RP-HPLC Method Development and Validation For Simultaneous Estimation of Paracetamol and Alprazolam in Bulk and Pharmaceutical Dosage Forms

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ABSTRACT

A new Reverse Phase High Performance Liquid Chromatographic (RP-HPLC) method has been developed for estimation of Paracetamol and Alprazolam in bulk and tablet dosage forms using UV-detector. A RP Cell Pack C18 column (250 mm × 4.6 mm, 5 µ particle size) using acetonitrile and water (80:20 % V/V) as mobile phase by maintaining flow rate of 1 mL/min at 236 nm as detection wavelength. The peaks were eluted at 4.8 and 6.2 mins for Paracetamol and Alprazolam, respectively. The method was validated in accordance with ICH guidelines, the linearity curve for Paracetamol was obtained over the range of 50-175 µg/mL, and it was found to be linear with y = 1961x + 9226 (r² = 0.999). The linearity curve for Alprazolam was obtained over the range of 0.25-1.5 µg/mL and was found to be linear with y =23328x + 939.3 (r² = 0.998). The percentage recoveries were found to be 99-101% and 99-102%, respectively. The system suitability parameters such as number of theoretical plates and tailing factor were found to be 7242, 1.56 for PAR and 6755, 1.15 for ALP. Hence the developed RP-HPLC method was found to be simple, accurate, economical, rapid and can be applied for routine analysis of these drugs in their combined formulations.

Keywords: Paracetamol, Alprazolam, Acetonitrile, RP-HPLC, Method development, Method Validation.
INTRODUCTION

In the present work a new Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method was developed for the simultaneous estimation of Paracetamol (PAR) and Alprazolam (ALP) in bulk and pharmaceutical dosage forms. PAR and ALP were commercially available in the combination dosage forms for the treatment of anxiety, depression and cold (Figure 1). Commercially available combined dosage forms (PAR+ALP) includes STS tablets (Emcure Laboratories).

![Chemical structures of Paracetamol (i) and Alprazolam (ii).](image)

**Figure 1:** Chemical structures of Paracetamol (i) and Alprazolam (ii).

PAR is an acetanilide derivative, it acts as analgesic, antipyretic and anti-inflammatory agent. It is chemically \( N-(4\text{-hydroxyphenyl}) \text{acetamide} \) having molecular formula \( \text{C}_8\text{H}_9\text{NO}_2 \) and molecular weight 151.163 g/mol. PAR is generally considered as the weak inhibitor of prostaglandins. The drug acts primarily in the Central Nervous System (CNS), by inhibiting miso forms of cyclooxygenase (COX-1, COX-2, and COX-3) enzymes involved in prostaglandin synthesis. It is mainly used in the treatment of fever and mild to moderate pain.

ALP is an anti-anxiety drug which belongs to the benzodiazepines class. It is chemically 8-Cloro-methyl-6-phenyl-4H-[1,2,4]triaz[4,3-a][1,4]benzodiazepine having the molecular formula \( \text{C}_{17}\text{H}_{13}\text{C}_1\text{N}_4 \) and molecular weight 308.769 g/mol. It affects the neurotransmitters present in the brain which are unbalanced and are not stable during anxiety. It works by enhancing the effects of a certain natural chemicals (GABA) in the body. It is used to treat anxiety disorders, panic disorders and anxiety that are caused due to stress and depression.

Literature survey revealed that development of various spectrophotometric\(^1\)-\(^4\), HPLC\(^5\)-\(^9\), HPTLC\(^10\), \(^11\), LCMS\(^12\)-\(^15\) and GCMS\(^16\) methods for estimation of PAR and ALP in various dosage forms in individual and in combination with other drugs. However, there was a UV-Spectrophotometric method\(^2\) for the simultaneous estimation of PAR and ALP in combined tablet dosage forms. To the best of our knowledge there is no method developed for the HPLC analysis of PAR and ALP in their combined dosage forms. Hence, in present work an attempt was made to develop a RP-HPLC method for simultaneous estimation of these combined formulations.
MATERIALS AND METHOD

Instrumentation:
Analysis was performed on a chromatographic system of Cyberlab HPLC system accomplished with UV-detector, quantitative HPLC was performed on an isocratic mode using Cap Cell Pack C18 column with 20 μL injection of sample loop. The output signal was monitored and integrated using Cyberlab LC 100 software.

Chemicals and reagents:
The API of PAR was procured from Dr. Reddy’s Laboratory, Hyderabad and ALP was procured from Cipla Laboratory, Malapur. Acetonitrile and water used were of HPLC grade, purchased from Merck Life Sciences Ltd, Mumbai. The drug formulations (STS: 500 mg of PAR and 0.25 mg of ALP) were purchased from local market.

Preparation of mobile phase:
A combination of acetonitrile and water (80:20 % V/V) was prepared, mixed and then degassed in ultra-sonic cleaner for 15 mins. The resultant solution was filtered through 0.45 µ membrane filter. It was used as diluent throughout the preparations of solutions.

Preparation of standard mixture of PAR and ALP:
Transfer 50 mg of PAR and 2.5 mg of ALP into 70 mL of diluent. Resulted solution sonicated for 15 mins and the volume made up to 100 mL with diluent. From the above solution pipette out 10 mL into 100 mL volumetric flask and make up the final volume with the diluent to get final concentrations 50 µg/mL and 2.5 µg/mL, respectively.

Preparation of sample solution:
Twenty tablets were weighed and average weight of each tablet was determined. The tablets were crushed into a fine powder. Accurately weighed and transferred tablet powder equivalent to 50 mg of PAR to a clean 100 mL of volumetric flask. Add 70 mL of diluent to dissolve and made up the volume with 100 mL. From that solution take 10 mL and made up to the mark. The solution was sonicated for 15 mins and filtered through 0.45 µ membrane filter and marked as sample solution.

Optimized chromatographic conditions:
RP-HPLC separation was achieved on Cell Pack C18 column (250 mm × 4.6 mm, 5 μ particle size). Method involves the combination of acetonitrile and water as mobile phase in the ratio of 80:20 %V/V. The elute detection was monitored at 236 nm using UV-detector. The flow rate was at 1.0 mL/min with the sample injection volume of 20 μL (Table 1). The mobile phase was filtered through 0.45 Millipore filter in glass apparatus and degassed by ultra-sonication. The components
were eluted at 4.8 mins for PAR and 6.2 mins for ALP, the chromatogram was showed in Figure 2.

Table 1: Optimized chromatographic conditions

| Parameters          | Chromatographic conditions                             |
|---------------------|--------------------------------------------------------|
| Column              | Cap Cell Pack C18 column (250 x 4.6 mm, 5 μ)           |
| Mobile phase (Ratio)| Acetonitrile: water (80:20 % V/V)                      |
| Elution mode        | Isocratic                                             |
| Flow rate           | 1 mL/min                                               |
| Detection wavelength| 236 nm                                                 |
| Injection volume    | 20 μL                                                  |
| Run time            | 10 min                                                 |
| Column temperature  | 25°C                                                   |

Figure 2: Optimized chromatogram in acetonitrile and water (80:20 % V/V).

RESULTS AND DISCUSSION

Method validation:
The method was validated as per ICH guidelines\textsuperscript{17-19} with respect to system suitability, linearity, accuracy, robustness, limit of detection and limit of quantification.

System Suitability\textsuperscript{20}:
From the standard stock solution a working standard solution of PAR (50 μg/mL) and ALP (0.25 μg/mL) was prepared and injected five times into the HPLC system. The column was equilibrated with the mobile phase for 30 min prior to the injection of the drug solution. The system suitability parameters such as theoretical plate number and tailing factor were found to be 7242, 1.56 for PAR and 6755, 1.15 for ALP.

Linearity:
A series of solutions of standard drug substance were prepared and injected into the HPLC system in the concentration ranging from 50-175 μg/mL for PAR and 0.25-1.5 μg/mL for ALP to demonstrate linearity. A calibration curve was plotted against amount of drug (μg/mL) v/s
chromatogram peak area (mV). Correlation coefficients ($r^2$) were found to be 0.999 & 0.998 for PAR and ALP respectively (Figure 3 & 4). The Linearity data was represented in Table 2.

Table 2: Linearity data of PAR and ALP

| Analyte | Concentration (µg/mL) | Peak area (mV) | Linear regression Equation |
|---------|-----------------------|----------------|---------------------------|
| PAR     | 50                    | 108091.1       | $y=1961x+9226$ $r^2 = 0.999$ |
|         | 75                    | 158168.2       |                           |
|         | 100                   | 202992.6       |                           |
|         | 125                   | 253359.8       |                           |
|         | 150                   | 300806.6       |                           |
|         | 175                   | 355607.7       |                           |
| ALP     | 0.25                  | 6785.9         |                           |
|         | 0.5                   | 12985.7        | $y=23328.6x+939.3$ $r^2 = 0.998$ |
|         | 0.75                  | 18234.5        |                           |
|         | 1                    | 23983.7        |                           |
|         | 1.25                  | 29664.8        |                           |
|         | 1.5                   | 36452.2        |                           |

Figure 3: Calibration curve for PAR (50-175 µg/mL).

Figure 4: Calibration curve for ALP (0.25-1.5 µg/mL).
Accuracy:
The accuracy was carried out by adding known amounts of standard drug to the analyte at three concentrations levels i.e., 50, 100 and 150 % to the target amount. At each level, three determinations were performed and the results were recorded. The accuracy was expressed in terms of percent analyte recovered which was determined by respective chromatograms (Figure 5-7). The method was found to be accurate and the % recovery was found to be 99-101% and 99-102% for PAR and ALP, respectively. The accuracy (% recovery) data was presented in Table 3 & 4.

Table 3: Accuracy data for PAR

| S. No. | Spiked level | Peak area | Peak height | % Recovery | % Mean |
|--------|--------------|-----------|-------------|------------|--------|
| 1      | 50%          | 295162    | 35021       | 100.8      | 100.1  |
|        | 50%          | 293853    | 35683       | 99.8       |        |
|        | 50%          | 293345    | 35942       | 99.8       |        |
| 2      | 100%         | 576369    | 70042       | 100.9      |        |
|        | 100%         | 579209    | 70861       | 101.1      | 100.5  |
|        | 100%         | 570405    | 70932       | 99.5       |        |
| 3      | 150%         | 864543    | 105882      | 99.1       |        |
|        | 150%         | 868347    | 105936      | 99.8       | 99.5   |
|        | 150%         | 869543    | 105856      | 99.7       |        |

Figure 5: Chromatogram of sample with 50% Standard addition.
Figure 6: Chromatogram of sample with 100% Standard addition.

Figure 7: Chromatogram of sample with 150% Standard addition.

Table 4: Accuracy data of ALP

| S. No. | Spiked level | Peak area   | Peak height | % Recovery | % Mean |
|--------|--------------|-------------|-------------|------------|--------|
| 1      | 50%          | 78345.3     | 20101       | 100.5      | 100.4  |
|        | 50%          | 79368.8     | 20612       | 100.5      | 100.4  |
|        | 50%          | 76543.4     | 20794       | 100.4      |        |
| 2      | 100%         | 157348.2    | 26670       | 100.6      | 100.7  |
|        | 100%         | 156832.4    | 26871       | 100.9      |        |
|        | 100%         | 156348.4    | 26538       | 100.8      |        |
| 3      | 150%         | 225036.2    | 32538       | 99.07      |        |
|        | 150%         | 226036.2    | 32901       | 100.6      | 99.9   |
|        | 150%         | 220146.5    | 32799       | 100.2      |        |

Precision:

Precision was determined in terms of repeatability. System precision and method precision was established in accordance with ICH guidelines. The system precision (Table 5) was determined by analyzing the standard solution of PAR and ALP where as the method precision (Table 6) was
determined by analyzing the samples of PAR and ALP. In both the cases the % RSD was found to be <2, which indicates the proposed method was precise.

**Table 5: System precision data of PAR and ALP**

| Injection | Retention time (mins) | Peak area (mV) | Peak height (mV) |
|-----------|-----------------------|----------------|------------------|
|           | PAR                  | ALP            | PAR              | ALP              |
| 1         | 4.80                 | 6.20           | 159899.2         | 15365.3          | 19725           | 1124            |
| 2         | 4.80                 | 6.20           | 158581.7         | 15812.2          | 19736           | 1156            |
| 3         | 4.80                 | 6.20           | 157476.1         | 15925.4          | 19563           | 1148            |
| 4         | 4.80                 | 6.20           | 155500.2         | 15244.8          | 19856           | 1165            |
| 5         | 4.80                 | 6.20           | 158891.6         | 15669.2          | 19256           | 1183            |
| 6         | 4.80                 | 6.20           | 154948.8         | 15753.4          | 19985           | 1179            |
| Mean      | 4.80                 | 6.20           | 157549.6         | 15628.38         | 19686           | 1159            |
| S.D       | -                    | -              | 1765.01          | 266.70           | -               | -               |
| % RSD     | -                    | -              | 1.1              | 1.7              | -               | -               |

**Table 6: Method precision of PAR and ALP**

| Injection | PAR Retention time | Peak area (mV) | ALP Retention time | Peak area (mV) |
|-----------|-------------------|----------------|-------------------|----------------|
|           | 4.80              | 168581.7       | 6.20              | 17509.1        |
| 2         | 4.80              | 163512.6       | 6.20              | 17581.8        |
| 3         | 4.80              | 162918.2       | 6.20              | 17567.5        |
| 4         | 4.80              | 163441.3       | 6.20              | 17881.2        |
| 5         | 4.80              | 164682.5       | 6.20              | 17639.1        |
| 6         | 4.80              | 165910.1       | 6.20              | 17911.7        |
| Mean      | 4.80              | 164841.06      | 6.20              | 17695.06       |
| SD        | -                 | 2125.17        | -                 | 172.12         |
| % RSD     | -                 | 1.2            | -                 | 0.97           |

**Robustness:**

This parameter was carried out to check the ability of proposed method to produce unaffected/unchanged results for deliberate changes in chromatographic conditions. The flow rate (1 ±0.2 mL/min) and detector wavelength (236 ±2 nm) changes were made in the optimized HPLC technique to determine the effect of deliberate variations in the optimized chromatographic parameters. The appropriate data was represented in Table 7 & 8.

**Table 7: Robustness data for PAR**

| Parameter       | Chromatographic conditions | Area 1 | Area 2 | Mean     | SD       | % RSD  |
|-----------------|-----------------------------|--------|--------|----------|----------|--------|
| Flow rate (mL/min) | 0.8 (Low)                  | 181482.2 | 182491.4 | 181986.8 | 713.61   | 0.39   |
|                 | 1.0 (Original)              | 153032.4 | 154216.2 | 153624.3 | 837.35   | 0.54   |
|                 | 1.2 (High)                  | 130605.8 | 131425.4 | 131015.6 | 580.11   | 0.44   |
| Wavelength (nm)  | 234 (Low)                   | 178091.4 | 177812.3 | 177951.8 | 197.31   | 0.11   |
|                 | 236 (Original)              | 155862.2 | 156918.2 | 156390.3 | 746.49   | 0.47   |
|                 | 238 (High)                  | 165935.8 | 166219.1 | 166077.4 | 200.32   | 0.12   |
Table 8: Robustness data for ALP

| Parameter         | Chromatographic conditions | Area 1     | Area 2     | Mean      | SD        | % RSD     |
|-------------------|----------------------------|------------|------------|-----------|-----------|-----------|
| Flow rate (mL/min)| 0.8 (Low)                  | 15653.5    | 15389.2    | 15521.3   | 106.88    | 1.20      |
|                   | 1 (Original)               | 17819.3    | 17624.5    | 17721.9   | 137.85    | 0.77      |
|                   | 1.2 (High)                 | 16623.2    | 16824.6    | 16723.9   | 142.41    | 0.85      |
| Wavelength (nm)   | 234 (Low)                  | 16682.9    | 16931.4    | 16807.1   | 175.53    | 1.04      |
|                   | 236 (Original)             | 17648.4    | 17919.5    | 17783.9   | 192.01    | 1.07      |

Detection Limit (DL) and Quantification Limit (QL):
The parameter DL was determined on the basis of height of the signal and noise of the response (3:1) for PAR and ALP. Similarly the parameter QL was determined on the basis of height of the signal and noise of the response. LOD was found to be 11.2 & 0.018 µg/mL for PAR and ALP respectively. LOQ was found to be 50 & 0.025 µg/mL for PAR and ALP respectively (Table 9). The respective chromatograms for DL and QL was showed in Figure 8 & 9.
Table 9: DL and QL data of PAR and ALP

| LOD (µg/mL) | LOQ (µg/mL) |
|------------|-------------|
| 11.2       | 50          |
| 0.018      | 0.025       |

Assay of formulation (PAR and ALP):

After preparation of appropriate standard and sample mixture solutions, a fixed volume of solution was injected into the HPLC system at optimized chromatographic conditions and the obtained chromatograms were evaluated for parameters determination of assay of PAR and ALP in their tablet formulations. 20 µL of standard and sample solution was injected with six replicates separately into the HPLC system and chromatograms were recorded. The percent drug found to be 100.9 % & 101.1 for PAR and ALP respectively (Table 10). The % RSD found to be within acceptable limits (<2).

Table 10: Assay results for optimized method

| Tablet formulation | Label claim amount (mg) | % Drug found ± SD (n=6) | % RSD |
|--------------------|-------------------------|-------------------------|-------|
| STS: PAR (500 mg)  | PAR- 500 mg             | 100.9                   | 1.1   |
|                    | + ALP (0.25 mg)          | ALP- 0.25 mg            | 101.1 | 1.7   |

CONCLUSION

A simple, accurate, rapid, sensitive and precise RP-HPLC method has been developed for the simultaneous estimation of PAR and ALP in tablet dosage form using UV-detector. A RP Cell Pack C18 column (250 mm × 4.6 mm, 5 µ particle size) with mobile phase consisting of acetonitrile and water in the ratio of 80:20 % v/v was used for separation and at 236 nm. The developed method was found to be satisfactory with good precision, linearity and accuracy. The optimized method was validated according to ICH (Q2R1) guidelines and all the results lie within the specified limits. Hence the proposed new RP-HPLC method was found valid, can be applied for routine analysis of simultaneous estimation of ALP and PAR in their combined pharmaceutical formulations.

ACKNOWLEDGEMENTS

Authors are thankful to the Principal and Management, Vijaya Institute of Pharmaceutical Sciences for Women, Vijayawada for their co-operation and also providing laboratory facilities to carryout research work.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.
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