Method Development, Validation and Stability Indicating Studies for Simultaneous Estimation of Anti-Hypertensive Drugs from Pharmaceutical Formulation by RP-HPLC

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ABSTRACT

Objective: Method development, validation & stability indicating studies for simultaneous estimation of Anti-Hypertensive drugs, Benidipine (BEN) and Metoprolol (MET) from pharmaceutical formulation by RP-HPLC.

Methods: For present work, reverse phase chromatography was selected as its suggested use for ionic and moderate to non-polar compounds. Reverse phase chromatography is simple, suitable, better regarding efficiency, stability, and reproducibility. C18 packed column, a 100 X 2.1 mm, ID column of 5.0 μm particle packing, was selected for separation of BEN and MET. Different solvent systems were tried and optimized in combinations as mobile phase. BEN (4 μg/ml) and MET (50 μg/ml) in 15mM ammonium formate-Methanol (15:85 v/v) was developed as it was showing good peak shapes and a significant amount of resolution. The mobile phase was flowed at 1.2 ml/min with detection of BEN analytes at 236 nm and MET analytes at 225 nm respectively.

Result: Method development was done. Specificity, linearity, accuracy, precision, robustness, limit of detection and limit of quantitation were used to accomplish validation. The method was found linear from 32.5 – 500 μg.mL⁻¹ for both BEN and MET individually. The percentage recovery of BEN when placed for period of 12 hours was found to be 100% in 0.1N/M NaOH at 60°C and Thermal (60°C); 12% degradation in 0.1N/M HCl at 60°C; Oxidation (3-6% H₂O₂) at room temperature whereas for MET was 100% in 0.1N/M NaOH, 0.1N/M HCl at 60°C, at thermal (60°C) as well as oxidation by 3-6% H₂O₂ at room temperature.

Conclusion: Developed analytical method for the simultaneous estimation of Benidipine (BEN) and Metoprolol (MET) in both bulk and tablet formulation has obliged the ICH guidelines including tailing factor (T), separation factors (α), theoretical plates (N), capacity factor (K), resolution (R) and RSD (%). The validated stress degradation studies under thermal, oxidative, alkali and acid ascertained few degradation products for Benidipine whereas the Metoprolol was unaffected with forced degradation studies.

Keywords: Benidipine, Metoprolol, Reverse-Phase High Performance Liquid Chromatography, Stability indicating method.

1. INTRODUCTION

New analytical technologies that are continuously being developed and also been used when it is appropriate to develop stability indicating method. The unknown impurity, which is observed during the analysis, pharmaceutical development, stress studies and formal stability studies of the drug substances and drug product, can be separated and analyzed by using various chromatographic techniques like reversed phase high performance liquid chromatography (RP-HPLC)¹-². Importantly, few publications reported the simultaneous analysis of both Benidipine and Metoprolol on C18 column³.
and has mentioned the details of capacity factor and resolution which specifically have great importance in system suitability as per ICH guidelines. As reported in few articles the Metoprolol was eluted with void volume/solvent front (ts) which is strictly not acceptable by ICH guidelines. In addition, the sensitivity of both Metoprolol and Benidipine were found negligible in UV detection. Considering it, attempt has been made to develop new, accurate, precise and robust reverse phase high performance liquid chromatographic (RP-HPLC) method has been successfully developed for the simultaneous estimation of both antihypertensives drugs Benidipine\(^{4,5}\) (3R)-1-Benzyl-3-piperidinyl methyl (4R)-2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydro-3,5-pyridinedicarboxylate (BEN, Fig. 1) and Metoprolol\(^{4,5}\) 1-(Isopropylamino)-3-(4-(2-methoxyethyl)phenox) propan-2-ol (MET, Fig. 2) in both standard and tablet formulation along with stability indicating studies or force degradation studies in 0.1 N HCl, 0.1N NaOH, 3% H\(_2\)O\(_2\), and thermal degradation at 50°C temperature.

A stability indicating method\(^{10-15}\) (SIM) is an analytical procedure used to quantitate the decrease in the amount of the active pharmaceutical ingredient (API) in drug product due to degradation. SIM measures the changes in active ingredients concentration without interference from other degradation products, impurities and excipients. Stress testing is carried out to demonstrate specificity of the developed method to measure the changes in concentration of drug substance when little information is available about potential degradation product. The addition of this analytical methods in the current practice would help the pharmaceutical industries in large to preserve the excellence of their products containing these active ingredients and also the enforcement agencies in general to monitor the quality of the marketed products.

Figure 1: Molecular structure of Benidipine
Figure 2: Molecular structure of Metoprolol

2. MATERIALS AND METHODS

Reagents and chemicals: Standard of Metoprolol and Benidipine were obtained from Intas Pharmaceuticals Pvt. Ltd., Ahmadabad. Benitowa® Beta (Akumentis Healthcare Ltd) tablets were purchased from medical store. BEN 4 mg and MET 25 mg were used. All chemicals and reagents used were a HPLC grade and purchased from Merck specialties Pvt., Ltd., Mumbai.

Benidipine (BEN) standard stock solution (40 μg/ml)
A sample of 40 μg of BEN was weighed and transferred to a 100 ml volumetric flask. Volume was made up to the mark with methanol-water (2:1 v/v). Take 10 ml from this solution, and transfer to 100 ml volumetric flask and volume was made up with methanol-water (2:1 v/v).

Metoprolol (MET) standard stock solution (500 μg/ml)
A sample of 50 μg of MET was weighed and transferred to a 100 ml volumetric flask. Volume was made up to the mark with methanol-water (2:1 v/v).

Preparation of standard solution of binary mixtures of BED (4 μg/ml) and MET (50 μg/ml)
Take 1 ml from the BEN stock solution and 1 ml from MET stock solution and transferred to 10 ml volumetric flask and volume made up to the mark by mobile phase which was used in trials.

Preparation of Sample Stock Solution (BEN 40 μg/ml, MET 500 μg/ml)
Exactly 10 tablets of Benitowa®Beta, were separately weighed, powdered and mixed in a mortar. An accurately weighed amount of the finely powdered Benitowa® Beta 4mg/50mg; Akumentis Healthcare Ltd tablets; equivalent to 4 mg of BEN and 50 mg of MET were separately made up to 100 ml with methanol and sonicated until they dissolved and make up volume with Mobile phase. The solution was filtered through Whatman filter paper no. 42.

Method Validation\(^{16-24}\)

Linearity/Calibration studies
Accurately measured aliquots of stock solutions equivalent to 32.15-500 μg, of BEN and MET, respectively were transferred separately into a series of 10 ml volumetric flasks. The final volume was adjusted with same mobile phase, and then 20 μL were injected into HPLC. A calibration curve (linearity graph) was plotted by calculating peak area against concentration.

Precision of the proposed method
Three similar concentrations of the mixture of BEN and MET (500, 250, 125 μg.L\(^{-1}\)) were analyzed three times, within the same day (intraday precision), using the procedure mentioned under (5.7.1). Also, the mentioned concentrations were analyzed on three successive days using the same procedure to determine the intermediate precision.

Robustness
Robustness was attempted by deliberately changing the chromatographic conditions to evaluate the difference in resolution, capacity factor, peak height and peak width (tailing factor). The flow rate of the mobile phase was changed by ±2 decimal; like 1.2 mL.min\(^{-1}\) was changed to 1.4 mL.min\(^{-1}\) and 1 mL.min\(^{-1}\) to evaluate the effect of the
flow rate; similarly the variation of organic modifier as Acetonitrile/methanol was changed by ±2% to 71% and 73% to monitor the peak area and retention time. Finally, the effect of wavelength was monitored by making deliberate variation 223 to 225 nm and the differences in system suitability parameters such as peak tailing, capacity factor, resolution and theoretical plates were evaluated.

**Forced degradation studies**

**Acid degradation**

Acid decomposition studies were performed by transferring 1 ml of stock solution in to 10 ml of volumetric flask. A volume of 2 ml of 0.1 N/M HCl solutions was added and mixed well and put for 12 hours at 60°C. After time period, the volume was adjusted with diluent to get 4 μg/ml for BEN and 50 μg/ml for MET.

**Base degradation**

Basic decomposition studies were performed by transferring 1ml of stock solution in to 10 ml of volumetric flask. A volume of 2 ml of 0.1 N/M NaOH solutions was added and mixed well and put for 12 hours at 60°C. After time period, the volume was adjusted with diluents to get 4 μg/ml for BEN and 50 μg/ml for MET.

**Oxidative degradation**

Oxidation decomposition studies were performed by transferring 1 ml of stock solution in to 10 ml of volumetric flask. A volume of 2 ml of 3 – 6 % H₂O₂ solutions were added and mixed well and put for 12 hours at room temperature. After time period, the volume was adjusted with diluents to get 4 μg/ml for BEN and 50 μg/ml for MET.

**Thermal degradation**

Thermal degradation studies were performed by transferring 1 ml of stock solution in to 10 ml of volumetric flask. The volumetric flask was stored in oven at 60˚C for 12 hours. Then, the volume was adjusted with diluents to get 4 μg/ml for BEN and 50 μg/ml for MET.

### 3. RESULTS AND DISCUSSION

**Selection of wavelength**

Standard solution of BEN (4μg/ml) and standard solution of MET (50μg/ml) were scanned between 200nm and 400nm using UV-visible spectrophotometer. Wavelength was selected from the overlay spectra of above solutions. UV detection was specifically carried out at 225nm for both selected BEN and MET as both compounds exhibit optimum absorption and showed good response at 225 nm. The flow rate was adjusted to 1.2 mL/min⁻¹ to achieve better resolution, and peak symmetry.

**Chromatographic Parameters**

Various chromatographic parameters are as follows,

1. Analytes: Benidepine (250ppm) + Metoprolol (500ppm)
2. Column: UltraSil-MCX; 5μ, 100 X 2.1mm. ID.
3. Mobile Phase: 15mM ammonium formate-Methanol (15:85 v/v)
4. Flow rate: 1.2mLmin⁻¹
5. Elution mode: Isocratic elution mode
6. Wavelength selected: 225nm
7. Temperature: Room temperature
8. Run time: 12 minutes
9. Retention time: Benidepine (1.22 min), Metoprolol (4.36 min)

**System suitability tests for BEN and MET**

System suitability test reveals the factors such as, theoretical plate (N), capacity factor (K’), resolution (R), separation factor (α), tailing factor (T). Mean±SD and RSD% and found to be in acceptable range for at least 6 successive injections of same analytes, as shown in Fig. 3 and Fig. 4. Table 1, represents the system suitability for BEN and MET.

| System suitability parameters | Benidepine (BEN) | Metoprolol (MET) | Acceptable Values |
|------------------------------|------------------|------------------|------------------|
| Theoretical plates (N)       | 189              | 709              | > 2000           |
| Capacity Factor (K’)         | 3.786            | 4.563            | > 1.5 - <10      |
| Resolution (K)               | --               | 6.26             | ≥ 2              |
| Selectivity/Separation factor (α) | 0.00           | 1.205            | > K’             |
| Asymmetry/Tailing factor (T) | 1.8              | 1.8              | ≥ 2              |
| Retention time (tᵣ)          | 1.19 min.        | 4.32 min.        | > K’             |
| Wavelength of Detection (nm) | 236 nm           | 225 nm           | > 200 nm         |
| Repeatability (%RSD)         | 1.88             | 1.65             | < 2              |
| Intra-Day Precision (%RSD)   | 1.12 – 2.15      | 0.25 – 1.78      | < 2              |
| Inter-Day Precision (%RSD)   | 0.82 – 2.04      | 0.25 – 1.12      | < 2              |
| Linearity range              | 32.5 – 500 µg.mL⁻¹ | 32.5 – 500 µg.mL⁻¹ | NA               |
| Regression equation          | Y= 16744x - 83701 | Y= 17805x + 102266 | NA               |
| SE of intercept (Sₑ)         | 111428.4996      | 79653.06         | NA               |
| SD of intercept (S₀)         | 249161.6997      | 178109.67        | NA               |
| Correlation Coefficient (r²) | 0.998            | 0.9991           | NA               |
| LOQ (µg.mL⁻¹)                | 49.10 µg.mL⁻¹    | 32.86 µg.mL⁻¹    | NA               |
| LOD (µg.mL⁻¹)                | 148.80 µg.mL⁻¹   | 99.58 µg.mL⁻¹    | NA               |
Repeatability
Implementing the procedure mentioned, the homologous mixture of both BEN and MET of same concentrations (500μg.mL$^{-1}$), were tested for six injections within the same day. The % RSD was calculated and found it is less than 2% shown in (Table 2).

| Sr. No. | Benidepine | Metoprolol |
|---------|------------|------------|
|         | Peak Area; Conc. 250 ppm | Peak Area; Conc. 250 ppm |
| 1       | 12415863   | 7807488    |
| 2       | 12463050   | 7820081    |
| 3       | 12679087   | 7881049    |
| 4       | 12669900   | 8012439    |
| 5       | 12064694   | 7631657    |
| 6       | 12635073   | 7929808    |

|         | Mean       | STD. DEV.  | RSD (%) |
|---------|------------|------------|---------|
|         | 12487944   | 235051.70  | 1.88    |
|         | 7847087    | 129649.49  | 1.65    |
Intraday Precision:
Implementing the procedure mentioned, the homologous mixture of both BEN and MET of three replicates of three different concentrations; 500 ppm, 250 ppm and 125 ppm were tested and evaluated within the same day (intra-day precision). The %RSD was calculated and found less than 2%; shown in Table 3 and Table 4.

Table 3: Intraday Precision data of BEN

| Sr. No. | Concentration (ppm) | Area   | Mean ± SD | %RSD |
|---------|---------------------|--------|-----------|------|
| 1       | 250 ppm             | 12415863 | 140348    | 1.12 |
|         | 250 ppm             | 12462050 |           |      |
|         | 250 ppm             | 12679087 |           |      |
| 2       | 250 ppm             | 12669000 | 224568.26 | 1.79 |
|         | 250 ppm             | 12264694 |           |      |
|         | 250 ppm             | 12635073 |           |      |
| 3       | 250 ppm             | 12249900 |           |      |
|         | 250 ppm             | 12124694 | 265995.45 | 2.15 |
|         | 250 ppm             | 12315073 |           |      |

Range of %RSD 1.12 - 2.15

Table 4: Intraday Precision data of MET

| Sr. No. | Concentration (ppm) | Area   | Mean ± SD | % RSD |
|---------|---------------------|--------|-----------|-------|
| 1       | 250 ppm             | 7807488 | 19921.100 | 0.25  |
|         | 250 ppm             | 7820081 |           |      |
|         | 250 ppm             | 7781049 |           |      |
| 2       | 250 ppm             | 8012439 | 47182.32  | 0.59  |
|         | 250 ppm             | 7531657 |           |      |
|         | 250 ppm             | 7929808 |           |      |
| 3       | 250 ppm             | 7881149 | 140492.86 | 1.78  |
|         | 250 ppm             | 8012439 |           |      |
|         | 250 ppm             | 7731650 |           |      |

Mean % RSD 0.25 - 1.78

Interday (intermediate) precision:
Implementing the procedure mentioned, the homologous mixture of both BEN and MET of three replicates of three different concentrations; 500 ppm, 250 ppm and 125 ppm were tested and evaluated in three successive days (interday/intermediate precision). The %RSD was calculated and found less than 2%; shown in Table 5 and Table 6.

Table 5: Interday (intermediate) Precision data of BEN

| Sr. No. | Concentration (ppm) | Area   | Mean ± SD | % RSD |
|---------|---------------------|--------|-----------|------|
| DAY 1   | 250 ppm             | 12615866 | 140766.44 | 1.12 |
|         | 250 ppm             | 12403058 |           |      |
|         | 250 ppm             | 12669087 |           |      |
| DAY 2   | 250 ppm             | 12219900 | 99276.58  | 0.82 |
|         | 250 ppm             | 12064694 |           |      |
|         | 250 ppm             | 12035055 |           |      |
| DAY 3   | 250 ppm             | 12269200 | 252006.03 | 2.04 |
|         | 250 ppm             | 12111691 |           |      |
|         | 250 ppm             | 12605071 |           |      |

Range of % RSD 0.82 - 2.04
Table 6: Interday (intermediate) Precision data of MET

| Sr. No. | Concentration (ppm) | Area     | Mean ± SD | % RSD |
|---------|---------------------|----------|-----------|-------|
|         | 250 ppm             | 7807480  | 19921.10  | 0.25  |
|         | 250 ppm             | 7820089  |           |       |
|         | 250 ppm             | 7781040  |           |       |
| DAY 1   | 250 ppm             |          |           |       |
|         | 250 ppm             | 8012439  | 54114.74  | 0.67  |
|         | 250 ppm             | 7929724  |           |       |
|         | 250 ppm             | 8012439  |           |       |
| DAY 2   | 250 ppm             |          |           |       |
|         | 250 ppm             | 7834451  | 89067.80  | 1.12  |
|         | 250 ppm             | 8012439  |           |       |
|         | 250 ppm             |          |           |       |
| DAY 3   | 250 ppm             | 7834451  | 89067.80  | 1.12  |
|         | 250 ppm             | 8012439  |           |       |
|         | 250 ppm             |          |           |       |

Range of % RSD 0.25 – 1.12

**Linearity**

Under linearity or calibration studies, a linear relationship between area under peak values and selected drug concentration (µg.mL.min⁻¹) was plotted for five-six chosen concentrations of Benidipine (shown in Fig.5) and (shown in Fig.6). The regression equations, correlation coefficient values (r), standard error of intercept (S_e), standard deviation of intercept (S_a), limit of detection (LOD) and limit of quantification (LOQ) have been calculated. The linearity of the calibration curves was validated by the high value of correlation coefficient, acceptable values of regression coefficient, standard deviation of the slope and standard deviation of the intercept; shown in (Table 7 and Table 8).

![Calibration curve of Benidipine](image1)

*Figure 5: Calibration curve of Benidipine*

![Linearity data of Metoprolol](image2)

*Figure 6: Calibration curve of Metoprolol*
### Table 7: Linearity data of Benidipine

| Sr. No. | Concentration (µg.mL⁻¹) | Area     | Average (Mean) |
|---------|--------------------------|----------|----------------|
| 1       | 250 PPM                  | 12415863 |                |
|         | 250 PPM                  | 12463050 | 12439456       |
| 2       | 125 PPM                  | 6227728  |                |
|         | 125 PPM                  | 6234428  | 6231078        |
| 3       | 62.5 PPM                 | 3119214  |                |
|         | 62.5 PPM                 | 3100114  | 3109664        |
| 4       | 31.25 PPM                | 1552933  |                |
|         | 31.25 PPM                | 1553931  | 1553432        |
| 5       | 15.62 PPM                | 776466   |                |
|         | 15.62 PPM                | 773221   | 774843         |

**Regression Equation**: Y = 49834x + 21482

**Correlation coefficient (R²)**: 0.999

**Std. Error of intercept**: 12200.22

**Std. Dev. of intercept**: 27280.52

**LOQ**: 1.80 ng.ml⁻¹

**LOD**: 5.47 µg.ml⁻¹

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### Table 8: Linearity data of Metoprolol

| Sr. No. | Concentration (µg.mL⁻¹) | Area     | Average (Mean) |
|---------|--------------------------|----------|----------------|
| 1       | 250 PPM                  | 7807488  |                |
|         | 250 PPM                  | 7820081  | 7813784        |
| 2       | 125 PPM                  | 3906802  |                |
|         | 125 PPM                  | 3906802  | 3906802        |
| 3       | 62.5 PPM                 | 1953477  |                |
|         | 62.5 PPM                 | 1953477  | 1953477        |
| 4       | 31.25 PPM                | 976724   |                |
|         | 31.25 PPM                | 976724   | 976724         |
| 5       | 15.62 PPM                | 488361   |                |
|         | 15.62 PPM                | 488361   | 488361         |

**Regression Equation**: Y = 31328x – 7741.1

**Correlation coefficient (R²)**: 1

**Std. Error of intercept**: 79653.06

**Std. Dev. of intercept**: 178109.67

**LOD**: 14.28 ng.ml⁻¹

**LOQ**: g.ml⁻¹
Limit of detection (LOD/LOQ)

Limit of detection represents the concentration of analyte at S/N ratio of 3.3 and limit of quantification (LOQ) at which S/N is 10 were determined and results are given in Table 7 and Table 8. Low values of LOD and LOQ indicate sensitivity of the applied method for determination of mentioned drugs in tablets.

Robustness for the chromatographic method

The flow rate of the mobile phase was changed from 1 mL.min\(^{-1}\) to 1.4 mL.min\(^{-1}\); results was shown in Fig. 7 and Fig. 8 as well as in Table 9 and Table 10.

Similarly, the effect of deliberate changes in organic modifier (Methanol) composition was evaluated. In this study, the percentage composition of methanol was altered by ±2% (shown in Fig. 9 and Fig. 10) in the previous set of gradients to evaluate the effects on the separation behavior of BEN and MET. Finally, the wavelength (shown in Fig. 11 and Fig. 12) was changed by ±2 nm wavelength and results were reported in Table 9 and Table 10.

From all above studies, after making deliberated changes in flow rate (± 0.2 mL.min\(^{-1}\)), organic modifier concentration; methanol (±2%) and wavelength (±2nm) have not made any significant changes in resolution, capacity factor and tailing factor. Nonetheless, it seems minute changes in robustness studies makes significant changes in theoretical plate counts. Robustness studies for BEN and MET displayed in Table 9 and Table 10.
Figure 9: Robustness studies for BEN (1.14 min) and MET (4.42 min) at Methanol 73%

Figure 10: Robustness studies for BEN (1.33 min) and MET (4.21 min) at methanol 71%

Figure 11: Robustness studies for BEN (1.19 min) and MET (4.32 min) at wavelength 223nm
Stability indicating method

Stability of both drugs are studied utilizing different parameter. In this study, the area of standard for stability and degradation of sample and standard were compare. Result shows BEN has highest degradation in oxidation and acid as compare to others. MET did not showed degradation in oxidation, acid and basic environment. The standard area of BEN and MET as well as peaks of all parameters were given in Fig 13-16. The percent degradation of all parameters is given below in Tables 11 and 12.
Figure 14: Force degradation data of BEN and MET at 0.1N HCl at 60°C

Figure 15: Force degradation data of BEN and MET at 0.1 N NaOH at 50°C.

Figure 16: Force degradation data of BEN and MET at 3% H2O2 at room temperature
Table 11: Stability indicating studies of BEN

| Conditions                  | Benidipine | Degradants of BEN |
|-----------------------------|------------|-------------------|
| Acid (0.1N/M HCl) + 60°C + 12 Hrs. | 88% | 12% |
| Base (0.1N/M NaOH) + 60°C + 12 Hrs. | 100% | 0% |
| Thermal (60°C) + 12 Hrs. | 100% | 0% |
| Oxidation (3-6% H2O2) + Room Temp. | 47.44% | 52.56% |

Table 12: Stability indicating studies of MET

| Conditions                  | Metoprolol | Degradants of MET |
|-----------------------------|------------|-------------------|
| Acid (0.1N/M HCl) + 60°C + 12 Hrs. | 100% | 0% |
| Base (0.1N/M NaOH) + 60°C + 12 Hrs. | 100% | 0% |
| Thermal (60°C) + 12 Hrs. | 100% | 0% |
| Oxidation (3-6% H2O2) + Room Temp. | 100% | 0% |

4. CONCLUSION

From results and discussion, it has been concluded that the developed analytical method for the simultaneous estimation of benidipine (BED) and metoprolol (MET) in both bulk and tablet formulation has obliged the ICH guidelines. As per the ICH guidelines, the developed method has complied the linearity range (calibration data), drug recovery studies (%), repeatability, precision studies (intraday and interday/intermediate), and robustness. Moreover, as per the ICH guidelines, the system suitability test performed for simultaneous estimation of benidipine and metoprolol have achieved all guidelines; including, tailing factor (T), separation factors (a), theoretical plates (N), capacity factor (k′), resolution (R) and RSD (%). The validated stress degradation studies under thermal, oxidative, alkali and acid ascertained few degradation products for benidipine whereas the metoprolol was unaffected with forced degradation studies. Hence, this developed and validated method for simultaneous investigation by reverse phase high performance liquid chromatography can be used for routine analysis of estimation of both or either benidipine and metoprolol from marketed formulation.

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