1. Seth SD, Seth V, editors. Text book of pharmacology. 3rd edition. Noida: Elsevier; 1998.
2. Murphy T, Nikolopoulos A, Schickaneder H. Process for the preparation of aceclofenac. 1999;EP 1082290 B1.
3. Zirngibl L, Gnehm R, Process for the preparation of a phenylacetic acid derivative. 1984;CH-682747.
4. Vila Casas A. New 2-((2, 6-dichlorophenyl) amine) phenylacetoxyacetyl derivatives, the process for preparing the same and their use in therapeutics. 1983;EP 0119932 B1.
5. Dockner T, Frank A, Karn H. Process for the preparation of 1(2-hydroxyethyl)-2-methyl-5-nitroimidazole of high purity. 1984; EP 0150407.
6. Flores Ramos R; Process for obtaining 2-[(2, 6-dichlorophenyl) amino] phenylacetoxy acetic acid. 1999; ES 2046141.
7. Hasan NY, Abdel-Elkawy M, Elzeany BE, Wagieh NE. Stability indicating methods for the determination of aceclofenac. Farmaco. 2003;58(2):91–99.
8. Kang W, Kim EY. Simultaneous determination of aceclofenac and its three metabolites in plasma using liquid chromatography-tandem mass spectrometry. J Pharm Biomed Anal. 2008;46(3):587-591.
9. Golhar MK, Joshi RR, Gupta KR, Wadodkar SG. Development and validation of spectrophotometric methods for determination of aceclofenac in tablets. Int. J. Chem Tech Res. 2011;3(2):786-790.
10. Aderibigbe SA, Adegoke OA, Idowu OS, Olaleye SO. Sensitive spectrophotometric determination of aceclofenac following azodye formation with 4-Carboxyl-2,6-Dinitrobenzene diazonium ion. Acta Pol. Pharm. 2012;69(2):203-211.
11. Shaikh A, Patil AT, Ingole AB. Sensitive LC method for the simultaneous determination of diacerein and aceclofenac in tablet dosage form. Int J Ind Chem. 2012;3(3):1-7.
12. Khana H, Alia M, Ahmad S, Ahmad N, Ahuja A, Baboota S et al. Validated UPLC/Q-TOF-MS method for simultaneous determination of aceclofenac, paracetamol and their degradation products in tablets. J. Liq. Chromatogr. 2012;35(1):109-128.
13. Godse VP. Deodhar MN, Bhosale AV, Sonawane RA, Sakpal PS, Borkar DD, et al. Reverse phase HPLC method for determination of aceclofenac and paracetamol in tablet dosage form. Asian J. Research Chem. 2009;2(1):37-40.

14. Musmade P, Subramanian G, Srinivasan KK. High-performance liquid chromatography and pharmacokinetics of aceclofenac in rats. Anal Chim Acta. 2007;585(1):103-109.
15. Jin Y, Chen H, GU S, Zeng F. Determination of aceclofenac in human plasma by reversed-phase high performance liquid chromatography. Se Pu. 2004;22(3):252-254.
16. Hinz B, Auge D, Rau T, Rietbrock S, Brune K, Werner U. Simultaneous determination of aceclofenac and three of its metabolites in human plasma by high-performance liquid chromatography. Biomed Chromatogr. 2003;17(4):268-275.
17. Lee HS, Jeong CK, Choi SJ, Kim SB, Lee MH, Ko GL, et al. Simultaneous determination of aceclofenac and diclofenac in human plasma by narrowbore HPLC using column-switching. J Pharm Biomed Anal. 2000;23(5):775–781.
18. Posac JR, Vázquez MD, Tascón ML, Acuña JA, de la Fuente C, Velasco E, et al. Determination of aceclofenac using adsorptive stripping voltammetric techniques on conventional and surfactant chemically modified carbon paste electrodes. Talanta. 1995;42(2):293-304.
19. Mahajan VK, Bari SB, Shirkhedkar AA, Surana SJ. Simultaneous densitometric TLC analysis of aceclofenac, paracetamol, and chlorzoxazone in tablets. Acta Chromatographica. 2008;20(4):625-636.
20. de Souza RL, Tubino M. Spectrophotometric determination of diclofenac in pharmaceutical preparations. J. Braz. Chem. Soc. 2005;16(5):1068-1073.
21. Kormosh ZO, Hunka IP, Bazel YR. Extraction and spectrophotometric determination of diclofenac in pharmaceuticals. J. Chin. Chem. Soc. 2008;55:356-361.
22. Ciapina EG, Santini AO, Weinert PL, Gotardo MA, Pezza HR, Pezza L. Spectrophotometric determination of diclofenac in pharmaceutical preparations assisted by microwave oven. Eclet. Quím. 2005;30(1):29-36.
23. Rodriguez JA, Islas G, Paez-Hernandez ME, Barrado E. Sequential injection spectrophotometric determination of diclofenac in urine and pharmaceutical formulations. J. Flow Injection Anal. 2008;25(1):39–43.
24. Panda SS, Patanaik D, Kumar BR. New Stability-Indicating RP-HPLC method for determination of diclofenac potassium and metaxalone from their Combined Dosage Form. Sci Pharm. 2012;80(1):127–137.
25. Muntean D, Vlase L, Ciciureanu R, Ciciureanu M. LC-MS/MS method for quantification of diclofenac from human plasma and its application to pharmakokinetics studies. Rev. Roum. Chim. 2011;56(1):19-24.
26. Emami J, Ghassami N, Talari R. A rapid and sensitive modified HPLC method for determination of diclofenac in human plasma and its application in pharmacokinetic studies. DARU. 2007;15(3):132-138.
27. Mohammad A, Sharma S. Separation of co-existing paracetamol and diclofenac. sodium on silica gel 'H' layers using surfactant mediated mobile phase: identification of diclofenac sodium from human urine. Farmacia. 2009;57(2):201-211.
28. Ensafi AA, Izadi M, Karimi-Maleh H. Sensitive voltammetric determination of diclofenac using room-temperature ionic liquid-modified carbon nanotubes paste electrode. Ionics. 2013;19(1):137-144.
29. Chethana BK, Basavanna S, Arthoba Naik Y. Voltammetric determination of diclofenac sodium using tyrosine-modified carbon paste electrode. Ind. Eng. Chem. Res. 2012;1(31):0287–10295.
30. Hassan SSM, Mahmoud WH, Elmosallamy MAF, Almarzooqi MH. Iron (II)-phthalocyanine as a novel recognition sensor for selective potentiometric determination of diclofenac and warfarin drugs. J Pharm Biomed Anal. 2005;39(1-2):315–321.

31. Mokhtaria A, Karimi-Malehb H, Ensafic AA, Beitollahi H. Application of modified multiwall carbon nanotubes paste electrode for simultaneous voltammetric determination of morphine and diclofenac in biological and pharmaceutical samples. Sensors Actuators Chem. 2012;169:96–105.
32. Aneshgar PD, Norouzi P, Ganjali MR, Dinarvand R, Moosavi-Movahedi AA. Determination of diclofenac on a dysprosium nanowire- modified carbon paste electrode accomplished in a flow injection system by advanced filtering. Sensors (Basel). 2009;9(10):7903–7918.
33. Ji W, Zhan J. Determination of diclofenac sodium by capillary zone electrophoresis with electrochemical detection. J Chromatogr A. 2000;868(1):101-107.
34. El-Saharty YS, Refaat M, El-Khateeb SZ. Stability-indicating spectrophotometric and densitometric methods for determination of aceclofenac. Drug Dev Ind Pharm. 2002;28(5):571-582.
35. Rockville. Food and Drug Administration (FDA) Guidance for Industry: PAT—A Framework for Innovative Pharmaceutical Development, Manufacturing and Quality Assurance; 2004.
36. International conference of harmonization of technical requirements for registration of pharmaceuticals for human use, ICH Q2 (R1) guidance on validation of analytical procedures: Text and Methodology Yokohama, Japan; 2005.
37. British pharmacopoeia. Commission office. British pharmacopoeia. The stationary office, London; 2010.



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