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# DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF ANAGLIPTIN AND METFORMIN HYDROCHLORIDE DRUG IN TABLET DOSAGE FORM

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## ABSTRACT

A simple, rapid, sensitive & selective stability indicating RP-HPLC method is developed for the determination of anagliptin and Metformin HCL in tablet dosage form. A RP-HPLC analysis was performed on KROMASIL $C_{18}$ column and its size 250×4.6mm, 5µm with using mobile phase methanol and 0.05M potassium dihydrogen phosphate with buffer pH4 in ratio (30:70) at 220nm detection wavelength. The gradient was optimized with flow rate 1 mL/min. the completed analytical method validation was successfully carried out as per ICH guidelines. The recovery study was carried out at 80% ,100% and 120% level of working concentration and result were in the range 100.1-101.2% for Metformin HCL and 100.2-100.9% for Anagliptin. The linearity was proven in concentration range 5-15µg/mL for anagliptin and 25-75µg/mL for Metformin HCL. The limit of detection (LOD) was found to be 0.069µg/mL for anagliptin & 1.56µg/mL for metformin HCL. The LOQ value was found to be 0.209µg/mL for anagliptin & 4.716µg/mL for metformin HCL. All validation parameters was in accepted range as per ICH guideline. furthermore, forced degradation study was also performed at various stress condition such as acidic, basic, oxidative and photolytic as per protocol of ICH. The developed method can be successfully used for the estimation of Anagliptin & Metformin HCL in tablet dosage form.

**Keywords: Anagliptin, Metformin HCL, stability indicating RP-HPLC method, validation**

## INTRODUCTION [1]

Antidiabetic drugs used to treat diabetes mellitus by lowering the glucose level in the blood. The type 2 diabetes mellitus which is characterized by polyphagia, polyuria, and polydipsia and need a lifetime treatment with antidiabetic drugs. Anagliptin (ANA) chemically N-[2-[[2-[(2S)-2-Cyanopyrrolodin-1-yl]-2-oxoethyl]amino]-2-methylpropyl-2-methylpyrazolo [1,5-a] pyrimidine-6-carboxamide 9 **(Figure 1)** is a Dipeptidyl peptidase 4 (DPP 4) inhibitor which is used in treatment of type 2 NIDDM **[2]**. Dipeptidyl peptidase 4 enzyme breaks down the incretions GLP-1 gastrointestinal hormone released in response to a meal by preventing GLP-1 inactivation they are able to increase the cell secretion determines glucagon release from neighbouring alpha cell homeostasis of blood glucose is maintained by hormone secretion from the pancreas. Glucose stimulates insulin secretion from beta cell but suppresses the release of glucagon hormone that raises blood glucose, from alpha-cells. This drives blood glucose levels towards level **[3, 4]**. This drug is not official in any of the pharmacopoeia.

Metformin HCL (MET), chemically 3-(diaminomethylidene)-1, 1-dimethylguanidine **(Figure 2)**. Metformin HCL is biguanide class of Antidiabetic drugs. That has been control blood glucose level of T2D patient for decrease and has been considered the first line treatment according to international guidelines. It is suppressing hepatic glucogenasis and glucose output from liver. It is official in Indian pharmacopoeia, British pharmacopoeia, European pharmacopoeia and united state pharmacopoeia.

Literature survey revealed that these antidiabetic drugs have been analyzed individually or in combination by many analytical methods like RP-HPLC, HPTLC and spectroscopic methods but no method has been reported for simultaneous estimation of Anagliptin & Metformin HCL for stability indicating RP-HPLC method in tablet dosage form.

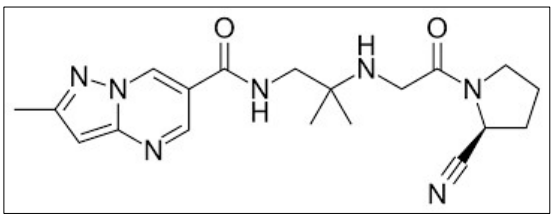


**Figure 1: Anagliptin**

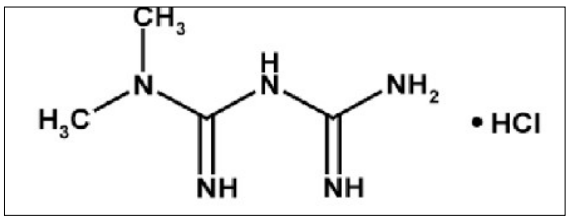


**Figure 2: Metformin HCL**

## MATERIAL AND METHOD

The API of anagliptin was obtained as gift sample from Intas Pharmaceutical Pvt LTD., Ahmedabad. Metformin HCL API was obtained as gift sample from nucleas lab, chtral. All solvent like HPLC grade water, methanol, ACN, were obtained from Merck specialties Pvt. Ltd. Mumbai, India.

**Selection and Detecction of Wavelength**

The selectivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. An ideal wavelength is the one that gives good response for the selected drug that is to be detected not interference of solvent effect. It gives maximum absorbance at 220nm in acetonitrile at 220nm drugs gives good peak point and not interference of solvent effect so; this wavelength was selected for estimation of anagliptin and Metformin HCL **(Figure 3)**.

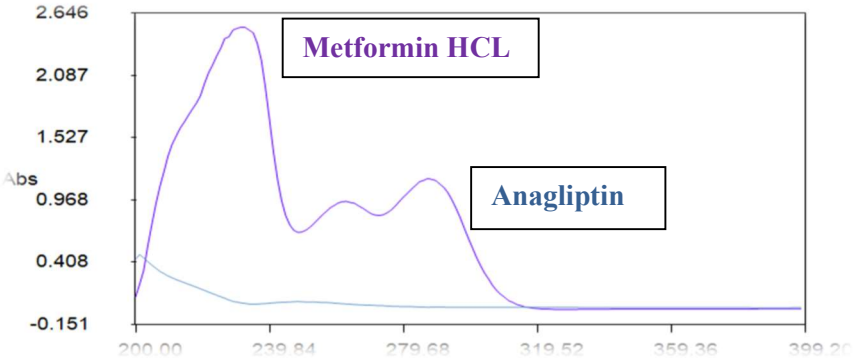


**Figure 3: Selection of analytical wavelength**

**Selection of mobile phase**

The potassium dihydrogen phosphate was prepared by dissolving accurately weight 6.8gm of potassium dihydrogen phosphate in 1000ml HPLC grade water in a 1L volumetric flask and pH was adjusted to pH 4 with ortho phosphoric acid. The prepared buffer pH was checked by using pH meter by ultra sonicating for 5 min solution degassed and obtained solution was filtered through 0.45µ millipore filter and mobile phase is prepared with ratio of buffer (pH 4): methanol (70:30 v/v) **(Figure 4)**.

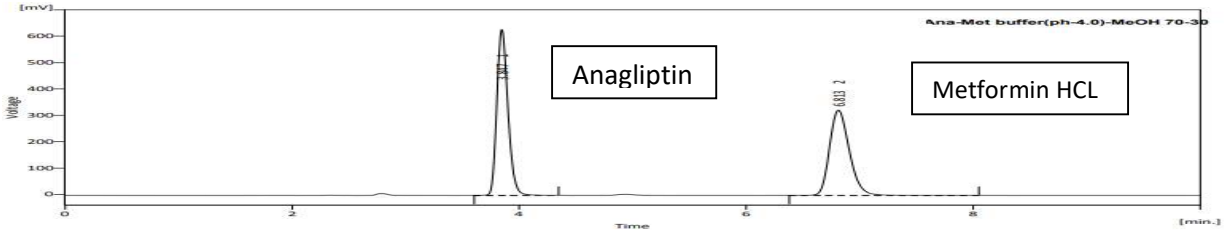


**Figure 4: Buffer pH 4: Methanol (70:30V/V)**

**Standard solution preparation**

**Preparation of standard stock and working standard stock solution of Anagliptin**

Weigh accurately 10mg of Anagliptin transfer 10ml volumetric flask dissolved in acetonitrile and it make up to mark to get 1000µg/ml(1000ppm) of Anagliptin standard solution. Pipette out 1ml of above solution transfer 10ml volumetric flask make up to mark with ACN to get 100µg/ml (100ppm).

**Preparation of standard and working standard stock solution of Metformin HCL**

Weighing accurately 10mg of Metformin HCL in 10ml volumetric flask make up to the mark with Acetonirtil and sonicated for 10 min. to get 1000µg/ml (1000ppm) from this 1000µg/ml solution Pipette out 5ml from above solution transfer into 10ml of volumetric flask and make up to with solvent to get 500µg/ml (500ppm).

**Method validation**

The purposed method was validated as per ICH guidelines Q2 (R1) **[8]**

The method was validated by the different validation parameters such as accuracy, precision, linearity, LOD, LOQ, robustness, rugudness.

**Linearity and Range**

Linearity is expressed in term of correlation co-efficient of linearity regression analysis. Calibration curve from working solution containing 500µg/mL MET and 100µg/mL ANA aliquotos 0.5, 0.75, 1, 1.25, 1.5mL were transferred in clean and dry 10mL vol. flask respectively and sonicated. The volume was made up to the mark with diluents. This yielded solution of 25, 37.5, 50, 62.5,75 µg/mL of MET and 5, 7.5, 10, 12.5,15 µg/mL of ANA respectively. an aliquot(20 µl) of each solution was injected under the operating chromatographic condition as described earlier calibration curve was prepared by plotting peak areas versus concentration and the regression equation was calculated each response was average of three determination **Figure 5** and **Figure 6** Result for linearity are shown **Table 2**.

**Precision**

**Repeatability**

The repeatability data of peak area measurement for Metformin HCL and Anagliptin based on six measurements of same solution. The mean area observed 4275.960 for Metformin HCL and 3930.223 for anagliptin with %RSD 0.56 for both. The repeatability shows that the % RSD values observed within the acceptance limit of NMT 2% results shown in **Table 3**.

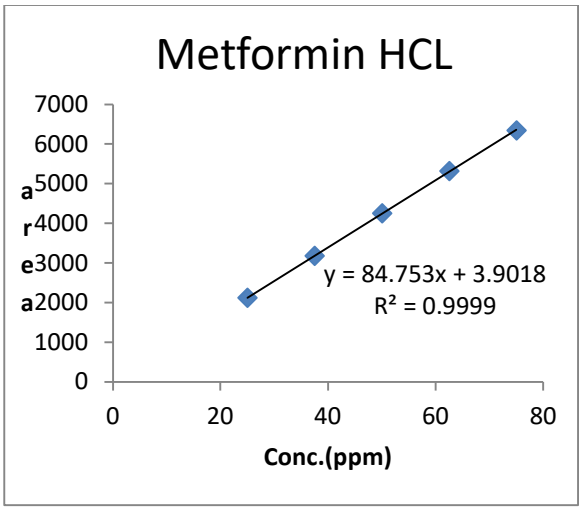


Figure 5: Calibration curve of Metformin HCL

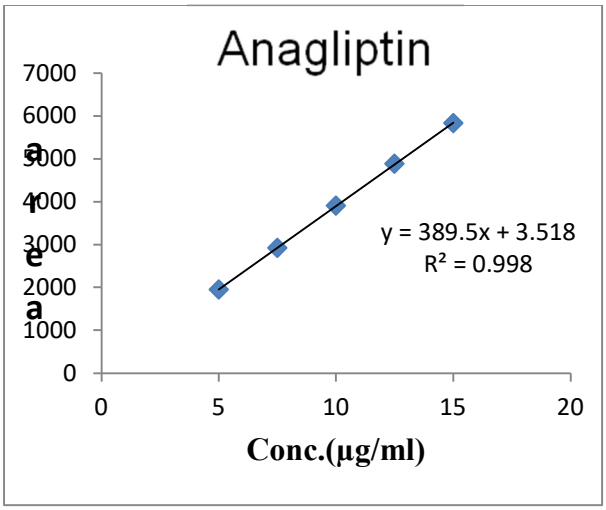


Figure 6: Calibration curve of Anagliptin

Table 2: Linearity Data for Anagliptin and Metformin HCL

| S. No. | Linearity Level | Concentration (µg/ml) | | Area ± SD(n=6) | |
|---|---|---|---|---|---|
| | | Metformin HCL | Anagliptin | Metformin HCL | Anagliptin |
| 1 | 50% | 25 | 5 | 2119.417±6.651 | 1948.216±1.10 |
| 2 | 75% | 37.5 | 7.5 | 3176.159±13.28 | 2919.663±3.54 |
| 3 | 100% | 50 | 10 | 4249.030±19.33 | 3905.805±7.22 |
| 4 | 125% | 62.5 | 12.5 | 5317.227±21.60 | 4887.495±9.84 |
| 5 | 150% | 75 | 15 | 6345.952±28.81 | 5833.596±16.15 |

Table 3: Repeatability Data for Anagliptin and Metformin HCL

| Metformin HCL | | | | | Anagliptin | | | | |
|---|---|---|---|---|---|---|---|---|---|
| S No. | Conc. (µg/ml) | Area | Mean ± S. D (n=6) | % RSD | Sr no. | Conc. (µg/ml) | Area | Mean ± S. D (n=6) | % RSD |
| 1 | 50 | 4231.529 | 4275.960±24.098 | 0.564 | 1 | 10 | 3889.629 | 3930.223 ±22.069 | 0.562 |
| 2 | | 4280.747 | | | 2 | | 3934.434 | | |
| 3 | | 4293.061 | | | 3 | | 3946.101 | | |
| 4 | | 4300.181 | | | 4 | | 3952.473 | | |
| 5 | | 4278.728 | | | 5 | | 3932.578 | | |
| 6 | | 4271.513 | | | | | 3926.123 | | |

**Interday and intraday precision**

The %RSD for Intra-day was found to be 0.170-0.595% for MET and 0.167-0.593% for ANA. The %RSD for Inter-day was found to be 0.450-0.801% for MET and 0.448-0.798% for ANA. The precision shows that the %RSD values observed within the acceptance limit of NMT 2.0% **(Table 4 and 5)**.

**Accuracy**

Accuracy was determined by calculating recovery of ANA and MET by the standard addition method known amounts of standard solution to pre-analyzed samples. Each solution was injected in triplet and recoveries were in between 100.1-101.2% for MET and 100.2-100.9% for ANA which is in accorandance with ICH guideline which prove method to be accurate **(Table 6)**.

**Table 4: Intraday precision data for Estimation of Anagliptin and Metformin HCL**

| S. No. | Metformin HCL | | | Anagliptin | | |
|---|---|---|---|---|---|---|
| | Conc. (µg/ml) | Area Mean ± S.D. (n=3) | % R.S.D | Conc. (µg/ml) | Area Mean ± S.D. (n=3) | % R.S.D |
| 1 | 25 | 2130.244 ± 3.630 | 0.170 | 5 | 1957.936 ± 3.270 | 0.167 |
| 2 | 50 | 4262.763 ± 25.35 | 0.595 | 10 | 3918.571 ± 23.24 | 0.593 |
| 3 | 75 | 6311.503 ±31.032 | 0.492 | 15 | 5802.795 ± 28.28 | 0.487 |

**Table 5: Interday precision data for Estimation of Anagliptin and Metformin HCL**

| S. No. | Metformin HCL | | | Anagliptin | | |
|---|---|---|---|---|---|---|
| | Conc. (µg/ml) | Area Mean ± S.D. (n=3) | % R.S.D | Conc. (µg/ml) | Area Mean ± S.D. (n=3) | % R.S.D |
| 1 | 25 | 2146.334 ± 9.65 | 0.450 | 5 | 1972.529 ± 8.84 | 0.448 |
| 2 | 50 | 4309.597 ± 34.53 | 0.801 | 10 | 3960.859 ± 31.16 | 0.798 |
| 3 | 75 | 6427.345 ± 41.06 | 0.639 | 15 | 5907.855 ± 37.44 | 0.634 |

**Table 6: Accuracy data of ANA and MET**

| Spike level | Amount of test solution (µg/ml) | | Amount of std.add (µg/ml) | | Amount recovered ±SD | | % recovered | | % RSD | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MET | ANA | MET | ANA | MET | ANA | MET | ANA | MET | ANA |
| 80% | 50 | 10 | 40 | 8 | 40.27 | 8.01 | 100.1 | 100.2 | 0.435 | 0.425 |
| 100% | 50 | 10 | 50 | 10 | 50.35 | 10.09 | 101.2 | 100.9 | 0.391 | 0.385 |
| 120% | 50 | 10 | 60 | 12 | 60.46 | 12.09 | 100.8 | 100.4 | 0.626 | 0.614 |

***Average of three determination**

## LOD and LOQ

The limit of Detection (LOD) and limit of quantitation (LOQ) were obtained by calculating using the standard formula as per the ICH guidelines, LOD=3.3(σ/S), LOQ=10(σ/S) Where σ is Standard deviation of the response and S is slope of the calibration curve. The LOD and LOQ for the drugs were estimated using the linearity data **(Figure 5 and 6)** repeated calibration curve 5 time and calculated deviation of the intercepts. The LOD for ANA was observed 0.069µg/mL and for MET 1.56µg/mL The LOQ for ANA was observed 0.209 µg/mL and for MET 4.716µg/mL the results were show in **Table 7**.

**Table 7: LOD and LOQ data of ANA and MET**

| LOD | | LOQ | |
|---|---|---|---|
| METFORMIN HCL | ANAGLIPTIN | METFORMIN HCL | ANAGLIPTIN |
| 1.56 µg/mL | 0.069 µg/mL | 4.716 µg/mL | 0.209 µg/mL |

## Robustness

The robustness study was performed to evaluate the influence of small but deliberate variation in the chromatographic condition. The robustness was checked by changing three small changes.

- Change flow rate by 10%. (i.e. 0.8ml/min and 1.2 ml/min)
- Change in mobile phase by 0.2 unit
- Change in pH by 0.2unit (i.e. pH 4.2. and pH 3.8)

After each sample solution was injected and peak area, tailing factor and retention time

were checked. The results are shown in the **Table 8**. Variation seen was within the acceptable range respect to peak asymmetry and theoretical plates, so the method was found to be robust.

**Table 8: Robust data for Anagliptin and Metformin HCL**

| METFORMIN HCL | | | | | | |
|---|---|---|---|---|---|---|
| **S. No.** | **Area at Flow rate (+0.2ml/min)** | **Area at Flow rate (0.2ml/min)** | **Area at pH (+0.2)** | **Area at pH (-0.2)** | **Area at Mobile phase (+2)** | **Area At Mobile phase (-2)** |
| **1** | **4128.035** | **4317.878** | **4244.493** | **4266.631** | **4337.648** | **4376.824** |
| **2** | **4148.470** | **4332.565** | **4265.786** | **4276.443** | **4345.126** | **4391.711** |
| **3** | **4138.681** | **4325.221** | **4251.423** | **4283.571** | **4367.524** | **4376.774** |
| **% R.S.D** | **0.247** | **0.170** | **0.255** | **0.199** | **0.357** | **0.196** |

| ANAGLIPTIN | | | | | | |
|---|---|---|---|---|---|---|
| **S. No.** | **Area at Flow rate (+0.2ml/min)** | **Area At Flow rate (-0.2ml/min)** | **Area at pH (+0.2)** | **Area at pH (-0.2)** | **Area at Mobile phase (+2)** | **Area At Mobile phase (-2)** |
| **1** | **3796.035** | **3967.548** | **3901.288** | **3920.826** | **3986.364** | **4022.647** |
| **2** | **3814.734** | **3980.774** | **3920.506** | **3930.116** | **3993.147** | **4036.049** |
| **3** | **3805.879** | **3974.117** | **3907.570** | **3936.578** | **4013.257** | **4022.601** |
| **% R.S.D** | **0.246** | **0.166** | **0.251** | **0.202** | **0.192** | **0.350** |

## System suitability

These tests are used to verify that the resolution and repeatability of the system were adequate for the analysis intended. The parameters used in this test were the chromatographic peak resolution (>2), HETP (>2000), tailing factor (<1.8). The repeatability of these parameters was checked by injecting six solutions of ANA and MET. The results were show in **Table 9**.

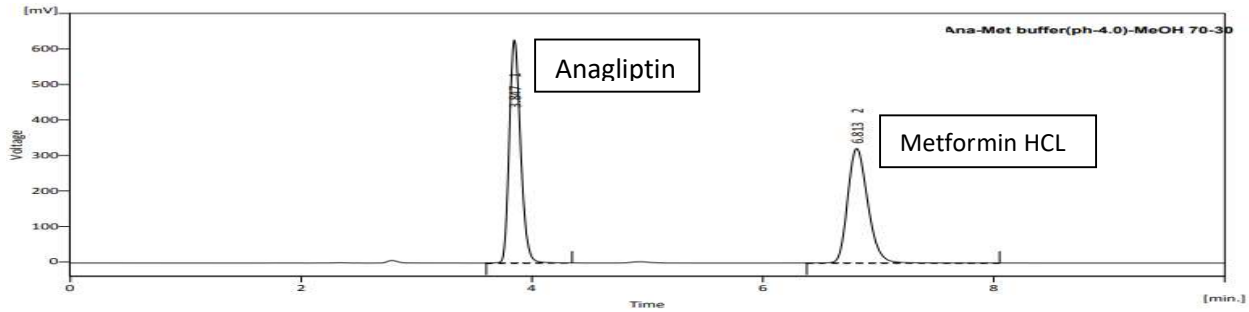


**Figure 7: Optimized Chromatogram of Anagliptin and Metformin HCL**

**Table 9: Observed value of system suitability parameter**

| S. No. | Parameters | Anagliptin | Metformin HCL |
|---|---|---|---|
| **1** | **Retention Time (min)** | **3.847** | **6.813** |
| **2** | **Theoretical plate(HETP)** | **6775** | **7381** |
| **3** | **Tailing factor** | **1.360** | **1.364** |
| **4** | **Resolution** | **11.769** | |

**Specificity**

Specificity was established by studying the resolution factor of drug peaks from the nearest resolving peak and among all other peaks. The specificity of method was evaluated by comparison between chromatogram of standard and test solution. There should be absence of any interfering peak with analyze peak **(Figure 8, 9 and 10)**.

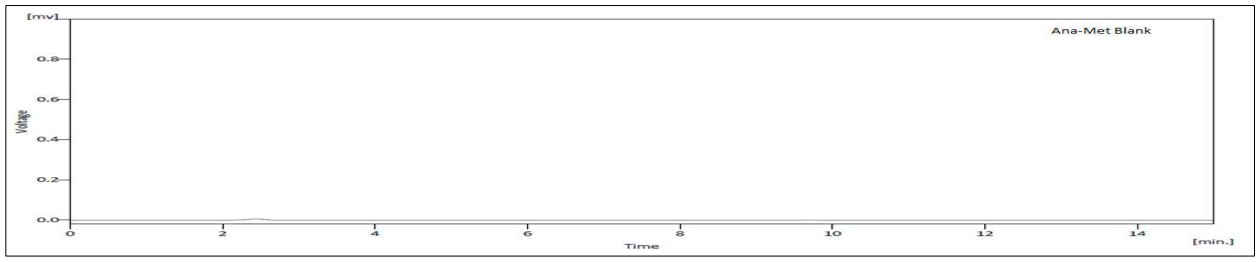


**Figure 8: Chromatogram of Anagliptin and Metformin HCL Blank**

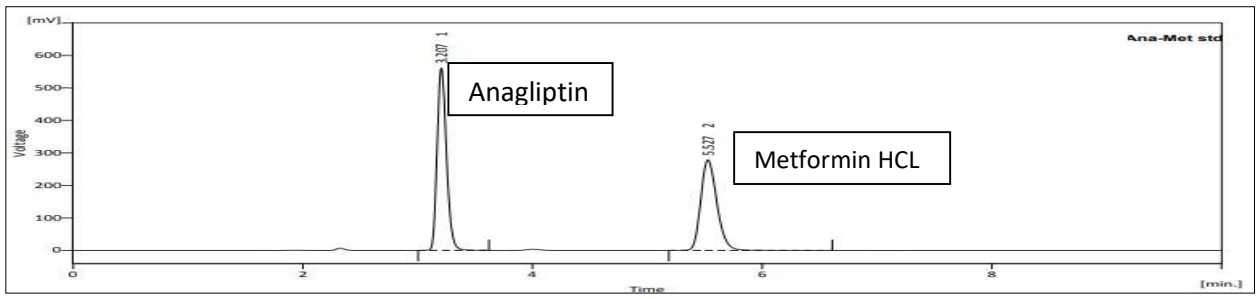


**Figure 9: Chromatogram of Anagliptin &Metformin HCL Standard**

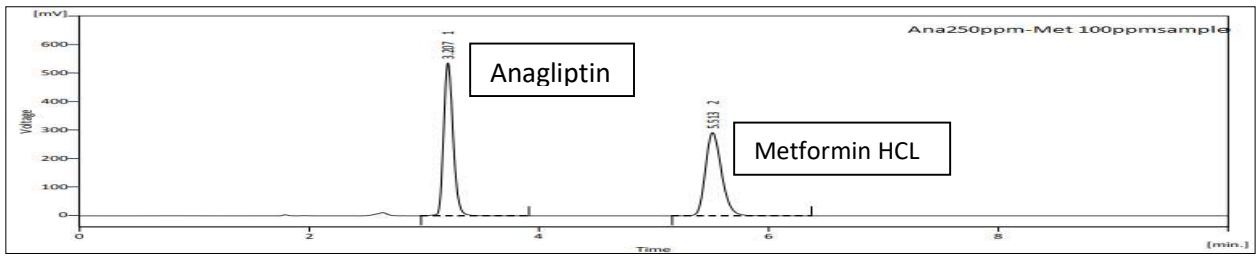


**Figure 10: Chromatogram of Anagliptin &Metformin HCL Sample**

**Analysis of marketed formulation**

An aliquot of 20µL from sample solution was injected under chromatographic condition and peak area measured and % assay was calculated from regression equation. Response was average of six determinations. The results are shown in **Table 10**.

**Table 10: Analysis of marketed tablet formulation**

| Drug name | Labeled (mg) | % Label claimed (% Assay* ± SD) (n=6) | %RSD |
|---|---|---|---|
| ANA | 100 | 101.374 ±0.176 | 0.173 |
| MET | 500 | 101.324±0.180 | 0.178 |

***Average of six determinations**

**Forced degradation studies**

Forced degradation study was intended to ensure the effective separation of ANA and MET and their potential degradation products which are generated under different condition as like acid hydrolysis, alkali hydrolysis, oxidative degradation, photolytic and thermal degradation.

**Acid hydrolysis**

Individually and combination 50mg and 10mg accurately weighed amount of MET and ANA were transferred in to 10mL vol. flask. Dissolve with 5mL of ACN and dilute up to with 0.1N HCL (5000µg/ml and 1000 µg/ml).and it was kept at room temperature for 4 hr. from this solution.1ml was taken and transferred into 10ml vol.flask and neutralized with 0.1 N NaOH and diluted up to with mobile phase(500ppm and100ppm).show in **Figure 11**.

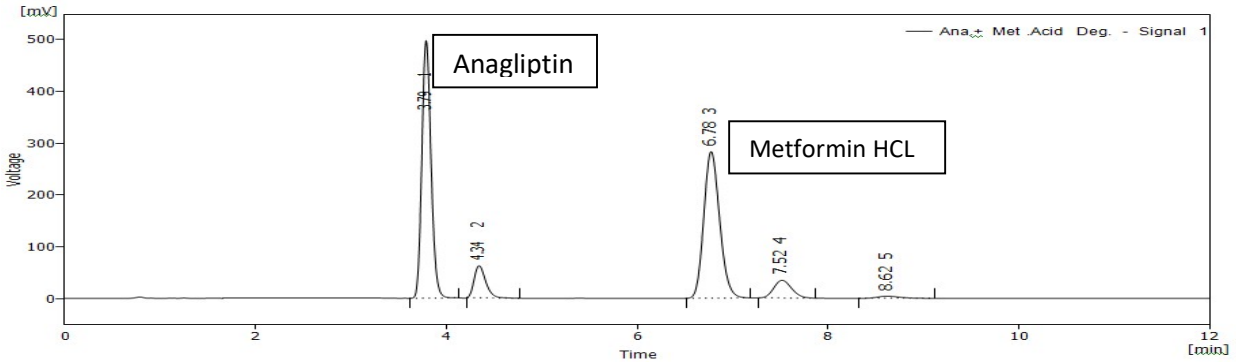


**Figure 11: Chromatogram of Acid Hydrolysis of mixture of ANA and MET at 220nm**

**Alkali Hydrolysis**

Individually and combination 50mg and 10mg accurately weighed amount of MET and ANA were transferred in to 10mL vol. flask. Dissolve with 5mL of ACN and dilute up to with 0.1N NaOH (5000µg/ml and 1000 µg/ml).and it was kept at R.T. for 4 hr. from this solution.1ml was taken and transferred into 10ml vol. flask and neutralized with 0.1 N HCL and diluted up to with mobile phase (500ppm and100ppm). Show in **Figure 12**.

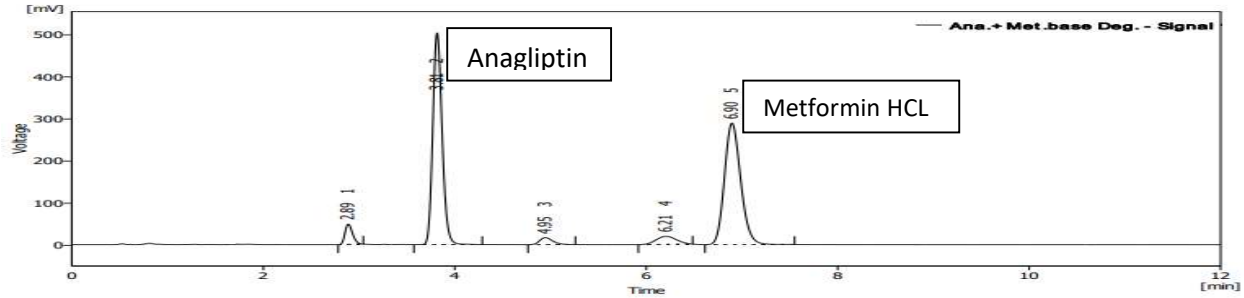


**Figure 12: Chromatogram of Alkali Hydrolysis of mixture of ANA and MET at 220nm**

**Oxidative Hydrolysis (3%$H_2O_2$)**

Individually and combination 50mg and 10mg accurately weighed amount of MET and ANA were transferred in to 10mL vol. flask. Dissolve with 5mL of ACN and dilute up to with 3% $H_2O_2$ (5000µg/ml and 1000 µg/ml).and it was kept at R.T. for 8 hr. from this solution.1ml was taken and transferred into 10ml vol.flask and diluted up to with mobile phase(500ppm and100ppm). Show in **Figure 13**.

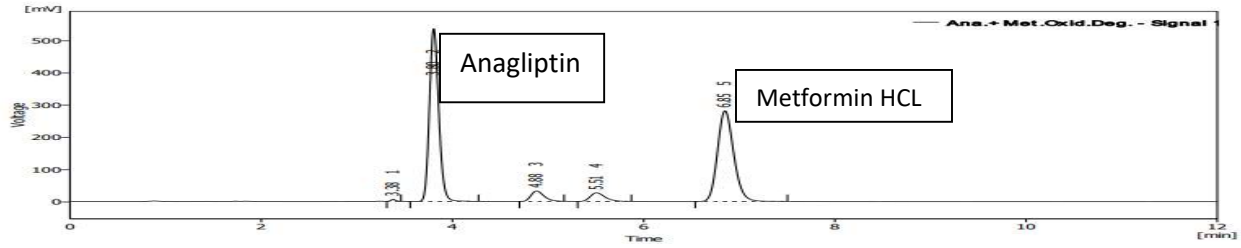


**Figure 13: Chromatogram of Oxidative Hydrolysis of mixture of ANA and MET at 220nm**

**Thermal degradation**

Individually and combination 50mg and 10mg accurately weighed amount of MET and ANA were transferred in to 10mL vol. flask. Dissolve and make up to with ACN (5000µg/ml and 1000 µg/ml).and it was kept at 80 °C. For 12 hr from this solution 1ml was taken and transferred into 10ml vol.flask and diluted up to with mobile phase (500ppm and100ppm). Show in **Figure 14**.

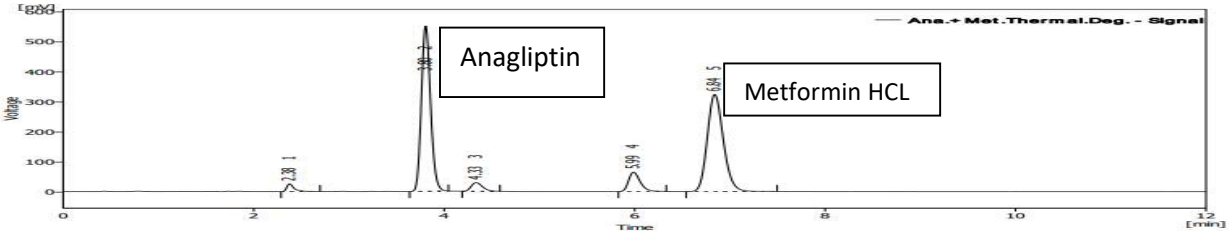


**Figure 14: Chromatogram of thermal degradation of mixture of ANA and MET at 220nm**

**Photo stability**

Individually and combination 50mg and 10mg accurately weighed amount of MET and ANA were transferred in to Petri dish and it was kept in UV chamber 254nm for 24 hr. After it were dissolved and make up to with ACN (5000µg/ml and 1000 µg/ml). From this solution 1ml was taken and transferred into 10ml vol.flask and diluted up to with mobile phase. This generated to 500ppm and100ppm. Show in **Figure 15**.

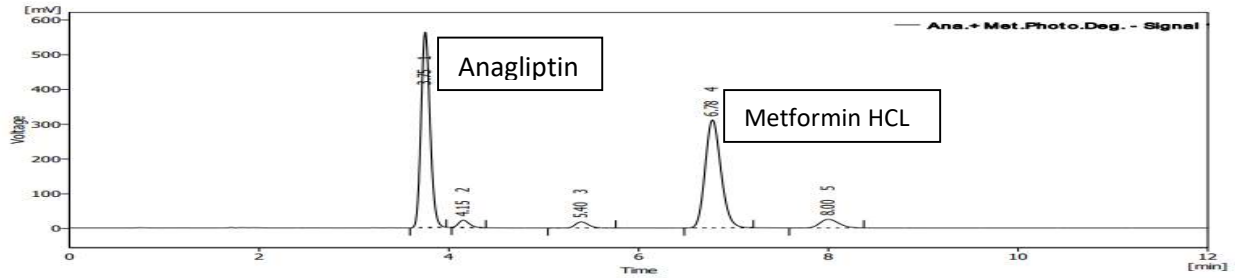


**Figure15: Chromatogram of Photolytic degradation of mixture of ANA and MET at 220nm**

## RESULT AND DISCUSSION

RP-HPLC method was developed for simultaneous estimation of anagliptin and metformin HCL using KROMASIL $C_{18}$ column and Methanol: Buffer pH 4 in volume 30:70 v/v mobile phase. RP-HPLC method was validated as per ICH guidelines it was found to be linear within the range correlation co-efficient for calibration curve of anagliptin and metformin HCL was found to be NLT 0.999 respectively. The accuracy of 80%, 100%, 120% levels. The percentage recovery for metformin HCL was 100.1-101.2% and 100.2-100.9% for anagliptin. The LOD for metformin HCL was found to be 1.56µg/ml also for anagliptin was found to be 0.069µg/ml and the LOQ of metformin HCL was 4.716 µg/ml and for anagliptin 0.209µg/ml as that indicating the sensitivity of method **(Table 6)**. The developed method was found to be precise as the %RSD value for intra-day and inter-day were found to be less than 2% and also the method was robust indicated by the %RSD value of less than 5%. The sample recoveries from all formulation agreed with their respective label claims, which suggested non-interference of formulation excipients in the estimation. The developed method was successfully separated Anagliptin and Metformin HCL from its degradation product formed under stress condition. Anagliptin and Metformin HCL degradant significant under acidic, basic and oxidative condition. Results were shown in **Table 11 and 12**.

**Table 11: Summary for Forced Degradation Studies of ANA and MET**

| S. No. | Stress condition | MET | | | | ANA | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | STD. | | Sample | | STD. | | Sample | |
| | | Area | % degradation | Area | % degradation | Area | % degradation | Area | % degradation |
| 1 | Acidic | 3316.59 | 15.99 | 3313.031 | 16.08 | 3321.42 | 13.70 | 3253.51 | 15.47 |
| 2 | Basic | 3454.84 | 12.48 | 3399.112 | 13.90 | 3457.10 | 10.18 | 3401.89 | 11.61 |
| 3 | Oxidation | 3593.06 | 8.98 | 3617.595 | 8.36 | 3598.69 | 6.50 | 3298.47 | 14.30 |
| 4 | Photolytic | 3667.91 | 7.09 | 3711.553 | 5.98 | 3549.23 | 7.78 | 3585.56 | 6.84 |
| 5 | Thermal | 3822.95 | 3.16 | 3691.383 | 6.49 | 3698.81 | 3.90 | 3788.62 | 1.56 |

**Table 12: Summary of validation parameters**

| S. No. | Parameters | | Metformin HCL | Anagliptin |
|---|---|---|---|---|
| 1. | Specificity | | Specific | |
| 2. | Linearity and Range | | 25-75 µg/ml | 5-15 µg/ml |
| 3. | Regression equation | | y = 84.75x + 3.901 | y = 389.5x + 3.518 |
| 4. | Correlation Co-efficient ($r^2$) | | 0.999 | 0.998 |
| 5. | Precision (%RSD) | Intraday | 0.170-0.595 | 0.167 -0.593 |
| | | Interday | 0.450-0.801 | 0.448 -0.798 |
| 6. | Accuracy | | Percentage recovery for Metformin HCL was 100.1-101.2%. and the Percentage recovery for Anagliptin was 100.2-100.9% | Accurate |
| 7. | Limit of Detection(LOD) | | 1.56 | 0.069 |
| 8. | Limit of Quantification(LOQ) | | 4.716 | 0.209 |
| 9. | Robustness (% RSD) | | The system suitability parameters were found well within the acceptance criteria as per system suitability | |

## CONCLUSION

A stability indicating RP-HPLC method has been developed and validated for the determination of ANA and MET in its tablet dosage form. The method was found to be specific as there was no interference of any co-eluting degradation product after stress degradation study. The degraded products are well resolved with indicating the methods can also be useful for determination of degraded products. The proposed methods are found to be simple, accurate, precise and robust. Hence, it can be used successfully for stability indicating assay method for simultaneous estimation of ANA and MET in its tablet dosage form.

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