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RESEARCH ARTICLE

DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF PARACETAMOL, ACECLOFENAC, AND THEIR RELATED SUBSTANCES IN BULK AND PHARMACEUTICAL DOSAGE FORMS

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ABSTRACT

The accurate quantification of related substances (RS) in pharmaceutical formulations is a critical aspect of ensuring drug safety, efficacy, and regulatory compliance. This review presents a comprehensive summary of the development and validation of a reverse-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of Paracetamol and Aceclofenac along with their pharmacopoeial and process-related impurities. The method was systematically optimized through solubility screening, buffer pH adjustment, gradient elution programming, and column selection, utilizing a pH-stable C18 stationary phase coupled with ion-pairing agents to achieve optimal peak resolution. Method validation was conducted following ICH Q2 (R1) guidelines, evaluating parameters such as specificity, linearity, sensitivity, precision, accuracy, and robustness. The method demonstrated excellent linearity ($R^2 > 0.999$), low limits of detection and quantification for all analytes, and high recovery rates within acceptable ranges. Importantly, critical resolution between closely eluting impurities, such as Aceclofenac Impurity-A and 4-chloroacetanilide, was successfully achieved, confirming the method's suitability for routine quality control. The study underscores the importance of robust analytical design in impurity profiling and offers a validated approach that can be adopted for regulatory submissions and real-time release testing in the pharmaceutical industry.

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INTRODUCTION

The aim of this research is to develop and validate a robust, specific, and stability-indicating RP-HPLC method for the simultaneous estimation of Paracetamol and Aceclofenac along with their pharmacopoeial impurities. Paracetamol and Aceclofenac are widely used in combination for analgesic and anti-inflammatory therapy, and their quality control requires rigorous analysis of related substances (RS), which may include toxic or reactive intermediates. According to solubility profiling, Paracetamol is freely soluble in methanol (1:10), propylene glycol (1:9), and ethanol (1:7), and moderately soluble in water. Aceclofenac showed maximum solubility in phosphate buffer at pH 7.4. Impurity profiling focused on key known impurities: for Paracetamol, these include 4-aminophenol and 4-chloroacetanilide—both toxicologically significant and listed in the BP monograph. For Aceclofenac, impurities A to I were considered, including structural variants such as the methyl/ethyl esters of diclofenac and aceclofenac, benzyl ester, acetic derivatives, and Indolinone analogs.

Method development involved systematic trials using various gradient programs and mobile phases. A mobile phase consisting of orthophosphoric acid buffer (pH 7.75) and acetonitrile-water (90:10) was used, enhanced with tetrabutylammonium hydroxide as an ion-pairing agent to improve the peak shapes of polar impurities like 4-aminophenol. The best chromatographic performance was achieved on a Phenomenex Kinetex C18 column (250 mm × 4.6 mm, 5 µm), designed for high pH stability and higher carbon load. The final method achieved satisfactory separation of critical impurity pairs, particularly Aceclofenac Impurity A and 4-chloroacetanilide, with a resolution ≥2.0. All analytes showed strong linearity across relevant concentration ranges (0.32–6.05 µg/mL for Aceclofenac and 1.04–19.5 µg/mL for Paracetamol), with correlation coefficients (R^2) exceeding 0.999. Limits of detection were as low as 0.04 µg/mL for Aceclofenac Impurity A and 0.13 µg/mL for 4-aminophenol. Accuracy studies showed recoveries between 94–107%, and robustness tests confirmed that the method is sensitive to slight pH variations, highlighting the critical need for buffer stability.

MATERIALS AND METHODS

Study Design: The study focused on the development and validation of a gradient RP-HPLC method for the simultaneous quantification of Paracetamol, Aceclofenac, and their related substances (impurities) in bulk drugs and marketed formulations. All procedures were performed according to ICH Q2 (R1) guidelines.

Table 1. Chemicals, Reagents, and Samples

Material	Source
Paracetamol and Aceclofenac standards	MedopharmPvt. Ltd., Chennai
Marketed tablets (Aceclofenac + Paracetamol)	Local pharmacy
HPLC grade Methanol and Acetonitrile	Research Lab Fine Chem Industries
Water (HPLC grade)	Research Lab Fine Chem Industries
Orthophosphoric acid (OPA)	Analytical grade
Tetrabutylammonium Hydroxide (40% MeOH)	Analytical grade

Table 2. Instruments Used

Instrument	Make/Model
HPLC System	SHIMADZU Prominence LC-2050C 3D
Analytical Column	Phenomenex Kinetex C18 (250 × 4.6 mm, 5 µm)
Balance	SHIMADZU AX200
Ultrasonic Cleaner	Life Acre Fast Clean System
Vacuum Filtration Unit	Standard filtration assembly
Software	LabSolutions (SHIMADZU)

Table 3. Chromatographic Conditions

Parameter	Specification
Column	Phenomenex Kinetex C18 (250 × 4.6 mm, 5 µm)
Mobile Phase A	0.112% OPA in purified water, pH adjusted to 7.75
Mobile Phase B	Acetonitrile:Water (90:10 v/v)
Ion Pairing Agent	Tetrabutylammonium hydroxide (40% in methanol), 1.15 g in 250 mL methanol
Final Diluent	Mobile Phase A:Mobile Phase B:Ion pair (30:60:10 v/v/v)
Flow Rate	1.0 mL/min
Column Temperature	40°C
Sample Temperature	10°C
Detection Wavelength	275 nm
Injection Volume	20 µL (standard)

Table 4. Gradient Elution Program

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0	85	15
25	50	50
35	10	90
40	15	85
45	85	15
55	85	15

Sample and Standard Preparation

Standard Solution

- 50 mg each of Aceclofenac and Paracetamol was weighed and dissolved in diluent to make a **2000 µg/mL stock solution**.
- Further dilutions were made using the same diluent to achieve calibration ranges.

Diluted Working Standard

- 2 mL of the stock solution was diluted to 10 mL.
- 1 mL of this intermediate solution was further diluted to 100 mL using diluent, yielding **4 µg/mL** working standard.

Sample Preparation (Tablet Formulation)

- 20 tablets (Aceclofenac 100 mg + Paracetamol 325 mg) were finely powdered.
- Powder equivalent to 50 mg Aceclofenac was transferred to a 25 mL volumetric flask.
- 10 mL of diluent was added and sonicated for 1 minute.
- Volume was made up to 25 mL with diluent and filtered through 0.45 µm nylon filter.

System Suitability Criteria (Table 5): System suitability was evaluated to ensure resolution between closely eluting analytes:

Component	Retention Time (min)	Resolution (USP)
Aceclofenac Imp-A	16.15	-
Aceclofenac	17.38	6.60
4-Chloroacetanilide	19.38	12.36

Acceptance Criteria

- Resolution between Aceclofenac Imp-A and 4-Chloroacetanilide ≥ 2.0
- Tailing factor < 2.0
- Theoretical plates > 2000

Impurity Profile and Identification

Paracetamol Impurities

- 4-Aminophenol
- 4-Chloroacetanilide
- Other known impurities (quinone, para-chloroaniline) from BP monograph

Table 6. Aceclofenac Impurities (per BP):

Impurity Name	RRT	RRF
Impurity A (Diclofenac Sodium)	0.93	1.39
Impurity B (Methyl ester)	1.90	0.97
Impurity C (Ethyl ester)	2.00	1.14
Impurity D (Aceclofenac Methyl ester)	1.85	0.92
Impurity E (Aceclofenac Ethyl ester)	1.95	0.87
Impurity F (Aceclofenac Benzyl ester)	2.10	0.74
Impurity G (Acetic derivative)	1.15	0.80
Impurity H (Diacetic derivative)	1.23	0.54
Impurity I (Indolinone)	1.63	0.21

Table 7. Linearity Range

Drug	Concentration Range (µg/mL)	Correlation Coefficient (R^2)
Paracetamol	1.04 – 19.50	0.9996
Aceclofenac	0.32 – 6.05	0.9996

Table 8. Limit of Detection (LOD) and Limit of Quantification (LOQ)

Compound	LOD (µg/mL)	LOQ (µg/mL)
Aceclofenac	0.08	0.32
Paracetamol	0.18	0.65
Aceclofenac Imp-A	0.04	0.35
4-Aminophenol	0.13	0.66
4-Chloroacetanilide	0.19	0.78

All LOQ repeatability values showed %RSD < 10%, confirming method sensitivity.

Precision and Accuracy

- **Repeatability**: 6 replicate sample injections (intra-day)
- **Intermediate Precision**: Performed by a different analyst, column, and system (inter-day)
-

Table 9. %RSD Results (Repeatability)

Component	%RSD
Aceclofenac Imp-A	2.27
Aceclofenac Imp-F	2.67
4-Aminophenol	7.75
4-Chloroacetanilide	0.00

Table 10. Accuracy (%Recovery at 50%, 100%, 150%)

Compound	Recovery (%) Range
Paracetamol	94.38 – 107.1
Aceclofenac	97.74 – 104.4
Major Impurities	93.69 – 106.33

Robustness Study: Deliberate variations in wavelength (273, 277 nm), flow rate (±0.2 mL/min), and column temp (±5°C) were tested.

Table 11

Parameter	Result (Acceptable Variations)
Wavelength variation	< ±0.05 in retention/area
Flow rate variation	No significant peak merging
Temperature variation	Acceptable shifts observed
pH Sensitivity	**Highly critical**—peak merging observed with minor pH shifts

RESULTS

Method Development and Optimization: A total of five RP-HPLC method development trials were performed, adjusting chromatographic parameters to optimize separation, resolution, and peak shape of Paracetamol, Aceclofenac, and their impurities.

Table 12. Trial Comparisons and Observations

Trial	Column	pH	Temp (°C)	Ion Pair	Key Findings
1	Waters C18 (250×4.6 mm)	7.5	Ambient	No	Poor baseline; peak broadening for Paracetamol impurities
2	Waters C18	7.5	15	No	Co-elution of Imp-A and 4-Chloroacetanilide
3	Waters C18	7.7	15	No	Partial resolution; broad peaks
4	Waters C18	7.75	10	Yes	Improved peaks; insufficient baseline
5	**Phenomenex Kinetex C18**	7.75	**10**	**Yes**	**Optimal resolution, shape, baseline stability**

Final Method Selected: Trial 5

- **Column:** Phenomenex Kinetex C18 (250×4.6 mm, 5 µm, pH-stable, high carbon load)
- **Mobile Phase A:** 0.112% OPA in water, pH 7.75
- **Mobile Phase B:**Acetonitrile:Water (90:10)
- **Ion-Pairing Agent:** Tetrabutylammonium hydroxide (1.15 g in 250 mL methanol)
- **Diluent:**A:B:Ion Pair = 30:60:10
- **Flow Rate:** 1.0 mL/min
- **Wavelength:** 275 nm
- **Column Temp:** 40°C
- **Sample Temp:** 10°C
- **Gradient Program:** (see Methods Section 2.5)

Impurity Identification and Retention

Paracetamol Impurities

- 4-Aminophenol (Toxic nephrotoxic intermediate)
- 4-Chloroacetanilide (Moderately toxic)

Table 13. Aceclofenac Impurities (BP Reference)

Impurity Name	Ret. Time (min)	RRT	RRF
Imp-A (Diclofenac)	17.10	0.93	1.39
Imp-B (Me ester)	34.93	1.90	0.97
Imp-C (Et ester)	36.77	2.00	1.14
Imp-D (Me-Aceclofenac)	34.01	1.85	0.92
Imp-E (Et-Aceclofenac)	35.85	1.95	0.87
Imp-F (Bn-Aceclofenac)	38.61	2.10	0.74
Imp-G (Acetic)	21.14	1.15	0.80
Imp-H (Diacetic)	22.61	1.23	0.54
Imp-I (Indolinone)	29.97	1.63	0.21
4-Aminophenol	2.94	0.16	0.52
4-Chloroacetanilide	15.63	0.85	0.59

Table 14. System Suitability Test (SST)

Component	Ret. Time (min)	Resolution (USP)
Aceclofenac Imp-A	16.15	—
Aceclofenac	17.38	6.60
4-Chloroacetanilide	19.38	12.36

Acceptance Criteria

- Rs > 2.0 between Imp-A and 4-Chloroacetanilide
- Baseline should be noise-free; peak symmetry should be ideal

All SST criteria were met

Linearity and Range

Calibration curves were prepared in 6 concentrations for both actives.

Table 15. Linearity Table – Aceclofenac

Concentration (µg/mL)	Area Response
0.32	7390
2.02	44983
3.03	67110
4.03	90020
5.04	114840
6.05	135389

Table 16. Linearity Table – Paracetamol

Concentration (µg/mL)	Area Response
1.04	9744
6.50	65516
9.75	98236
13.00	132267
16.25	168937
19.50	200201

- **Regression equation:** y = 22522x – 289.88
- **R^2 (Paracetamol and Aceclofenac):** 0.9996
- **Range confirmed as LOQ–150%** for all analytes

Table 18. Sensitivity (LOD & LOQ)

Analyte	LOD (µg/mL)	LOQ (µg/mL)
Aceclofenac	0.08	0.32
Paracetamol	0.18	0.65
Aceclofenac Imp-A	0.04	0.35
4-Aminophenol	0.13	0.66
4-Chloroacetanilide	0.19	0.78

All LOQ-level responses showed %RSD < 10%, proving acceptable precision at low concentrations.

Precision

Table 19. Repeatability (Intra-Day)

Impurity	%RSD
Aceclofenac Imp-A	2.27
Aceclofenac Imp-F	2.67
4-Aminophenol	7.75
4-Chloroacetanilide	0.00

Table 20. Intermediate Precision (Inter-Day, Analyst/System Change)

Impurity	%RSD
Aceclofenac Imp-A	1.012
Aceclofenac Imp-H	9.68
4-Aminophenol	0.00
4-Chloroacetanilide	0.00

Accuracy (Recovery Studies): Recovery studies were performed at LOQ, 50%, 100%, and 150%.

Table 21.

Compound	Recovery at LOQ (%)	50%	100%	150%
Paracetamol	107.10	97.06	95.48	94.38
Aceclofenac	97.74	99.84	104.40	103.23
4-Aminophenol	104.56	101.32	106.06	105.93
4-Chloroacetanilide	98.42	97.77	100.41	101.69

All recoveries fall within ICH range (90%–110%)

Robustness: Robustness was tested by varying wavelength, flow rate, column temperature, and buffer pH.

Table 22.

Parameter Variation	Effect on Peak Area/RT	Acceptable?
Wavelength (±2 nm)	≤ ±0.05 variation	Yes
Flow (0.8, 1.2 mL/min)	Minor variation	Yes
Temp (35°C, 45°C)	Acceptable	Yes
pH ±0.1	**Unacceptable (Peak merging)**	**No**

Conclusion: Method is **robust**, except **highly sensitive to pH variation**

Application to Marketed Formulation

Tested on tablet dosage form containing Paracetamol 325 mg and Aceclofenac 100 mg.

- Sample prepared from 20 tablets
- Assay results:

Table 23

Drug	Label Claim (mg)	Assay Result (%)
Paracetamol	325	98.70
Aceclofenac	100	99.84

- **No interference** from excipients or degradation products was observed.

CONCLUSION

The present study successfully established a scientifically sound, stability-indicating, and regulatory-compliant RP-HPLC method for the simultaneous quantification of Paracetamol, Aceclofenac, and their associated related substances in bulk and pharmaceutical formulations. Method development was approached systematically through solubility screening, pH optimization, ion-pair reagent utilization, and column selection. The final method employed a gradient elution using a high pH-tolerant Phenomenex Kinetex C18 column, with orthophosphoric acid buffer (pH 7.75), acetonitrile, and tetrabutylammonium hydroxide enhancing resolution and peak shape—particularly for polar and closely eluting impurities.

The method demonstrated strong validation characteristics

- **Linearity** across therapeutic and trace impurity levels (R^2 = 0.9996 for both drugs).
- **Low detection and quantification limits**, enabling sensitive impurity profiling (e.g., LOD = 0.04 µg/mL for Aceclofenac Imp-A).
- **High recovery** (94–107%) and low %RSD values confirming method **accuracy and precision**.
- **Resolution ≥ 2.0** between critical pairs such as Aceclofenac Imp-A and 4-Chloroacetanilide.
- **Robustness** under variations in flow rate, temperature, and wavelength—with sensitivity noted specifically to buffer pH shifts.

Application to a marketed fixed-dose combination product demonstrated excellent specificity, with no interference from formulation excipients or degradation products, and yielded assay results within 98–100% of label claim. This method not only meets all ICH Q2 (R1) guidelines but also aligns with pharmacopoeial expectations for impurity testing, making it suitable for comprehensive quality control and regulatory documentation.

Abbreviations

Abbreviation	Full Form
RS	Related Substances
RP-HPLC	Reverse Phase High-Performance Liquid Chromatography
ICH	International Council for Harmonisation
OPA	Orthophosphoric Acid
RRT	Relative Retention Time
RRF	Relative Response Factor
LOD	Limit of Detection
LOQ	Limit of Quantification
RSD	Relative Standard Deviation
SST	System Suitability Test
%RSD	Percent Relative Standard Deviation
UV	Ultraviolet
µg/mL	Micrograms per Milliliter
nm	Nanometer
°C	Degree Celsius
BP	British Pharmacopoeia
Me	Methyl
Et	Ethyl
Bn	Benzyl

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