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A Validated Stability Indicating Reverse Phase Liquid Chromatography Method for Metformin HCL and its Impurities in Bulk and Pharmaceutical Dosage Form.

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ABSTRACT

A simple and stability indicating liquid chromatographic method was developed for the determination of purity of metformin hydrochloride drug substance, drug product in bulk samples and pharmaceutical dosage form in the presence of its impurities and degradation product. The mobile phase used consisted of Buffer: acetonitrile (90:10 %v/v) with pH 3.6 (adjusted with 0.006M phosphoric acid) and flow rate 1.0ml/min in isocratic mode. The separation was carried out by UV detector at wavelength 218nm using Inertsil ODS 3V (250 mm X 4.6mm id, 5 µ) column. This new method was validated in accordance with USP requirements for new methods for assay determination, which include accuracy, precision, specificity and linearity. The current method demonstrates good linearity over the range of 50 to 200% of metformin hydrochloride. The accuracy of the method was found to be from 97.11 to 98.36%. The precision of this method reflected by relative standard deviation of replicates was found to be 0.27%.Validation of the same method for impurities determination was also performed according to USP requirements for quantitative determination of impurities which include accuracy, precision, linearity, selectivity and limit of quantification.

INTRODUCTION

Metformin HCl chemically known as Imididicarbonimidic diamide, N-N- dimethyl mono hydrochloride. Metformin impurities Related compound A (RCA), Related compound B (RCB) and Related compound C (RCC) are chemically known as 1- Cyanoguanidine (RCA), 1- methyl biguanidine hydrochloride (RCB) and N, N dimethyl (1, 3, 5) triazine 2, 4, 6 triamine (RCC). Metformin HCL is a biguanidine antihyperglycemic agent and for treating non insulin dependent diabetics mellitus (NIDDM). Its mechanism of action differs from other class of oral antihyperglymic agents. It decreases blood glucose levels by decreasing hepatic glucose production, decreasing intestinal absorption of glucose, and improving insulin sensitivity by increasing peripheral glucose uptake and utilization [1]. USP and BP describe a non aqueous titration as a method for metformin HCL analysis and another separate high performance liquid chromatography method for the analysis of its impurity [2, 3]. However stability indicating test method for the analysis of metformin HCL and its impurities (RCA, RCB and RCC) is therefore needed. Many high performance liquid chromatography methods such as simultaneous determination of Glimipride, pioglitazone and metformin HCL by Reverse phase High performance liquid chromatography method in tablet formulation [4], simultaneous determination of metformin HCL, Gliclazide and pioglitazone by Reverse phase High performance liquid chromatography in tablet formulation [5] were developed and validated. Several other methods for High performance liquid chromatography and spectrophotometric determination of metformin HCL [6,7,8,9,10,11,12] also were reported. However all these methods, are not employed for stability indicating for the determination of metformin HCL and its potential impurities. Development and validation of an analytical method for metformin HCL and its related compound (1- cyanoguanidine) in tablet formulation was reported [13]. In this method only one impurity has been analysed. Development and validation for simultaneous determination of metformin HCl and its impurities in tablet formulations [14] has been reported. However they used a hydrophilic interaction liquid chromatography technique which requires a special column, and expert analysts in this field which is not available in some laboratories. The objective of the current work is therefore to develop a simple RP -HPLC, stability indicating method for analysis of metformin HCL and its related compounds in tablet formulation.

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Validation of the current method was conducted for the both determination of metformin HCL in tablet formulation (assay) and for quantitative determination of its three impurities (RCA, RCB and RCC). Validation of the method for metformin HCL analysis was performed according to the equivalent of USP for assay determination which include accuracy, precision specificity, linearity and range while validation of the method for RCA, RCB, RCC were performed according to the requirements of USP for quantitative determination of impurities which include accuracy, precision, specificity, linearity, range and LOQ.

EXPERIMENTAL

Chemicals

Acetonitrile HPLC grade obtained from Rankem, sodium heptane sulphonate and sodium chloride are obtained from Merck (Mumbai, India). Related compound metformin hydrochloride and metformin are obtained from Edict Pharmaceuticals, Kelambakam, Chennai

Apparatus

HPLC system (waters 2489) with a pump, autosampler, column, oven and UV detector was employed. The Empower software was used. The chromatographic analysis was performed on an Inertsil ODS-3V (5mm) (250mm length, 6mm inner diameter) column. The column was kept at 30^0C temperature.

Preparation of stock solution

Impurity stock solution was prepared by dissolving each 1mg of related compounds (A, B, C) in 100ml of diluents to obtain a solution having a concentration of each related compounds (0.5mg/ml).

Resolution solution of metformin HCL and its related compound were prepared by adding 5ml of impurity stock solution in 20ml of diluents. Samples of formulated metformin HCL (tablet) were prepared by dissolving a quantity of tablet equivalent to 125 mg of metformin HCL in 100ml of diluents. A portion of sample solution was centrifuged at 3500rpm for 10 minutes 5ml of supernatant liquid was transferred to 50 ml volumetric flask and add diluents upto the mark (125mg/ml) in order to detect impurities and degradents which may present in the sample of Metformin HCl tablets.

For assay the sample solution was prepared as follows. 5 tablets of glyburide/ metformin HCL were accurately weighed to find out the average weight and powdered. The tablet powder equivalent to 250 mg of metformin HCL was transferred into 50 ml volumetric flask and made up the volume with diluent. Portion of sample solution was centrifuged at 3500 rpm for 10 min. 5ml of supernatant liquid was transferred into a 100ml volumetric flask and made up the volume with diluent 2.

Method validation

Precision

The precision of the method was verified by method precision and system precision. System precision was checked by using a standard suitability solution. The standard suitability solution was prepared and injected. Peak area responses for six replicate injections of the standard solutions were recorded. Method precision was checked by injecting six individual preparations of metformin in glyburide and metformin HCL (1.25mg/250mg tablets) sample spiked with of its three impurities. The chromatograms were recorded. %RSD areas for three impurities were calculated and system suitability was calculated.

Limit of detection and Limit of quantification

Standard and impurity stock solutions were diluted volumetrically to determine the limit of detection and limit of quantification. LOQ and LOD were determined from injections of prepared solutions and using the following formula.

LOD = 3.3X Residual standard deviation/ slope
LOQ = 10X Residual standard deviation/ slope

Linearity

Linearity test solutions for the assay method were prepared from metformin stock solution of eight concentration levels from LOQ to 400% of assay analytes concentration (0.095, 0.119, 0.143, 0.167, 0.191, 0.239, 0.359 and 0.479µg/ml). The peak area versus concentration data was treated by least square regression

analysis. Linearity test solutions for related substances method were prepared by diluting stock solution to the required concentrations. The solutions were prepared at seven concentration levels from LOQ to 200% of the specification levels (LOQ, 30, 50, 80, 100, 150 and 200%).

Accuracy

Accuracy of the assay method was evaluated in triplicate using three concentration levels of 50, 100 and 200% on sample (metformin in glyburide and metformin HCL tablets). Standard addition and recovery experiments were conducted on sample to determine accuracy of the related substances method. Study was carried out in triplicate using four concentration levels LOQ, 50, 100 and 200%. The percentage of recoveries for metformin and its impurities were calculated.

Robustness

The robustness is a measure of method capacity to remain unaffected by small, deliberate variations in method parameters and provides an indication of method reliability during normal use. To determine the robustness of the developed method, experimental conditions were deliberately altered and system suitability parameters for metformin and its impurities were calculated. The flow rate of the mobile phase was 1.0ml/min. To study the effect of flow rate on the resolution, flow was changed by 0.1 unit from 0.9 to1.1ml/min. The effect of column temperature on resolution was studied at 35°C and 25°C instead of 30°C. The effect of the percent acetonitrile strength on resolution was studied by varying acetonitrile from -2 to +2%. The effect of pH of the buffer on resolution was studied by varying from -0.2 to + 0.2.

Solution stability

Solution stability of metformin in the assay method was carried by leaving both the test solutions of sample and reference standard in tightly capped volumetric flask at room temperature for 48 hrs. The same sample solutions were assayed for 12hr interval upto the study period. Solution stability of metformin and its impurities in the related substance method was carried out by leaving spiked sample solutions in tightly capped volumetric flask at room temperature for 48 hrs. Content of impurities A, B and C was determined for every 12 hr interval up to the study period.

Ruggedness

Ruggedness is a measure of reproducibility of test results under normal variation of the operating condition, for e.g. analyst to analyst, system to system, column to column and day to day. The method precision ruggedness (reproducibility or intermediate precision) of the assay method was determined by injecting six individual preparation of metformin (125µg/ml) level against reference standard. The intermediate precision of the assay method was evaluated by different analyst. Intermediate precision of the impurities method was determined by injecting six individual preparation of Glyburide and metformin (1.25mg/ 250mg tablets) spiked with 1.25 µg/ml of its known impurities by using different analyst and different day. %RSD was calculated.

Specificity (forced degradation study)

Specificity is the ability of the method to means the analyte response in the presence of its potential impurities. The specificity of the developed reversed phase liquid chromatographic method for metformin was carried out in the presence of its impurities namely A, B and C. Stress studies were performed for glyburide and metformin tablets to provide an indication of the specification of the proposed method. Degradation was attempted with a stress condition of UV light, Acid (1N HCL), base (1N NaoH), oxidation (3% H_2O_2) and heat (105°C) to evaluate the ability of the proposed method to separate metformin from its degradation product. For heat and light studies, study period was 3 days whereas for acid, base and oxidation it was 3hours. Peak purity test was carried out for the metformin peak by using PDA detector in stress sample.

Filter study

A filter study was performed to determine the suitability of filter used and to determine the amount of filtrate to be discarded before a sample solution was collected for analysis. Accuracy sample at 100% solution was used for the filter study. This sample was divided in to six portions. One portion of the prepared sample was centrifuged (3500 rpm for 10 minutes), the clear supernatant from the centrifuge tube injected and analysed. The centrifuged sample was used as a control for the filter study. Second portion of sample was filtered through 0.45µ nylon filter by discarding the first 1ml, 2ml, 3ml and 5ml of the filtrate. Accuracy 100% sample has been considered as 0.45 µ PVDF filter.

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Method development

Xterra RP_{18} (150mm X 4.6mm, 5µ) column was tested using a mixture of buffer: acetonitrile as a mobile phase. (Different volumes fractions) Buffer pH 4.58 adjusted with 0.006M phosphoric acid. The use of these different volumes of mobile phase, however, gives, peak tailing was found to be metformin peak, baseline was interfering with peak and peak shape was not good. However, a mixture of buffer (pH 3.65 adjusted with 0.006 phosphoric acid) with acetonitrile in the ratio of 90:10%v/v and Inertsil C_8 (250 mm X 4.6mm id, 5 µ) column was used. As a result, USP plate count was found to be 3500 and only one related compound peak (RC-A) was found at 3.06 minutes. Next, to increase the run time was 40.0minutes and spiked with known impurities (RCB, RCC). Metformin related compound C peak was found to be 20.042 minutes and related compound B peak was missing. Instead of Inertsil C_8, symmetry C_{18} column, the same mobile phase, flow rate 1.0ml/min were used. The resolution between metformin HCL and related compound B was found to be very less. Then Inertsil ODS 3V (250 mm X 4.6mm id, 5 µ) column, same mobile phase and flow rate was used. The USP plate count for metformin HCL was found to be 11442 and USP resolution between metformin HCl and metformin related compound B was found to be 2.81 minutes. After this optimization this method was employed for the separation of and its related compounds (RCA, RCB & RCC) a good separation with adequate resolution was obtained Fig -1.

Method Validation

The proposed method was validated as per USFDA guidelines [15].

After method development, validation of the current test method for metformin HCL was performed in accordance with USP requirement for assay determination (category 1 analytical methods for the quantitation of active ingredient in finished pharmaceutical products which include accuracy, precision, specificity, linearity and range

Precision

The precision of the related substances method was verified by method precision and system precision. The %RSD for the areas of impurities A, B& C in related substance method precision study were found to be 1.94, 2.97 and 3.63% respectively. The % RSD of the assay results obtained in the system precision study was found to be 0.27% for metformin HCL. The %RSD values for known and unknown impurities of six sample preparations should not more than 10.0%. It indicated good precision of the method. System suitability parameters values were found to be with in the limit. The values were presented in table-1.

Table 1: Data for System precision

System suitability Parameter	Values	USP limit
Tailing factor	1.7	Not less than 7
USP resolution	3.28	Not less than 3000
**RRT for RC-A impurity	0.27	-
RRT for RC- B impurity	0.89	-
RRT for RC-C impurity	2.8	Not more than 1.5
*%RSD for Area	0.27	

*%RSD- Percentage relative standard deviation, **RRT-Relative retention time

Linearity

Linearity calibration plot for the assay method was obtained over the range from 50 to 200% and correlation co-efficient was found to be 0.9991. The results showed than the excellent correlation existed between the peak area and concentration of the analyte. Linear calibration plot for the related substance method was obtained over the calibration range from LOQ to 200%. The correlation coefficient was found to be (r^2) 0.9994, 0.9923 and 0.9924 for RCA, RCB and RCC respectively. The linear regression data showed that the method was linear over the concentration range of LOQ to 200%. The reports of analysis were shown in table-2.

Table 2: Linear regression data for metformin HCl and its related compounds

Compound	Mass range	Liner regression	Correlation coefficient
Metformin HCL	0.095 to 0.479	0.9989	Y=34,009.832x-50.202
Related compound A	0.006 to 0.257	0.9994	Y=121667.178x+106.788
Related compound B	0.031 to 0.248	0.9923	Y=30179.65x-329.913
Related compound C	0.082 to 0.274	0.9904	Y=113336.07x-834.07

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Limit of detection and limit of quantification

The determined limit of detection, limit of quantification and precision at LOQ values for metformin and its impurities were shown in table -3. The LOD and LOQ values as shown demonstrated that the method was sensitive for the determination of metformin related substance.

Table 3: LOD and LOQ data for metformin HCL and its impurities

Compound	Mass range	Slope	Residual SD	LOQ	LOD
Metformin HCL	0.003 to 0.098	29186.964	296.249	0.105	0.0334
A	0.003 to 0.0103	124368.45	70.838	0.0056	0.0018
B	0.003 to 0.099	33758.068	102.724	0.0304	40.01
C	0.003 to 0.110	102537.017	867.391	0.0845	50.0279

Accuracy

The percentage recovery of metformin from drug product was ranged from 97.11 to 98.36%. The percentage recovery of impurities in metformin and glyburide tablets sample varied from 81.30 to 12.30%. The % recovery values for impurities and metformin HCL were shown in table-4. Hence the method was acceptable with respect to accuracy.

Table 4: Evaluation of Accuracy Studies

	Percentage Recovery			
Amount spiked	Metformin	Impurity A	Impurity B	Impurity C
*LOQ	-	102.69±5.28	81.30±1.24	88.25±0.75
50	97.11±0.07	98.07±0.71	98.00±1.06	-
100	97.58±0.16	97.03±1.13	100.66±0.76	95.48±1.37
200	98.36±0.27	96.06±0.34	98.83±1.39	97.33±0.91

*LOQ- Limit of quantification

Robustness

In all the deliberate varied chromatographic conditions (flow rate, column temperature, mobile phase ratio of organic solvent and pH) the resolution between all pairs of compounds were greater than 2.0 and the relative standard for the peak area response from five replicate of standard solutions should not be more than 5.0%. Based on the above discussion, it is concluded that the method was unaffected by small deliberate variations in flow rate, column temperature, mobile phase ratio and buffer pH.

Solution stability

The results of standard solutions at 48 hours percentage differed by less than 10.0% when compared to the initial standard concentration and hence the acceptance criteria of concentration % difference should be not more than 10.0% was found. Therefore the standard solution can be used upto 48 hours after its preparation if it was stored at room temperature. The results of spiked sample solution upto 48 hours percentage differed by less than 20.0% was found. When compared that of the initial and hence the acceptance criteria of % impurity difference should not be more than be 20.0% was found. The solution stability experiments data confirm that the standard solution and spiked sample solution were stable for 48 hours.

Ruggedness

The relative standard deviation (RSD) for the peak area response from five replicate injection of standard solution was found to be 0.19 for analyst –I and 0.90 for analyst –II. The USP resolution between metformin related compound B and metformin HCL was found to be not less than 1.5 from system suitability solution. (3.28 for analyst-I and 3.05 for analyst –II). The %RSD values for impurities were found to be 1.94, 2.97 and 3.63 for impurity A, B, and C respectively (analyst-I) and the % RSD values for impurities were found to be 1.42, 6.37 and 3.53 for impurity A, B and C respectively (analyst – II) (%RSD values for impurities should not be more than 10.0%). The ratio for the average impurities values obtained from two analysts was found to be within the acceptance criteria of 0.80 to 1.20 for all the impurities. Therefore the method was found to be rugged.

Results of forced degradation studies

Metformin sample solution was found to degrade significantly in base and peroxide stress condition. Mild degradation was observed in acid stress condition. It was found to be stable under photolytic and thermal stress conditions. During the forced degradation, it was observed no secondary peak arising from degraded samples interfered with the elution of the metformin peak. Peak purity analysis was using the photodiode array detector

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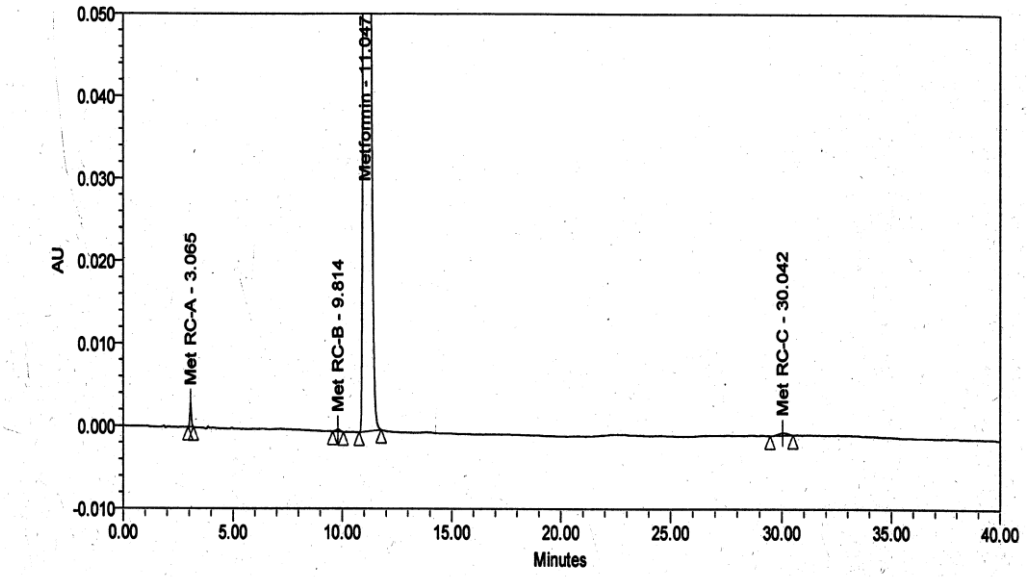
demonstrated metformin homogeneity. All known impurities did not interfere with the metformin peak. The study validated that the method was specific and stability indicating. The reports of analysis were shown in table-4.

Table 5: Summary of forced degradation studies

Sample name	Stress condition	%Degradation	Purity angle	Purity threshold
	*NA	NA	0.043	0.223
	5ml of IN HCL		0.039	0.223
Control sample	heated on a water bath at 70˚C for 3 hours	0.89		
Acid stress	5 ml of IN NaOH heated on a water bath at 70℃ for 1 hour	3.64	0.068	0.211
Base stress Peroxide stress	5 ml of 3% H_2O_2 Heated on a water bath at 80˚C for 60min.	2.68	0.039	0.223
UV light stress	Stress under UV light for 72 hrs	Nil	0.039	0.223
Heat stress	Heated in a oven at 105˚C for 72 hrs	Nil	0.042	0.228

*NA ------ not applicable



Peak Results

	Name	Vial	Inj	RT (min)	Area (µV*sec)	% Area	USP Tailing	USP Plate Count	USP Resolution
1	Met RC-A	2	1	3.065	14568	0.36	1.12	8716	
2	Met RC-B	2	1	9.814	3631	0.09	1.00	14596	29.14
3	Metformin	2	1	11.047	4033022	99.31	1.86	10442	3.18
4	Met RC-C	2	1	30.042	9694	0.24	0.94	25253	30.02

Figure1: Optimized Chromatogram for Metformin and its impurities

Filter study

The assay and percentage of impurities found in the filtered fractions of sample were comparable to the assay and the impurities found in the centrifuged portion of sample, there was no significant difference in assay and percentage impurities between the different volumes filtered. Therefore, the 0.45µ PVDF and nylon filters were suitable for use in the validation and discarding of 4ml of the sample solution as filtrate, as stated in the method, was a suitable volume to discard before collecting for analysis by HPLC.

CONCLUSION

A simple, accurate and precision stability indicating HPLC analytical method was developed and validated for the analysis of metformin HCL in tablet formulation. The current method has the ability to separate metformin HCL from its related compounds (RCA, RCB and RCC). Low LOD and LOQ for related compounds using this method enables the detection and quantification of this impurity at low concentration.

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