QUANTIFICATION OF HYDRAZINE HYDRATE IN IMATINIB MESYLATE AT GENOTOXIC LEVEL BY CHROMATOGRAPHIC METHOD

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(Received 29 June 2019) (Accepted 15 November 2019)

ABSTRACT

Hydrazine hydrate has genotoxic effect in nature and so it should be controlled down as Potential Genotoxic Impurity (PGI). Being polar molecule, hydrazine hydrate ($N_2H_4{\cdot}H_2O$) has no chromophores present in structure which can follow Lambert beer law, thus it is difficult to analyze. The present work described an accurate and highly sensitive reversed-phase liquid chromatography-UV derivatization method for determination of hydrazine in imatinib mesylate drug substance. The method of quantification was developed by attaching chromophores to hydrazine with derivatization, which helped to increase sensitivity. The derivatization of hydrazine hydrate was performed using 1% methanolic solution of benzaldehyde which acts as derivatizing agent. The derivatized product 1,2-dibenzylidenehydrazine gives maximum absorbance at 300 nm and at this wavelength no interference of solvents and other impurities are noted. Limit of detection for developed method was 0.002 µg/g. The developed method was validated to determine hydrazine content and can be used in quality control for commercial batch release of imatinib mesylate drug substances with a genotoxic specification limit level 1.87 µg/g by HPLC.

Keywords: Hydrazine, Imatinib Mesylate, Potential Genotoxic Impurity (PGI), Chromatographic Method, Method Validation

INTRODUCTION

Hydrazine hydrate is a colorless flammable liquid which has pungent ammonia-like odour and is very dangerous to handle in solution[1,2]. Hydrazine hydrate is highly reactive and posses carcinogenic activity in nature, inspite of these limitations it is used in manufacturing of numerous intermediates and pharmaceutically active ingredients in bulk productions[3,4]. Structural characteristics present in hydrazine hydrate are mainly responsible for the genotoxicity. Moreover, metabolites produced from metabolism of hydrazine hydrate have synergistic effect on genotoxic potential of hydrazine hydrate[5]. At highly reactive methyl diazonium ions and methyl free radicals are formed when hydrazine hydrate is intercalate with DNA which further cause cellular damage[6]. In addition hydrazine hydrates reacts with endogenous formaldehyde and tends to produce formaldehyde hydrazone which is also genotoxic in nature[7]. Alkylating compounds such as diazomethane produced as metabolites of hydrazine hydrate and also account for the known genotoxicity[8]. Hydrazine hydrate has been employed as reducing agent in various reactions, for example Knorr synthesis,[9,10] Gabriel synthesis[11] and Wolff-Kishner reaction[12] and should be controlled at Therapeutic Threshold Concentration (TTC) limit[13,14]. Estimation of hydrazine hydrate is very difficult as it does not possess chromophores which can be detected on UV spectroscopy or on HPLC nor ionizable group for LC-MS nor carbon atoms for flame ionization detection (GC). Due to these reasons, derivatization has a strategic advantage in development of highly selective and sensitive method for determination of hydrazine hydrate. For determination and quantification of hydrazine hydrate, various methods are reported such as GC-MS,[15,16] LC-MS/MS[4,17,18]. High performance Liquid chromatography[19-21] and ion chromatography.[22] Most of the methods use derivatization for estimation of hydrazine hydrate e.g, Zhang et.al[23]. developed a method having 0.25 ppm detection limit using 2-hydroxy-1-naphthalaldehyde as a derivatizing agent, while Tamas et.al[24]. have developed a method using benzaldehyde as a derivatizing agent in allopurinol with solid phase extraction for sample preparation, but no method is available for hydrazine to quantify at a TTC level by HPLC.

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https://doi.org/10.53879/id.56.12.11971

Imatinib mesylate is a tyrosine kinase inhibitor used to treat patients with hematological malignancies or malignant sarcomas such as gastrointestinal stromal tumors, chronic myeloid leukemia, acute lymphoblastic leukemia and gastrointestinal stromal tumors[25,26]. Imatinib mesylate is designated chemically as 4-[(4-methylpiperazin-1-yl) methyl]-N-(4-methyl-3-phenyl) benzamide methane sulfonate and is a specific inhibitor of the tyrosine kinase in Bcr-Abl^{+} cell.[27] It is an approved drug for treatment of gastrointestinal stroma tumor since 2002[28] and for chronic myelogenous leukemia since 2011[29] by United States Food and Drug Administration. Recently, we have reported the development and validation of residual solvent determination by headspace gas chromatography in imatinib mesylate API[30]. Reduction of nitro group during the synthesis of imatinib can be achieved by several reducing reagents such as Fe/HCl, $SnCl_2$/HCl, hydrazine hydrate/Raney Ni and hydrazine hydrate/$FeCl_3$/C. The use of Fe/HCl and $SnCl_2$/HCl as reducing agent were not preferred, as due to presence of metallic hydroxides emulsion formation occurs during isolation process of imatinib. $SnCl_2$ is an expensive reagent and also toxic[31]. In comparison to other reduction process, the reduction with hydrazine hydrate produces harmless byproducts such as nitrogen gas and water. Route of synthesis of imatinib has been described. In the synthesis stage-1, hydrazine hydrate plays an important role as reducing agent (Fig.1). The genotoxicity of hydrazine hydrate is proven.

The aim of our study was to develop an accurate, sensitive, robust, selective and reproducible method which can analyze hydrazine in imatinib mesylate API. In the developed method, selection of derivatization reagent was a critical parameter, by which derivatized product must give strong UV absorption to increase sensitivity of analytical method[23]. To optimize selectivity of the developed method complete conversion of hydrazine to derivatized 1,2-dibenzylidenehydrazine to confirm true sample hydrazine content mark checked. The derivatized product having higher resolution from API and related impurities and product has been confirmed by LC-MS. By these strategies, development of a sensitive derivatization method for hydrazine hydrate content determination in imatinib mesylate API using reversed-phase HPLC-UV was developed for the 1,2-dibenzylidenehydrazine product to achieve the resolution requirements needed for genotoxic level analysis. The optimized method was selective, sensitive, accurate, robust, linear, precise and reproducible.

MATERIALS AND METHODS

Chemicals and Reagents

Active pharmaceutical ingredient imatinib mesylate samples were synthesized in Cadila Healthcare Ltd. (Ahmedabad, Gujarat, India), For route of synthesis refer Fig.1. Hydrazine hydrate (>99%) and benzaldehyde solution (>99%) were purchased from Sigma-Aldrich (Darmstadt, Germany). Glacial acetic acid, ACN (HPLC grade) and methanol were obtained from Merck (Darmstadt, Germany). High purity HPLC Grade water was obtained by Milli-Q water purification system (Millipore, Darmstadt, Germany).

Chromatographic conditions and equipment

HPLC analysis was conducted on a Shimadzu Prominence HPLC-DAD system (Japan) equipped with a SIL-20ACauto sampler, LC-20AT binary pump, CTO-10AS thermostatic column compartment and SPD-M20Aphotodiode array. An Inertsil ODS-3V HPLC column (5.0 µm, 4.6 × 250 mm) from GL Sciences Inc. (Japan) was used for the analysis. A Sartorious micro balance CP225D (Germany) and Sonicator (PCI Analytics, India) were used for the sample preparation and the derivatization reaction.

LC–MS conditions

An electrospray LC–MS system (Shimadzu Prominence HPLC coupled with Triple Quadropole Mass Spectrometer LCMS-8040 with Lab Solution software, version 5.72, Japan) was used for identification of derivatized product. A Chromatography was performed on Inertsil ODS-3V HPLC column 250-mm, 4.6 mm and 5 µm particle size column from GL Sciences Inc. (Japan) using mobile phase includes, Solvent A was water and solvent B was mixture of acetonitrile:water-9:1 %V/V with pump flow rate of 1.5 mL/min. The LC isocratic mode consisting premix ratio of Solvent A-30% and Solvent B-70%. The column temperature was maintained at 40°C. Methanol was used as a diluent. Injection volume was 50 µL. The analysis was carried out by using electro spray ionization mode (+ve). Capillary voltage at 3500 V and collusion Energy -35V. Desolvation temperature is 250°C with nebulizing gas flow rate 180 L/h. LCMS Chromatograms are shown in Fig. 2.

Derivatization solution preparation

1 mL of benzaldehyde solution diluted to 100 mL with methanol (1% benzaldehyde solution in methanol).

Acetic acid solution preparation

0.06 mL of acetic acid solution diluted to 100 mL with methanol (0.06% acetic acid solution in methanol).

Standard solution preparation

Standard stock solution of 1.5 mg/mL hydrazine was prepared with methanol. Dilution was made to prepare second stock of 0.15 µg/mL hydrazine in methanol. From the second stock solution take was 5 mL solution into 50 mL volumetric flask. To that 1 mL of benzaldehyde solution and 2 mL acetic acid solution were added and heated at 50°C-55°C for 60 min. Samples were removed, cooled to room temperature and made up with methanol and injected directly to HPLC column.

Test solution preparation

400 mg of imatinib drug substance was taken in 50 mL volumetric flask add 5 mL methanol was added to dissolve and 1 mL of benzaldehyde solution and 2 mL acetic acid solution were added and heated at 50°C-55°C for 60 min. Samples were removed, cooled to room temperature and made up with methanol and injected directly to HPLC column.

RESULTS AND DISCUSSION

Optimization of derivatization reagent

The selection and optimization of a derivatization agent is a critical parameter to designing this method for hydrazine hydrate analysis. To develop sensitive, selective and efficient method, the derivatization agent should maximize conversion of free hydrazine hydrate to the derivatized 1,2-dibenzylidenehydrazine. The derivatized 1,2-dibenzylidenehydrazine product should have a strong absorption in UV at 300 nm, so its maxima should be far away from the solvents and reagents interferences of the API. In high pressure liquid chromatography, the 1,2-dibenzylidenehydrazine reaction is widely suitable because the resultant 1,2-dibenzylidenehydrazine product is lipophilic in nature and should be highly retained on reverse-phase chromatographic column i.e. C18, C8 and C6. The benzaldehyde is an economical commercially available and effective derivatizating agent for multiple batch analysis of imatinib mesylate. Fig. 1 illustrates the proposed reaction of one benzaldehyde molecule with one hydrazine molecule to form a 1,2-dibenzylidenehydrazine product. Methanol served as a solvent to dissolve the imatinib mesylate, derivatizing reagent benzaldehyde and derivatized product. The reaction was optimized for reproducibility, selectivity and sensitivity.

A number of reaction conditions, such as pH, reaction temperature and reaction time were optimized through different studies, the results of which are collectively summarized in Fig. 2a. & Fig.2b, showing the effect of time and temperature on the derivatization reaction.

While there were uneven results found in reaction efficiency in basic pH (achieved by adding 1% NaOH in methanol), however in acidic pH achieved by adding 0.06% acetic acid in methanol reproducible results obtained due to accurate reaction efficiency, In addition, increased temperatures at 50°C facilitated the reaction in forming the derivatized 1,2-dibenzylidenehydrazine product. Reaction kinetics at 50°C was monitored from 15 min to 4 h (Fig. 2), showing that 60 minutes was sufficient to reach completion of the reaction. The excess amount of benzaldehyde in solution did not interfere with the chromatography and analysis of hydrazine hydrate. Based on these studies, the optimal reaction conditions were found to be 50°C in a water bath for 60 min using 1% methanolic benzaldehyde solution as the derivatizing solution with 0.06% methanolic acetic acid solution. Derivatized 1,2-dibenzylidenehydrazine product was confirmed by LCMS as per section 2.3 conditions. For

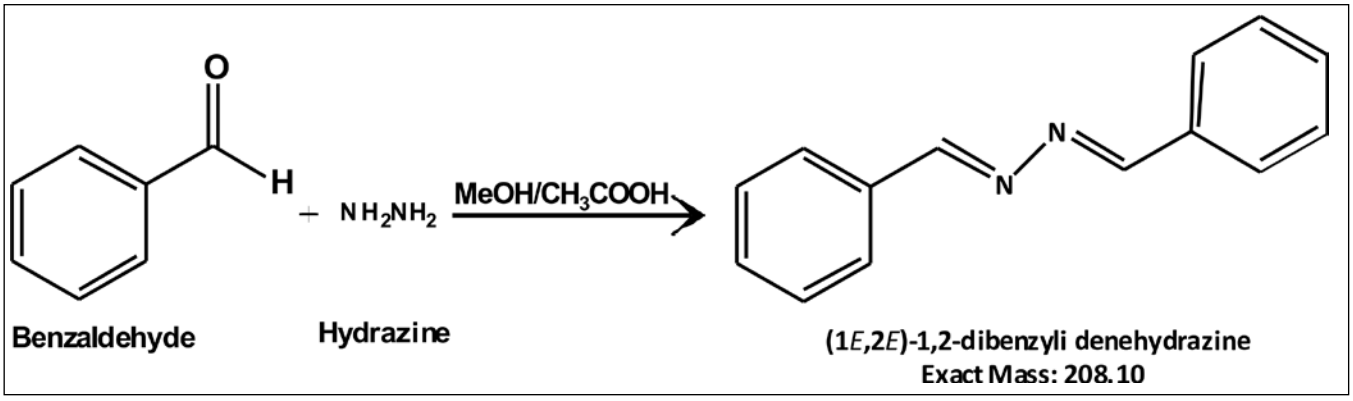

Fig. 1: Reaction scheme of benzaldehyde with hydrazine in acidic condition for derivatization

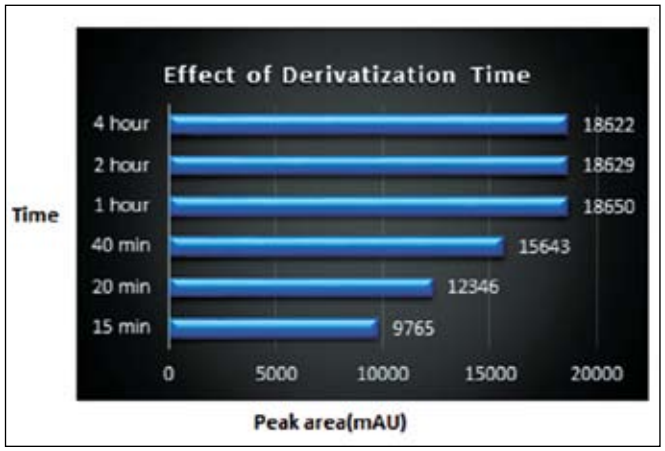

Fig. 2a: Reaction kinetic study with increasing time in acidic condition at 50°C for derivatization

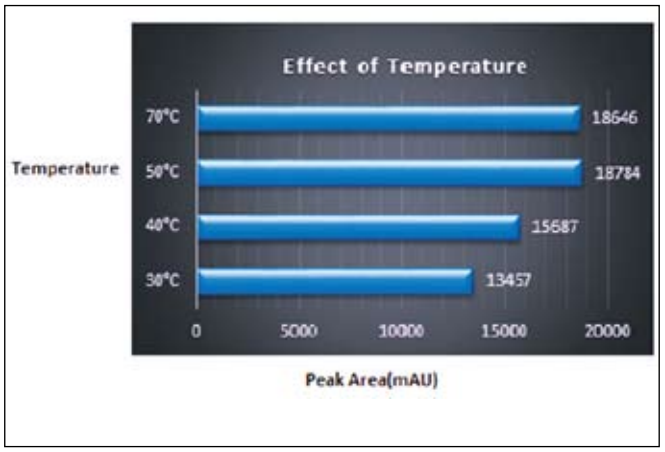

Fig. 2b: Reaction kinetic study with increasing temperature in acidic condition for derivatization

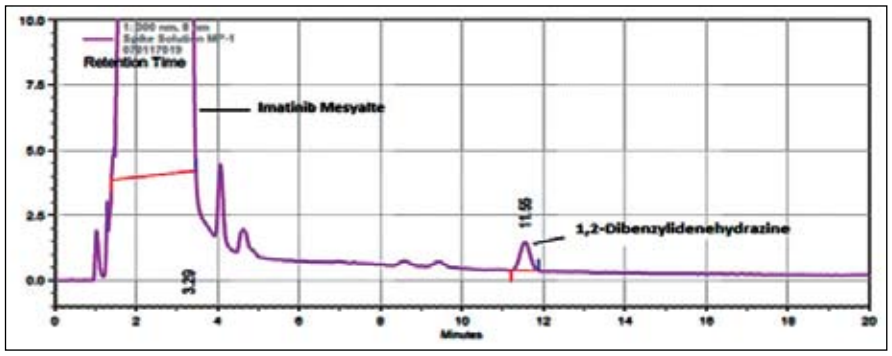

Fig.3. Chromatogram of spike solution having 0.015 µg/g hydrazine spiked in 8000 µg/g imatinib mesylate

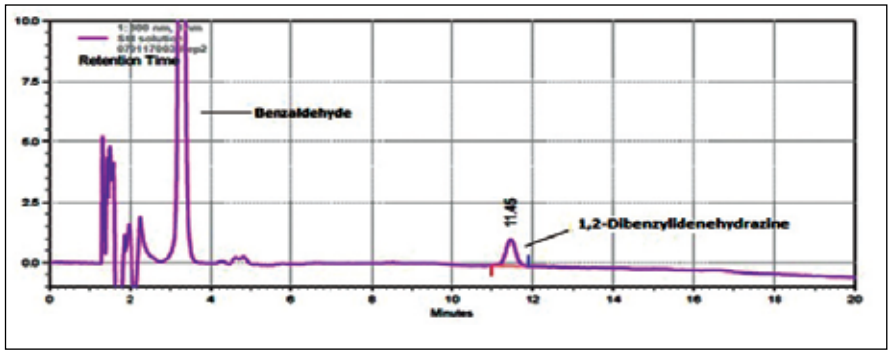

Fig.4. Chromatogram of standard solution having 0.015 µg/g derivatized 1,2-dibenzylidenehydrazine

LCMS chromatogram, refer Fig. 2, which shows molecular ions at m/z 209.05 (M^++H) confirming that resultant product was 1,2-dibenzylidenehydrazine.

Chromatographic method development and optimization

A reversed-phase chromatographic method was developed to analyze the derivatized 1,2-dibenzylidenehydrazine product. A number of reverse phase columns of different make and different stationary phase chemistries were screened in the method development process such as Alltima C18, Waters Symmetry C18, X-bridge Shield C18, Eclipse XDB Phenyl, Hypersil BDS C18, Zorbax SB-Phenyl and YMC Triat C18. In the best chromatographic method, imatinib API matrix and related substance elute near the dead time and far away from the derivatized 1,2-dibenzylidenehydrazine product. Inertsil ODS-3V was selected because of high retention time of the 1,2-dibenzylidenehydrazine product and optimum resolution from the API peak and derivatizing agent (benzaldehyde) peak as seen in Fig. 3. & Fig. 4, respectively. Fig. 3. depicts chromatogram of spike solution which shows a well separation between imatinib mesylate and 1,2-dibenzylidenehydrazine peak (resolution was 23.6). Fig. 4 is a chromatogram of standard solution shows good separation between benzaldehyde and 1,2-dibenzylidenehydrazine peak. The peak shape and resolution of the 1,2-dibenzylidenehydrazine product was excellent with this column. Finally, in reversed-phase LC method water was used as mobile phase-A, while acetonitrile: water-90:10%V/V was used as mobile phase-B). A low rate of 1.5 mL/min was used with an injection volume of 50 µL while the column temperature was set at 40°C. Detection wavelength of 300 nm UV was selected for detection of

derivatized 1,2-dibenzylidenehydrazine. Total run time was 20 min. Isocratic flow was selected in the ratio of MP-A: MP-B-30:70%V/V. The HPLC method parameters are summarized in Table I and system suitability parameters are summarized in Table II. Peak purity results are summarized in Table III.

Table I: Summary of final HPLC method parameter conditions

Parameter	Conditions
HPLC Column	Inertsil ODS-3V, 5µm, 4.6 × 250 mm
Mobile Phase	A: water
	B: Acetonitrile:Water-90:10 %V/V
Injection Volume	50µL
Isocratic	Solvent A:Solvent B-30:70
Flow Rate	1.5mL/min
Column Oven Temp.	40°C
UV Wavelength	300nm
Run Time (min)	20 minutes

Table II: Results for system suitability parameters by RP-HPLC

Parameters	Observed results (n=6)	Acceptance criteria	Remarks
Theoretical plates	11223	> 2000	Method passes the system suitability test
Tailing factor	1.03	T ≤ 1.5	
Repeatability (% RSD)	1.70	%RSD <5	
Resolution	23.6	Rs <2	

Table III: Results of peak purity

Parameters	Observed results	Acceptance criteria	Remarks
Purity angle	7.519	Purity angle should be less than purity threshold	Peak is pure
Purity threshold	7.986		

METHOD VALIDATION

The developed method has been validated for content of hydrazine hydrate in imatinib mesylate by HPLC as per ICH guideline. The method was validated for its specificity, linearity, range, accuracy and precision to demonstrate that the method is suitable for its intended use as per ICH Q2 (R1) guideline. Method validation data are summarized in Table IV.

Specificity

The specificity of this method was demonstrated by separation of imatinib mesylate API. The resolution of the 1,2-dibenzylidenehydrazine derivative from imatinib and benzaldehyde is greater than 2.5. The representative chromatograms are shown in Fig. 3 and Fig. 4.

METHOD PRECISION

Repeatability of the method was checked by analyzing six replicate samples of 8000 µg/g imatinib mesylate spiked with 0.0150 µg/g of hydrazine hydrate (at 100% level). The %RSD was calculated for hydrazine hydrate for its content. The percent relative standard deviation of recovery of six replicate injections at spike level was 0.87%.

Sensitivity

Sensitivity of the method was proved by establishing the limit of detection (LOD) and limit of quantitation (LOQ) for hydrazine hydrate with a signal-to-noise ratio of 3:1 and 10:1, respectively. Accuracy at LOQ level was verified by injecting three individual preparations of imatinib mesylate spiked with hydrazine hydrate at LOQ level and by calculating % recoveries of hydrazine hydrate content. The limit of detection at 0.0020 µg/g showed an S/N ratio range from 4-6, while a range of 15-20 of S/N ratio was observed for quantification limit of 0.0040 µg/g. The quantification limit was also validated with sample matrix where 0.0040 µg/g of hydrazine was spiked in imatinib mesylate. The average percent of recovery of three replicate injections at LOQ level was 101.3% with a %RSD of 0.87%.

Linearity and range

To establish linearity of the method, solutions were prepared by diluting the hydrazine hydrate impurity second stock solution to obtain the required concentrations at six different levels ranging from LOQ to 150% (i.e. LOQ (0.0040), 0.0075, 0.0120, 0.0150, 0.0180 and 0.0225µg/g). The correlation coefficient, slope and y-intercept of the calibration curve were calculated. The method exhibits good linearity and range with a linear regression fit of R^2 = 0.9930 with a best fit equation of y=1147892.335x+1018.6, linearity overlay chromatograms are shown in Fig.5. The method has been demonstrated to be linear in a range of 25% to

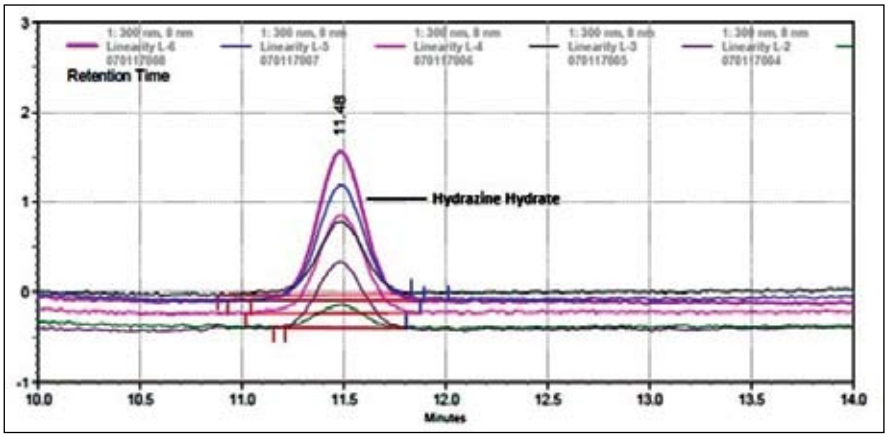

Fig. 5: Overlay linearity chromatogram of derivatized hydrazine hydrate covered concentration of 0.0040 to 0.0225µg/g

Table IV: Validation data summary of the developed HPLC method

Parameters	Results		
Calibration curve range	0.0040 - 0.0225 µg/g		
Regression line equation	Y = 1147892.3353x+1017.611		
Slope	1147892.3		
Intercept	1017.6		
Steyx	1021		
Correlation coefficient (r^2)	0.9930		
Accuracy	Concentration	% Recovery	% RSD
25% Level	0.0040 µg/g	101.32	0.87
50% Level	0.0075 µg/g	105.24	0.58
100% Level	0.0150 µg/g	99.55	0.96
150% Level	0.0225 µg/g	97.31	0.35
Method Precision-100%(n=6)		-	1.47

150% level from 0.0040 µg/g to 0.0225 µg/g.

ACCURACY

For the determination of accuracy of method, recovery study was carried out by analyzing the spiked samples. The known amounts of hydrazine hydrate was spiked in triplicate at four different concentration levels of 0.0040, 0.0075, 0.0150 and 0.0225 µg/g to a previously analyzed imatinib mesylate drug substance sample. The percentage of recoveries for hydrazine hydrate was calculated. The accuracy and precision was validated on a imatinib mesylate spiked with hydrazine hydrate at four concentration levels covering the specific range with 6 replicates for 0.0150 µg/g and 3 replicates for 0.0040, 0.0075 and 0.0225 µg/g. The imatinib mesylate was prepared at a concentration of 8000 µg/g. The percent recovery was calculated by spiking hydrazine in imatinib mesylate API. The individual percent recoveries for all preparations were from 97.3–105.2% and the %RSD for all injections was 1.2%.

RESULT

The hydrazine hydrate content was determined by developed and validated method for commercial batch of imatinib mesylate API in triplicate. The hydrazine hydrate contents were not detected in any of the three batches (Table V).

Table V: Hydrazine hydrate content in the commercial batch of imatinib mesylate API

Batch Sr. No	Hydrazine content (parts per million)
Batch set-1	ND
Batch set-2	ND
Batch set-3	ND

CONCLUSION

A sensitive and accurate method for the quantification of hydrazine hydrate in imatinib mesylate using an efficient derivatization reaction and RPLC-UV has been developed. 1% methanolic benzaldehyde solution plays a key role as derivatizing agent, which generated the derivatized product 1,2-dibenzylidenehydrazine that meets the ideal requirements of analytical strategies for quantification. The derivatization effectively shifts the resultant 1,2-dibenzylidenehydrazine product away to higher wavelengths in the UV spectrum where API matrix components & solvents do not interfere with the analysis. A specific LC-UV method using an Inertsil ODS-3V column was tailored to achieve the desired chromatography with the 1,2-dibenzylidenehydrazine product and was demonstrated for suitable specificity, linearity/range, accuracy and precision. The LOQ of the method was determined to be 0.0040 µg/g (%w/w) and was adequate for sensitive quantification of hydrazine hydrate in imatinib mesylate API.

ACKNOWLEDGEMENTS

The authors would like acknowledge the management of Cadila Healthcare Ltd. for support and encouragement. ZRC Communication No.574.

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