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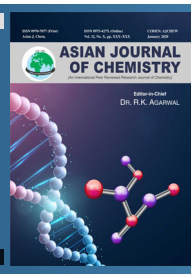

A Simple, Specific, Mass Compatible and Validated Gas Chromatographic Method for the Estimation of Piperidine-3-amine Content in Linagliptin Finished and Stability Samples without Derivatization

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A new simple, selective, highly sensitive, specific (stability indicating), robust, rugged and mass compatible gas chromatographic method and sample with direct injection-mode was developed for the quantitative determination of amino-3-piperidine (3-aminopiperidine, 3-AMP) in linagliptin. As the CAD-LC method did not proved for specificity and pre-derivitizations are challenging task for quality control (QC), a simple GC method has been developed. Compared to LC-CAD method, which is not proved for specificity and pre-column derivitization methods having the limitations to analyze the reaction monitoring in-process samples and degradation samples, the present method is selective, simple, cost effective, QC friendly, widely available GC technique with direct injection and high in sensitivity. Also this method is mass compatible, specificity proved by forced degradation, method was validated as per ICH guidelines. Mass balance was proved by analyzing the stressed samples for net degradation by HPLC and assay by HPLC methods. This GC method also provides an advantage to analyze the in-process samples to monitor the progress of the synthetic process, where the reaction monitoring samples are unpurified or non-isolated samples and contains many process related impurities (reference) and solvents, which will have interference with 3-AMP in LC-CAD and pre-column derivitization methods. This method involves simple sample preparation process and direct injection with GC-FID technique. Hence, this method can be used to analyze the finished product samples, degradation samples, stability samples and reaction monitoring samples. The method was developed with widely available GC column (diphenyl dimethyl polysiloxane as stationary phase, 30 m length, 0.53 mm internal diameter & 5.0 µm thickness), helium as carries gas, FID detector set at 240 ºC, column oven starts at 200 ºC and starts increases after 2 min with 20 ºC/min and holds up to 11 min, which will ensure the column bake. The solvents used for the process were well separated from 3-AMP peak. Mass balance was reported > 99%. The limit of quantification and limit of detection values for 3-AMP were 0.002% (0.4 µg/mL) and 0.007% (1.4 µg/mL) of targeted concentration (20 mg/mL), respectively. The method was precise at LOQ and at specification level with %RSD values 2.8 and 4.7, respectively. Linearity was established in the range of 0.0014 mg/mL (LOQ) to 0.045 mg/mL for 3-AMP with correlation coefficient ($r^2 > 0.9995$). The percentage recoveries were obtained between 99.9% and 104.4%. The range of the method was established from LOQ (0.0014 mg/mL) to 150% (0.045 mg/mL) of the specification targeted limit of 0.15% (0.03 mg/mL). The standard and spiked sample solutions were studied up to 2 days and are stable at room temperature. The forced degradation studies were performed by using HCl, NaOH, H_2O_2 thermal, UV radiation and water. A mild degradation bout 0.25% was observed in base degradation condition, which was confirmed with mass number by GC-MS analysis. Validation parameters were evaluated according to the International Conference on Harmonization (ICH) Q2 guidelines.

Keywords: Linagliptin, Amino-3-piperidine, Forced degradation, Orthogonal method.

INTRODUCTION

Linagliptin (LIP) is an orally-active inhibitor of dipeptidyl peptidase-4 (DPP-4) enzyme and acted as an oral diabetes medicine that helps control blood sugar levels. Linagliptin is used together with diet and exercise to improve blood sugar control in adults with type 2 diabetes mellitus [1], however not applicable for treating type 1 diabetes [2].

(*R*)-Piperidin-3-amine dihydrochloride (3-AMP) is widely used as a key starting material in the synthetic process to get the desired isomer of linagliptin and other DPP-4 inhibitors [3-6]. It is necessary to estimate and control the impurity as per

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regulatory bodies [7]. A few analytical methods are available for the determination of linagliptin and related impurities, including the process related and raw material related impurities [8-11] and another study reports about enantio-separation of linagliptin [12]. Jadhav *et al.* [13] discussed about determination of enantiomer in litagliptin with metformin from oral dosage-tablet forms.

Literature survey reveals that there is no validated, specific method was available for the evaluation of raw material (3-AMP) along with major degradents of linagliptin. As 3-AMP is non-chromophoric in nature, one sensitive method [14] reported for the determination of enantiomer content for the quality establishment of 3-AMP raw-material with pre-column derivitization using chiral separations [15]. This method is intended and validated only for the estimation of unwanted other isomer (*S*-isomer) in 3-AMP (*R*-isomer) as a raw-material. But this method will be critical for linagliptin analysis due to linagliptin and other possible related impurities will also undergo derivatization, which will interfere in LC method, hence chromatographic interference will impact the accuracy of the result.

Another method reported only for 3-AMP content determination in linagliptin sample by CAD detector by LC [16], where specificity of the method was not proved along with all other possible impurities and degradation impurities. There will be limitation in CAD to analyze the reaction mass sample analysis to monitor 3-AMP conversion in the synthetic process. The solvents used and other impurities arose in the synthetic process have more chance to interfere with 3-AMP in CAD analysis.

Even CAD method is a novel approach, LC with CAD detector is challenge for the QC analyst and CAD is very costly and limitedly available detector for the general QC laboratories, which is very often in use. The method transfer and implementation and life cycle of the method in the QC analysis also a challenging task. Since there is no specificity proved, validated method available in the literature for the quantification of potential impurity 3-AMP. Present work focuses on the development, forced degradation study and validation of simple, cost effective, QC friendly, easy to use and specific method for the determination of 3-AMP in linagliptin samples.

By considering above essentiality, a user friendly for QC personal, rapid, simple and mass compatible GC method [17, 18] with direct liquid injection technique has been developed, applied, validated and proved as specific method for the determination of 3-AMP content in linagliptin samples. It can be considered as orthogonal method [19-21] for 3-AMP content against CAD method. Several methods have been reported for the quantification of pharmaceutical compound by GC methods [22-27]. Because of GC technique, having the capability of showing non-interference from all the other process related impurities, which are majorly non-volatile and can be monitored by LC and will not show any response or detection in GC. This method can be applicable for other drug substances also, which contains 3-AMP as raw a material or process impurity or a dergadent.

Hence, a stability indicating simple, highly sensitive thermal gradient GC method was developed and fully validated as per ICH guidelines, where all the impurities have shown the non-interference from 3-AMP. Stability indicating capability of the method was evaluated by stress testing of samples followed by GC-MS studies and conformed the mass number of degradent. This GC method shows greater range from LOQ to 150% of the specification of 0.15% and high sensitivity than CAD method (Table-1).

A simple, rapid GC method was developed for the quantification of one of the non-chromophoric raw material (3-AMP), which is mild degradent as well in one of the harsh stress condition. The GC method was developed by using widely available GC 5% phenylmethyl polysiloxane stationary phase, Example: DB-5), 30meters length, 0.53 mm, 5.0 µm thickness column (which is non-polar, low bleed and with a high temperature limit), helium as carries gas, FID detector set at 240 ºC, column oven starts at 200 ºC and starts increases after 2 min hold with 20 ºC per min and holds up to 11 min.

Degradation samples were analyzed in GC method to evaluate 3-AMP impurity content and conforms the 3-AMP mass number by GC-MS study. Both the studies were helped to find the material (mass) balance of drug substance from degradation (stress) study. It is essential to know 3-AMP content in the synthetic process to monitor the progress and for the completion of reaction (reaction monitoring) and in final samples to get the quality of the final material of linagliptin sample to meet the specification as per ICH.

This method is used to monitor 3-AMP in reaction monitoring for synthetic process along with related substances method and assay method in-order to prove material balance. A RP-HPLC method has been developed with UV detection mode for the determination of assay and quantification of all other potential impurities, which are successfully separated from each other.

EXPERIMENTAL

Working standard of (*R*)-Piperidin-3-amine dihydrochloride (3-AMP) and samples of linagliptin were supplied by Dr. Reddy′s Laboratories Limited, Hyderabad, India.

Instruments: The GC instrument (Agilent technologies) consisting of auto sample manager was used. Output signal was monitored using FID detector and data processed using Empower software. Cintex digital water bath was used for hydrolysis studies. Photo stability studies were carried out in

TABLE-1
COMPARISON OF RESULTS WITH CURRENT PROPOSED GC METHOD WITH FID DETECTOR AND REPORTED LC METHOD WITH CAD DETECTOR

Parameter	3-AMP in proposed method GC-FID	LC with CAD
LOD	0.4 µg/mL (equivalent to 0.002% of sample concentration)	1.35 µg/mL (equivalent to 0.027% of sample concentration)
LOQ	1.4 µg/mL (equivalent to 0.007% of sample concentration)	2.73 µg/mL (equivalent to 0.055% of sample concentration)

a photostability chamber (Sanyo, Leicestershire, UK). Thermal stability studies were performed in a dry air oven (Cintex, Mumbai, India).

Chromatographic conditions: GC system was equipped with auto sampler and FID detector. The chromatographic column used was DB-5, 30meters length, 0.53 mm, 5.0 µm thickness. Liquid injector temperature set at 220 ºC, injection volume with 1.0 µL, split ratio is 1:5 and Helium was used as carrier gas with constant flow rate 3.0 mL/min.

Initial oven programme started at 200 ºC and hold for 2 min, raised to 240 ºC at the rate of 20 ºC/min and hold for 11 min. Total analysis time was 15 min.

Preparation of standard, test solutions and impurity stock solution: Test sample solutions of linagliptin were prepared by dissolving appropriate amount in diluent (methanol) to meet final concentration of 20 mg/mL.

Methanol was selected as diluent due to the advantage of freely soluble nature of sample and early elution of diluent (methanol) peak in GC to get non-interference and better separation from the analyte peak in the chromatogram, which provides the stable baseline with low level baseline noise.

Primary 3-AMP impurity stocks solutions were prepared by dissolving appropriate amount in diluent and pooled impurity stock was prepared by further diluting stock solution with diluent to get the known amount of 3-AMP at specification level.

Forced degradation and GC-MS studies for identification of degradents: The specificity of developed GC method was carried out in the presence of their other process related impurities. Linagliptin drug substance solutions at test concentration of 20 mg/mL were subjected to expose for chemical stress conditions of acid (0.5N HCl at 70 ºC), base (0.5 N NaOH at room temperature), hydrolytic (70 ºC), oxidation (3% H_2O_2 at RT) and analyzed in GC method for 3-AMP content. Further diluted the stressed samples to test concentration of 0.5 mg/mL with diluent and analyzed as per related substances by HPLC in-house method conditions. Physical stress at UV light as per ICH (1.2 M Lux-hours) [28,29] and heat stress at 105 ºC to evaluate the ability of the proposed method to separate respective active from their degradation products. For heat and light studies, study period was continued till 10 days where as for hydrolytic, acid the stress time was about 30 and 16 h for base stress and 10 min for oxidation.

Initially, the above stress samples were analyzed for assay determination and related substances by HPLC in-house method. The net degradation was observed > 5% in acid, base and peroxide degradations in related substances by HPLC method. Then all the stressed samples were analyzed for 3-AMP content in the developed GC method and evaluated the specificity of method.

GC-MS study for identification of 3-AMP: GC-MS system (Shimadzu 2010 GC-MS equipped with single quadrupole mass analyzer and operating with GCMS solutions software, Shimadzu, Japan) was used for identification of degradent formed during forced degradation studies. The analysis was performed in electron impact ionization mode, the ion source voltage was 70 eV and the source temperature was 250 ºC.

Method validation: The proposed method was validated as per ICH guidelines [30,31].

Precision: Repeatability was assessed by injecting six individual test preparations as per test method in to GC system, which were prepared by spiking accurate volume of 3-AMP pooled stock solution to meet (30 µg/mL) 0.15% of target concentration (20 mg/mL) of linagliptin. The %Mean and %RSD of 3-AMP was evaluated.

As a part of ruggedness, intermediate precision of method was also evaluated by different analyst, different GC instrument with different column and the analysis was performed on different day-2.

Limit of detection (LOD) and quantitation (LOQ): The LOD and LOQ for 3-AMP were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. A precision study was also carried out at the LOQ level by injecting six individual preparations and calculating the RSD (%) of the area.

Linearity: Linearity test method was performed by preparing stock solutions followed by further dilutions of 3-AMP at six concentration levels ranging from LOQ to 150% of target concentration. Peak area *versus* concentration data was treated by least-squares linear regression analysis and slope, y-intercept, coefficient of correlation, bias at 100% specification level was evaluated.

Accuracy: The accuracy of test method for 3-AMP was evaluated in triplicate preparations of test sample spiked 3-AMP at each level ranging from 50% to 150% and recovery at LOQ was performed by preparing three test solutions spiked with 3-AMP. %Added *vs.* %found and % recovery for 3-AMP was evaluated.

Robustness: To determine the robustness of the developed method, experimental conditions were deliberately altered, ±10% of flow rate, ± 5 ºC of column initial temperature were altered from original conditions, system suitability, tailing factor and retention time (RT) of 3-AMP was monitored.

Solution stability: Solution stability was determined by leaving solutions of standard and spiked test sample in tightly capped volumetric flasks for a period of 48 h at room temperature during, which they were analyzed at 24 h intervals and its stability was determined by analyzing the freshly prepared standard solution at 24 h intervals till 2 days and the results were compared with those obtained from freshly prepared standard solutions.

RESULTS AND DISCUSSION

Method development and optimization: The main aim of this method development is to develop a simple, rapid, sensitive and specific method for the estimation of 3-AMP in linagliptin release and stability samples. A simple and widely available conventional column was used to minimize the analytical expensive for the analysis. GC column with diphenyl dimethyl polysiloxane phase (example, DB-5/AT-5) is a common column for the estimation of residual solvents for many of the products.

Initial trails provides the longer retention time and more tailing for 3-AMP with lover initial oven operating temperature at 40 ºC. Different trails were conducted with different column oven temperatures. Improved symmetry of the peak was observed and tailing was decreased to close extent of 1.1 by increasing

the oven temperature. Split ratio and injection volumes were also optimized to get the lower base line noise and peak symmetry.

Moderate retention time and good separation from process solvents (Fig. 1) and diluent provides the confident on the method to analyze linagliptin samples for 3-AMP content. There was no interference observed from the intermediates, process related impurities and other degradation impurities with 3-AMP.

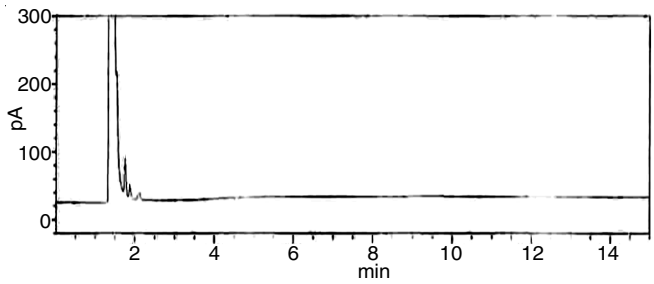

Fig. 1. Chromatogram of spiked sample with process related residual solvents

Methanol was selected as diluent and same was used for injection syringe washing post- and pre-analysis of samples. No carryover of the analyte was observed during the analysis. Satisfactory S/n ratio with >10 was attained for 3-AMP at LOQ (0.007%) of target concentration *i.e.* 20 mg/mL. With finalized chromatographic conditions, which is mass compatible method, also showed satisfactory separation from other degradation impurities. The present method also have the choice to run on GC with mass detector (GC-MS) to prove the specificity of the method. The *m/z* was conformation for 3-AMP standard by using this method.

Forced degradation and GC-MS studies: Significant degradation of linagliptin was achieved by acid, base, H_2O_2 and water hydrolysis, which was confirmed by related substances by HPLC method. No major degradation was observed in thermal and photolytic (light) degradation. Mass balance was calculated for all the degradation samples. A mild degradation was observed in base stress (Fig. 2) study with *m/z* value of 100 (Fig. 3).

Mass balance (% assay and net degradation) was performed by diluting stressed at assay concentration of 100 µg/mL and calculated against reference standard. The stressed sample were also analyzed for *S*-isomer (S-LIP) content by diluting the samples to 1.0 mg/mL with methanol (diluent) and found that

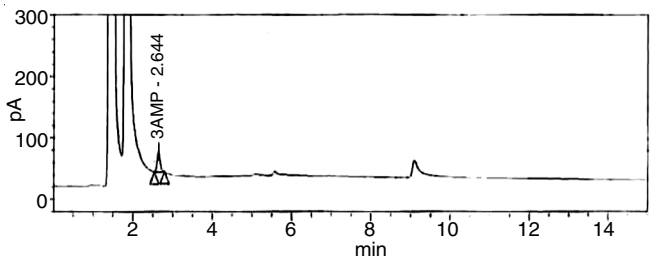

Fig. 2. Base stress sample chromatogram

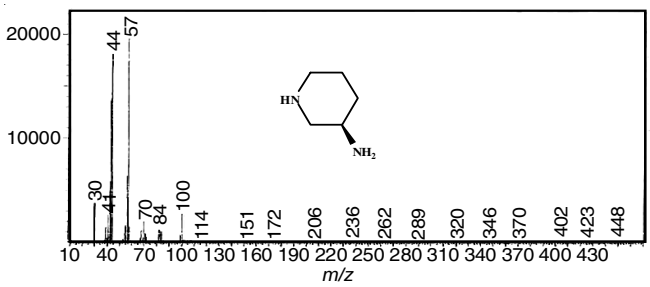

Fig. 3. GC-MS chromatogram of base stress sample (*m/z* 100, 3-AMP imp)

S-isomer was not detected, which is also proved with same stress conditions by validated chiral HPLC method [12]. Hence *S*-linagliptin content was excluded from the results and mass balance calculations. The non-interference of related impurities and deg-radation products with 3-AMP was ensured and thus confirms the stability-indicating power of method (Table-2).

Stability studies and reaction monitoring: Stability studies for linagliptin drug substance in primary packing (nitrogen filled polybag, along with silica gel) up to 6 months accelerated condition 40 ºC/75%RH were analyzed and impurity trending was evaluated. No potential degradents were detected in this GC method and rest of known impurities were monitored by HPLC.

Precision: The %RSD for 3-AMP content in method precision and intermediate precision study was < 1.4%. Results in Table-3 demonstrated that the method is precise and stood rugged, resisting day-to-day, system-to-system, column-to-column and analyst-to-analyst variations.

LOD and LOQ: S/N ratio with > 10 for LOQ of 0.007% and S/N ratio with > 3 for LOD of 0.002% of test concentration were achieved. Mean values of precision (%RSD < 2.8) at LOQ of 3-AMP are reported in Table-4 and recoveries at LOQ

TABLE-2
FORCED DEGRADATION STUDIES

Sample name	Stress condition	% Degradation by HPLC**	%3-AMP by GC***	Assay by HPLC**	% Mass Balance*
Unstressed	As is test sample	0.10	ND	99.9	NA
Acid stressed	0.5N HCl 70 °C-30 h	6.92	ND	92.1	99.1
Base stressed	0. 5N NaOH RT-16 h	5.04	0.25	94.3	99.4
Oxidation stressed	3% H_2O_2 RT-10 min	5.57	ND	92.9	98.6
Water stressed	70 °C 30 h	5.48	ND	95.2	100.8
Thermal stressed	105 °C –10 Days	0.17	ND	99.2	99.5
Photo stressed	1.2 M Lux	0.09	ND	99.7	99.9
Photo stressed	Visible region	0.10	ND	99.5	99.7

* Mass Balance = %Degradation by HPLC + %3-AMP by GC + % assay by HPLC.
**% Degradation calculated from total of other related impurities quantified by in-house validated HPLC method.
***0.25% of 3-AMP was observed in GC method in base degradation sample, which is confirmed by GCMS, in other all deg. samples it is ND.

TABLE-4
LOD, LOQ, PRECISION & ACCURACY

Name	%LOD	% LOQ	Precision at LOQ %RSD	Recovery @ LOQ	Recovery @ 50%	Recovery @ 100%	Recovery @ 150%
3-AMP	0.002%	0.007%	**2.8**	104.4	100.0	102.6	104.4

Obtained USP s/n > 3 for % LOD and > 10 for % LOQ. Acceptance criteria for %RSD for LOQ Precision is NMT 15.0%, Recovery at LOQ, 50%, 100% & 150% should be within 85-115%.

TABLE-5
LINEARITY AND REGRESSION

Name	Slope	y-Intercept	Correlation coefficient	Bias at 100% level
3-AMP	1838.0	-0.8	0.9996	-1.5

For Linearity: Correlation coefficient > 0.997 and bias should be < ± 5.0%

TABLE-3
PRECISION AND INTERMEDIATE PRECISION OF 3-AMP

Name	Precision (%)	Intermediate precision (%)
Preparation-1	0.155	0.156
Preparation-2	0.151	0.161
Preparation-3	0.149	0.151
Preparation-4	0.147	0.149
Preparation-5	0.152	0.148
Preparation-6	0.157	0.152
Average:	0.152	0.153
STDEV:	0.004	0.005
%RSD:	2.4	3.2

Acceptance criteria: %RSD for precision and intermediate precision NMT 10.0%.

level to 150% level are in the range of 99.9 to 104.4%. This shows the method's extraction efficiency and sensitivity towards recovery and sensitivity.

Linearity: Linear calibration plot for 3-AMP method was obtained over the calibration ranges tested, *i.e.*, LOQ to 150% of the specification level. The correlation coefficient obtained was greater than 0.9995 for 3-AMP. The slope and y-intercept and bias values are also provided in Table-5, which confirmed good linearity between peak areas and concentration. The %bias shows the exactness of the results at lower levels also.

Accuracy and range: Recovery of 3-AMP in spiked studies was ranged from 99.9% to 104.4% and this satisfactorily fulfills the recovery of analyte (Table-4). Range of the method for analyte was verified by performing precision at lower limit *i.e.* LOQ and higher limit *i.e.* 150% of target concentration and results found satisfactory.

Solution stability: Established the stability of standard and spiked sample solutions up to 2 days on bench top at room temperature (RT) condition, compared the results with freshly prepared standard at different time intervals and was found stable till 2 days.

Robustness: In all the varied chromatographic conditions, (flow rate variation and column oven initial temperature variation) the tailing factor and similarity factor were within acceptable limits (< 1.5 and < 1.1, respectively).

Conclusion

A simple, highly sensitive, QC user friendly, rapid, mass compatible GC method has been developed for quantitative determination amino-3-piperidine (3-aminopiperidine, 3-AMP) in linagliptin was found to be precise, accurate, linear, rugged, robust and specific. The method is stability-indicating and can be used for routine analysis of reaction monitoring, stability and finished production samples.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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