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A rapid stability indicating LC method for nevirapine using RR-LC

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ABSTRACT

A simple, sensitive isocratic RR-LC method has been developed for the quantitative determination of Nevirapine Related compounds in bulk drug, used for the HIV Aids. The developed method is also applicable for the Assay determination. Efficient chromatographic separation was achieved on a C18 stationary phase with simple mobile phase combination delivered in a gradient mode and quantification was carried out using ultraviolet detection at 220 nm at a flow rate of 2.0 mL min^{-1}. In the developed RRLC method the resolution between Nevirapine and its two potential impurities was found to be greater than 4.0. Regression analysis shows an r value (correlation coefficient) of greater than 0.999 for Nevirapine and it's all the two impurities. This method was capable to detect all two impurities of Nevirapine at a level of 0.01 % with respect to test concentration of 0.24 mg mL^{-1} for a 10 µL injection volume. The inter and intraday precision values for all two impurities and for Nevirapine was found to be within 2.0 % RSD at its specification level. The method has shown good and consistent recoveries for Nevirapine two impurities (99.0-102.5%). The test solution was found to be stable in diluent for 48 h. The drug was subjected to stress conditions of exposure to acid hydrolysis and reduction. Humidity, Photolysis and thermal degradation. Considerable degradation was found to occur in acid hydrolysis stress conditions. The stress samples were assayed against a qualified reference standard and the mass balance was found close to 99.5 %. The developed RR-LC method was validated with respect to linearity, accuracy, precision and robustness.

KEYWORDS

Column liquid chromatography;
Nevirapine anhydrous;
Forced degradation;
Validation.

INTRODUCTION

Nevirapine 11-Cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido[3,2-b:2′,3′-e][1,4]diazepin-6-one (Figure 1) is a Anti retro viral product. The generic name of Nevirapine is viramune, it is prescribed for the treatment of HIV Aids. Viramune is prescribed for advanced cases of HIV. HIV-the human immunodeficiency virus that causes AIDS-undermines the immune system over a period of years, eventually leaving the body defenseless against infection. Viramune is generally prescribed only after the immune system has declined and infections have begun to appear. It is always taken with at least one other HIV medication such as Retrovir or Videx. If taken alone, it can cause the virus to become resistant. Even if used properly, it may be effective for only a limited time.

Like other drugs for HIV, Viramune works by impairing the virus's ability to multiply.

Few analytical methods were reported in literature

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for the quantification of Nevirapine in human plasma [1-6]. As far as we are aware there is no stability-indicating LC method for determination of related compounds and for quantitative estimation of Nevirapine. In this paper we described validation of related compounds method for accurate quantification of Nevirapine two impurities in bulk drug samples along with method validation as per ICH norms. Intensive stress studies were carried out on Nevirapine accordingly a stability-indicating method was developed, which could separate various degradation products.

The present drug stability test guideline Q1A (R2) issued by International Conference on Harmonization (ICH)[7] suggests that stress studies should be carried out on a drug to establish its inherent stability characteristics, leading to separation of degradation products and hence supporting the suitability of the proposed analytical procedures. It also requires that analytical test procedures for stability samples should be stability indicating and they should be fully validated.

Accordingly, the aim of present study was to establish inherent stability of Nevirapine through stress studies under a variety of ICH recommended test conditions and to develop a stability-indicating assay method.

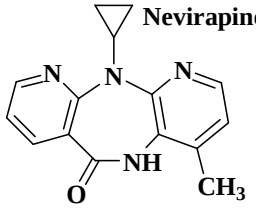
EXPERIMENTAL

Chemicals

Samples of Nevirapine and its related impurities were received from Hetero Laboratories Ltd, Hyderabad, India (Figure 1). HPLC grade acetonitrile and was purchased from Merck, Darmstadt, Germany. Analytical reagent grade Mono basic ammonium phosphate and sodium hydroxide were purchased from Merck, Darmstadt, Germany. High purity water was prepared by using Millipore Milli-Q plus water purification system. All samples and impurities used in this study were of greater than 99.6% purity.

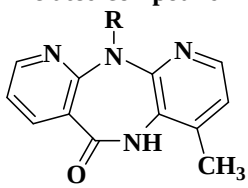
Equipment

The LC system, used for method development, forced degradation studies and method validation was Agilent 1200 RRLC. The output signal was monitored and processed using Chemistation software on Pentium computer (Digital equipment Co).



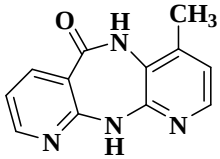
11-Cyclopropyl-4-methyl-5,11-dihydro-6H-dipyrido [3,2-b:2',3'-e][1,4] diazepin-6-one:

Related compound-A



R = C_2H_5: 11-ethyl-4-methyl-5,11-dihydro-6H-dipyrido[3,2-b:2',3'-e] [1,4]diazepin-6-one

Related compound-B



R = H: 4-methyl-5,11-dihydro-6H-dipyrido[3,2-b:2',3'-e][1,4]diazepin-6-one

Related compound-C:

R = CH_2-CH_2-CH_3: 4-methyl-11-propyl-5,11-dihydro-6H-dipyrido[3,2-b:2',3'-e][1,4]diazepin-6-one:

Figure 1: Structures and labels of Nevirapine and its impurities

Chromatographic conditions

The chromatographic column used was XDB-C18, 50X4.6 with 1.8μm particles. The mobile phase contains a mixture of buffer and acetonitrile in the ratio of 4:1 (v/v). Buffer consists of 2.8g of Mono basic ammonium phosphate dissolve in 800 mL of water and adjusted to pH 7.0 using sodium hydroxide solution then adds 200 mL of water.

The flow rate of the mobile phase was 2.0mLmin^{-1}. The column temperature was maintained at 40^{0}C and the detection was monitored at a wavelength of 220 nm. The injection volume was 10μL. mobile phase was used as diluent.

Preparation of solutions

Preparation of standard Solutions

A Stock solution of Nevirapine (0.24 mg mL^{-1}) was prepared by dissolving appropriate amount in the diluent. Working solutions of 240 and 24μg mL^{-1} were prepared from above stock solution for related compounds determination and assay determination, respectively. A stock solution of impurities (mixture of related compound-A and B) at a concentration of 0.24 mg mL^{-1} was also prepared in diluent.

Analytical method validation

The developed chromatographic method was

validated for selectivity, linearity, range, precision, accuracy, sensitivity, robustness and system suitability[13,14,15].

Selectivity

Selectivity of the developed method was assessed by performing forced degradation studies[7-12]. The terms selectivity and specificity are often used interchangeably. Selectivity is the ability of the method to measure the analyte response in the presence of its potential impurities. According to ICH[7] stress testing of the drug substance can help the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedure used. Photo stability testing should be an integral part of stress testing. The standard conditions for photo stability testing are described in ICH Q1B[9]. The specificity of the developed LC method for Nevirapine was determined in the presence of its impurities namely imp-1, imp-2, imp-3 and degradation products. The stress conditions employed for degradation study includes light (carried out as per ICH Q1B), heat (80^0C), acid hydrolysis (1N HCl). For heat and light studies, study period was 48 hrs where as for acid and reduction it was 48 hrs. All stressed samples of Nevirapine (heat (80^0C), acid hydrolysis (1N HCl), were analysed.

Assay studies were carried out for stress samples against qualified reference standard and the mass balance (% assay + % of impurities + % of degradation products) was calculated. Assay was also calculated for bulk samples by spiking all two impurities (related compound-A and B) at the specification level (i.e. 0.15% of analyte concentration which is 240μg mL^{-1}).

Analytical method validation

Precision

Precision was determined through repeatability (intra-day) and intermediate (inter-day) precision. The precision of the related compounds method was checked by injecting six individual preparations of (240μg mL^{-1}) Nevirapine Anhydrous. The % RSD for percentage of each impurity was calculated.

Linearity and range

To establish linearity of the method, calibration solutions were prepared from stock solution at six concentration levels for chromatographic purity method-concentration levels ranging from LOQ to 150% (with respect to test concentration of 240μg mL^{-1}, LOQ, 50, 80,100,120 and 150%) were prepared by diluting the impurity stock solution to the required concentrations. Average peak area at each concentration level was subjected to linear regression analysis with the least square method. Calibration equation obtained from regression analysis was used to calculate the corresponding predicted responses. The residuals and sum of the residual squares were calculated from the corresponding predicted responses. The % y-intercept for chromatographic purity method was calculated. Analytical range of the method was established from the analysis of sensitivity curves. Upper and lower levels of range were also established.

Sensitivity

Sensitivity was determined by establishing the Limit of detection (LOD) and Limit of quantification (LOQ) Related compound-A and B estimated at a signal-to-noise ratio of 3:1 and 10:1 respectively, by injecting a series of dilute solutions with known concentration. The precision study was also carried out at the LOQ level by injecting six individual preparations of related compound-A and B and calculated the % RSD for the areas of each impurity.

Accuracy

For determination of accuracy, recovery study was carried out by spiking analysis. A known amount of the impurity stock solutions were spiked to the previously analysed samples at LOQ,50,100 and 150% of the analyte concentration (240μg mL^{-1}). The percentage of recoveries related compound-A and B were calculated. Each concentration level was prepared for three times.

Robustness

Robustness study was conducted by making small but deliberate changes in the optimized method parameters. Critical sources of variability in operating procedure such as percent organic strength, buffer strength, temperature of the column were identified. By deliberate change in experimental conditions the resolution between Nevirapine , related compound-A and B was evaluated. The flow rate of the mobile phase was 2.0 mL min^{-1}. To study the effect of flow rate on the resolution, 0.2 units changed i.e 1.8 and 2.2 mL min^{-1}. The effect of column temperature on resolution was studied

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at 35^{0}C and 45^{0}C instead of 40^{0}C. In the all above varied conditions, the components of the mobile phase were held constant.

Solution stability and mobile phase stability

The solution stability of Nevirapine in the assay method was carried out by leaving the test solutions of sample in tightly capped volumetric flasks at room temperature for 48 h. The same sample solutions were assayed 6 h interval up to the study period against freshly prepared standard solution. The mobile phase stability was also carried out by assaying the freshly prepared sample solutions against freshly prepared reference standard solutions 6 h interval up to 48 h. Prepared mobile phase was kept constant during the study period.

The solution stability of Nevirapine and its impurities in the related compounds method was carried out by leaving spiked sample solution in tightly capped volumetric flask at room temperature for 48 h. Content of related compound-A and B were determined every 6 h interval up to the study period. Mobile phase stability was also carried out for 48 h by injecting the freshly prepared sample solutions for every 6 h interval. Content of related compound-A and B was checked in the test solutions. Mobile phase prepared was kept constant during the study period.

RESULTS AND DISCUSSION

Method development and optimization

All the impurities and Nevirapine solutions were prepared in diluent at a concentration of 100 ppm and scanned in UV-visible spectrometer; all the 2 impurities and Nevirapine were having UV maxima at around 220 nm. Hence detection at 220nm was selected for method development purpose.

United States pharmacopeia has given a HPLC method for the determination of Nevirapine, but in that method the total run time was 60 min. keeping these disadvantages in view, a rapid resolution LC method was developed with in 10 min for the quantification of nevirapine and its related compounds.

The primary target of this work was to develop a stability indicating chromatographic method for the determination of Nevirapine. and its impurities Imp-A and Imp-B. To get separation of nevirapine from its impurities, and degradation products chromatographic method was developed using different stationary phases like C18, C8 and Cyano; different mobile phases containing buffers like phosphate, sulphate and acetate with different pH (2-8) and using organic modifiers like acetonitrile and methanol in the mobile phase.

The chromatographic separation was achieved on XDB-C18, 50X4.6 with 1.8μm particles). To decrease the interactions of Nevirapine with stationary phase column (due to hydrophobicity) mobile phase was selected with higher percentage of acetonitrile. Different ratios were tried to optimize the retention time of Nevirapine and resolution between the impurities. Satisfactory results (retention time of Nevirapine is ~1.541 min and the resolution between all the impurities is >4) were obtained with optimized conditions

In the optimized conditions Nevirapine anhydrous, related compound-A and B were well separated with a resolution of greater than 4 and the typical retention times of related compound-A and B and Nevirapine were about 1.009, 1.541 and 2.629 min respectively. The system suitability results were given in TABLE1.

Buffer pH and % acetonitrile played a major role in achieving the separation between all two impurities and Nevirapine anhydrous.

Analysis was performed for different batches of bulk drug samples (n=3) Results were given in TABLE 2.

Method validation

Precision

The %RSD of area of related compound-A and B in precision study were within 2.0 %. Confirming the good precision of the developed analytical method.

Sensitivity

The limit of detection of related compound-A and B was 0.003 and 0.003% (of analyte concentration, i.e.240 μg mL^{-1}) respectively for 10μL injection volume. The limit of quantification of related compound-A and B was 0.01 and 0.01% (of analyte concentration,

TABLE 1: System suitability report

Compound	USP Resolution (R_S)	USP tailing factor	No of theoretical plates USP tangent method (N)
Related compound-B	--	1.164	1735
Nevirapine anhydrous	5.060	1.108	2941
Related compound-A	8.519	1.068	5504

i.e.240µg mL^{-1}) respectively for 3µL injection volume. The % RSD for area of related compound-A and B were below 2.0% for precision at LOQ level.

Linearity

Calibration curve obtained by the least square regression analysis between average peak area and concentration showed linear relationship with a regression coefficient of 0.999 over the calibration ranges tested.

The results of linearity and range obtained for the two potential impurities were tabulated in the TABLE 3. Linear calibration plot for related compounds method was obtained over the calibration ranges tested, i.e. LOQ to 0.225% for related compound-A and B. The correlation coefficient obtained was greater than 0.999 for all two impurities.

Accuracy

The percentage recovery of related compound-A and B in bulk drug samples ranged from 99.0 to 102.5.

TABLE 2: Batch analysis

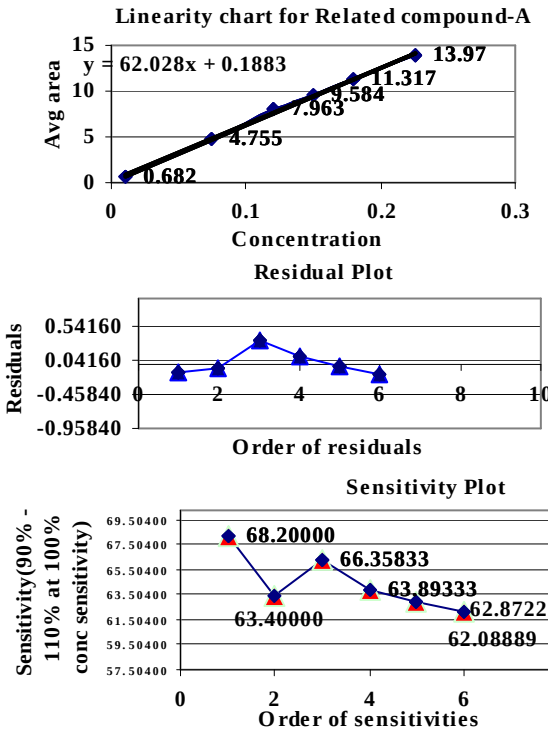
Batch no:	Related compd. -A	Related compd. -B	Related compd. -C	Maximum single unknown impurity	Total Impurities	Assay by HPLC
As per new developed method						
01	0.02	0.01	ND	0.01	0.04	99.8
02	0.02	0.01	ND	0.01	0.04	99.7
03	0.02	0.01	ND	0.01	0.04	99.8
As per USP method						
01	0.02	0.01	ND	0.01	0.05	99.8
02	0.02	0.01	ND	0.01	0.04	99.6
03	0.02	Below LOQ	ND	0.01	0.04	99.8

Where ND = Not Detected

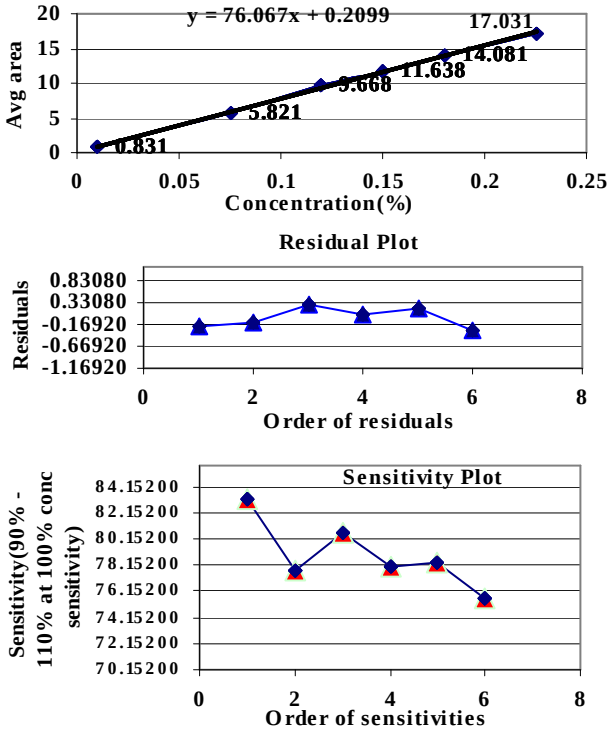
TABLE 3: Linearity results for related compounds estimation

	Related compound-A	Related compound-B	Nevirapine anhydrous
Trend line equation	y = 62.02704x +0.188	y = 76.06831x +0.210	y = 65.3178x + 0.119
Linearity range	0.01-0.225%	0.01-0.225%	0.01-0.15%
Regression coefficient	0.9992	0.9993	0.9991
Slope	62.02704	76.06831	65.3178
Intercept	0.188	0.210	0.119
%Intercept	1.97	1.80	1.76
Residual sum of squares	0.1733	0.2560	0.0937

Linearity graph for related compound -A (TABLE 3)



Linearity graph for related compound -B (TABLE 3)



HPLC chromatogram of spiked sample with all two impurities in Nevirapine bulk drug sample is shown in figure.

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TABLE 4: Results of accuracy study for related compounds

Added (µg/mL) (n= 3)	% Recovery of related compound-A	% Recovery of related compound-B
0.024	100.3	99.0
0.18	101.0	102.5
0.36	100.9	100.0
0.54	101.9	100.0

n =3, Number of determinations

TABLE 5 : Results of robustness study

S. no.	Parameter	Variation	Resolution (R_s) Between related compound-B and nevirapine anhydrous	System precision
1	Temperature (± 5^{0}C of set temperature)	(a) At 35°C	4.639	0.45
		(b) At 40°C	5.141	0.47
2	Flow rate (± 10% of the set flow)	(a) At 1.8 ml min^{-1}	4.919	0.47
		(b) At 2.2 ml min^{-1}	4.941	0.25

TABLE 6 : Summary of forced degradation results

Stress condition	Time	% Assay of active substance	Mass balance (%Assay + %impurities + % Degradation products)
Acid hydrolysis (1N HCl at 80°C)	2 h	86.3	98.8
Base (1n NaOH, heating for o hours at 80^{0}C)		99.7	99.8
Humidity(100%RH)	48hrs	99.8	99.8
Thermal (80°C)	48hrs	99.7	99.7
Light (photolytic degradatio	1200KLUX	99.7	99.7
Oxidation (30% H_2O_2 for tw ating at 80^{0}C)		99.8	99.8

Robustness

Close observation of analysis results for deliberatly changed chromatographic conditions (flow rate and column temperature) revealed that the resolution between closely eluting impurities, namely related compound-A Nevirapine was greater than 4.0, illustrating the robustness of the method (TABLE 5).

Solution stability and mobile phase stability

The %RSD of assay of Nevirapine during solution stability and mobile phase stability experiments was within 1.0. No significant changes were observed in the content of related compound-A and B during solution stability and mobile phase stability experiments. The solution stability and mobile phase stability experiments data confirms that sample solutions and mobile phase used during assay and related substance determination were stable up to the study period of 48 h.

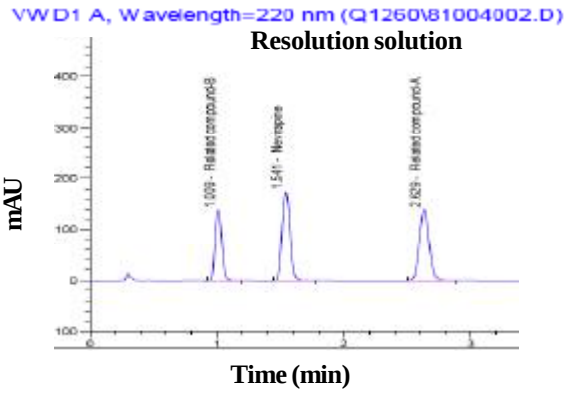

Figure 2: Typical chromatogram of Nevirapine spiked with impurities

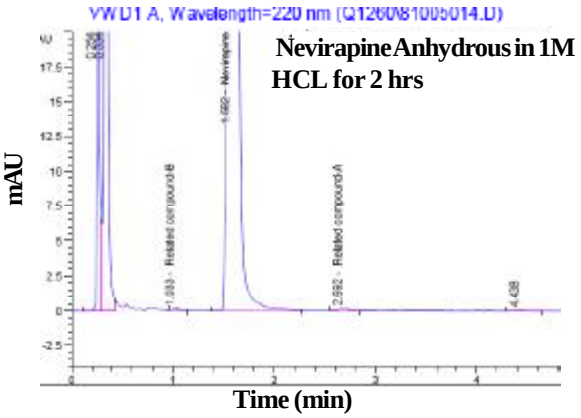

Figure 3: Typical chromatograms of stressed Nevirapine samples

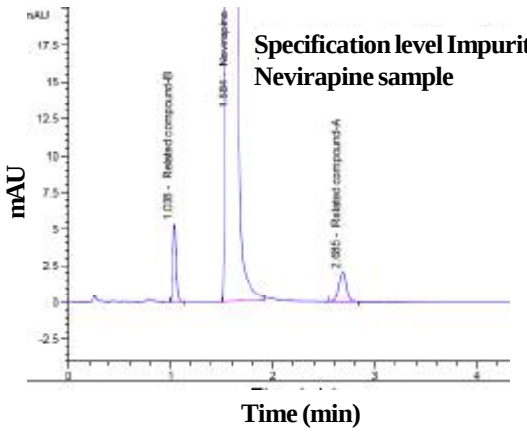

Figure 4: Specification level Impurities spiking in 100% Nevirapine sample

Results of forced degradation studies

Degradation behavior

Stress studies on Nevirapine under different stress conditions suggested the following degradation behavior.

Degradation in acidic solution

Nevirapine is highly sensitive to bases and was degraded into unknown impurities by acid hydrolysis in 1 N HCL. The drug was exposed to 1 N HCL at 80^0C temperature for 2 h. Nevirapine has shown significant sensitivity towards acid treatment . The drug gradually undergone degradation with time and degraded into unknown (~12.4%).

Degradation in basic solution

The drug was stable to the effect of 1 N NaOH. When the drug was exposed to 1 N NaOH at 80^0C temperature for 2 h, no degradation was observed.

Degradation in oxidative condition

The drug was stable to the effect of 30% H2O2. When the drug was exposed to 30% H2O2 at 80^0C temperature for 2 h, no degradation was observed.

Photolytic conditions

The drug was stable to the effect of photolysis. When the drug powder was exposed to light for an overall illumination of 1.2 million lux hours and an integrated near ultraviolet energy of 200-watt hours/square meter (w/mhr) (in photo stability chamber), no degradation was observed.

Thermal degradation

The drug was stable to the effect of temperature. When the drug powder exposed to dry heat at 80^0C for 2 days, no degradation was observed.

CONCLUSION

The gradient RR-LC method developed for quantitative and related compounds determination of Nevirapine in bulk drug is precise, accurate and specific. The method was completely validated showing satisfactory data for all the method validation parameters tested. The developed method is stability indicating and can be used for the routine analysis of production samples and also to check the stability of Nevirapine samples.

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