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Determination of Methyl 2-Amino-3-Nitrobenzoate Genotoxic Impurity in Candesartan Cilexetil Drug Substances using HPLC Technique

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ABSTRACT

A sensitive, cost-effective, reproducible HPLC method was developed and validate for the quantitative determination of genotoxic impurity methyl 2-amino-3-nitrobenzoate present in candesartan cilexetil drug substance by applying the concept of threshold of toxicological concern, a limit of 46.88 µg/g was calculated based on the maximum daily dose of the drug substance. The impurity was separated on Kromasil C18 250 x 4.6 mm, 5 µm analytical columns with a mobile phase consisting of the buffer pH 3.0 and acetonitrile with the gradient program at a flow rate 1.0 ml/min. The effluent was monitored by UV detection at 235 nm with column temperature maintained at 30 °C and the injection volume 10 µl. Validation activity was planned and completed based on the ICH guideline. The LOD and LOQ value were found to be 1.55 µg/g and 4.70 µg/g and accuracy results were well in the range 99.02 to 102.68 %. The linearity curve showed the correlation coefficient of 0.9999 and method very sensitive.

Keywords: Impurity; HPLC; TTC; Validation; ICH guidelines

INTRODUCTION

Candesartan cilexetil is a non-peptide tetrazole derivative drug, chemically described as 2-ethoxy-3-[21-(1H-tetrazol-5-yl)biphenyl-4ylmethyl]-3H-benzoimiadazole-4-carboxylic acid 1-cyclohexyloxycar-bonyloxy ethyl ester [1]. Its molecular formula is $C_{33}H_{34}N_6O$ and molecular weight 610.67 [2]. The drug is used mainly for the treatment hypertension and commercially available in 4 mg, 8 mg, 16 mg and 32 mg of tablet strength either individually or combination with other antihypertensive drugs [3-6].

Methyl 2-amino-3-nitrobenzoate is the most important intermediate used in the synthesis of candesartan cilexetil [7, 8]. This is identified as a genotoxic impurity in candesartan cilexetil according to the guidelines [9, 10]. Based on the threshold of toxicological concern (TTC) and the maximum daily dosage of candesartan cilexetil the evaluation limit of its genotoxic impurity is calculated 46.88 µg/g [9]. The chemical structures of candesartan cilexetil and methyl 2-amino-3-nitrobenzoate are presented in figure 1 [7].

Detailed literature survey reveals that the many HPLC methods are available for the determination of the drugs individually or in combination with other drugs [11-17]. GC-MS method describes the determination of genotoxic impurity in candesartan cilexetil drug [18]. There was no HPLC method available for quantitative determination of methyl 2-amino-3-nitrobenzoate in candesartan cilexetil. The objective of present study was to develop and validate a specific and sensitive HPLC method for the quantitative determination of genotoxic impurity methyl 2-amino-3-nitrobenzoate present in candesartan cilexetil drug substance.

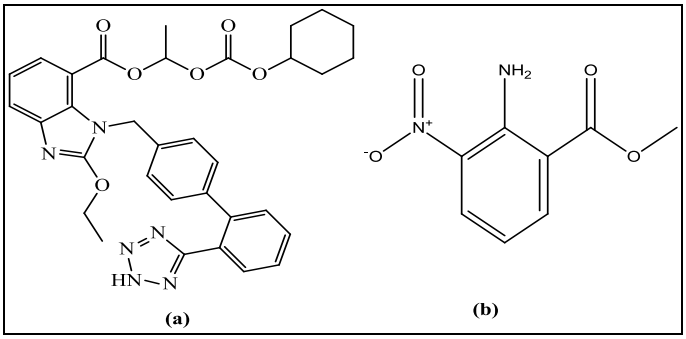


Figure 1: Chemical structure of (a) Candesartan cilexetil and (b) Methyl 2-amino-3-nitrobenzoate

EXPERIMENTAL SECTION

Chemical and reagents

HPLC grade of water, orthophosphoric acid, methanol, and acetonitrile were purchased from Merck, Mumbai India. AR grade of Sodium dihydrogen phosphate monohydrate was purchased from Rankem, Mumbai India. All pure drug substances and impurities are used for research purpose were procured in-house Macleods pharmaceutical LTD.

Instrumentation

The HPLC system consisted of Shimadzu model LC 2010 $C_{HT,}$ UV and PDA detector. The output signals were monitored and integrated using chromeleon software. Sartorius analytical balance and Pico^{+} pH meter were used.

Chromatographic conditions

The chromatographic separation was achieved on a gradient method using Kromasil C18 (250 x 4.6 mm, 5µm) column. The mobile phase was consisting of buffer pH 3.0 as mobile phase A and the acetonitrile as mobile phase B. The flow rate of mobile phase was 1.0 ml/min. The run time for standard solution was kept 15 min and for blank, impurities and sample was kept 60 min. The HPLC gradient program set as, time (min) / % mobile phase B: 0.01/50, 15/50, 20/75, 40/75, 45/50 and 60/50. The column temperature was maintained at 30 °C and the detection was monitored at 235 nm. The injection volume was 50 µL and acetonitrile was used as diluent.

Preparation of buffer pH 3.0

Dissolved 1.38 g sodium dihydrogen phosphate monohydrate in 1000 ml of water, pH is adjusted to 3.0 with orthophosphoric acid.

Preparation of standard and sample solutions

A stock solution of methyl-2-amino-3-nitrobenzoate impurity was prepared by dissolving the appropriate amount of methyl-2-amino-3-nitrobenzoate impurity in diluent. The working concentration of 0.94 µg/ml impurity solution was prepared from the stock solution and used as a standard solution. The sample solution (20 mg/ml) was prepared by weighing 400 mg of drug substances and transferred to 20 ml volumetric flask.

RESULTS AND DISCUSSION

Method development

The method was developed by considering the main parameters like the selection of wavelength, HPLC column, mobile phase, column oven temperature, flow rate, injection volume and diluent. The solubility was checked for candesartan cilexetil, methyl-2-amino-3-nitrobenzoate, and other impurities in water, methanol, acetonitrile and the combination of water: methanol, water: acetonitrile in different ratios. All compounds had a good solubility in acetonitrile than others diluent. Hence, Acetonitrile was selected as diluent.

The standard solution was prepared and injected into the HPLC system with PDA detector and a spectrum was obtained. The maximum absorption wavelength of the solution had shown about 235.2 nm (Figure 2), hence 235 nm was selected for the quantification of this impurity in the candesartan cilexetil drug substances.

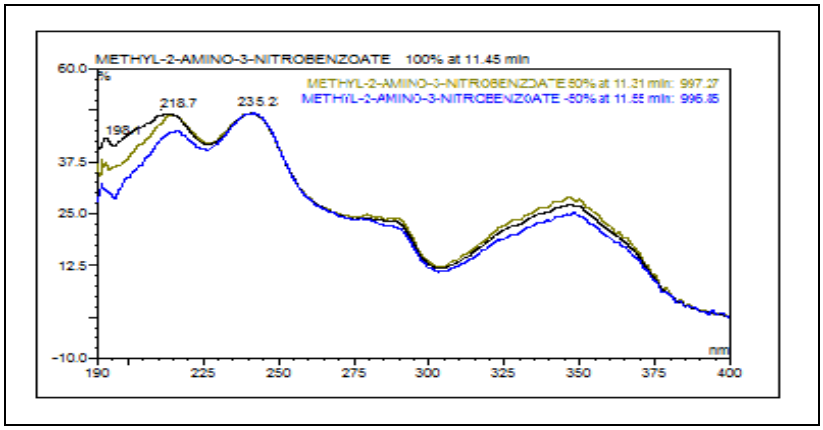


Figure 2: Absorption spectrum of methyl-2-amino-3-nitrobenzoate

The selection of HPLC column carried out by conducted trials on various packaging material of ODS, C8 and C18 in different length, internal diameter, particle size and pore size manufactured by different industries. After performing trials the decisive separation was accomplished on Kromasil C18 (250 x 4.6mm) 5µm HPLC column.

Development has started with a mixture of water, acetonitrile and methanol with the isocratic program. After taking several trials and solubility / polarity basis 0.01mM sodium dihydrogen phosphate monohydrate was decided as the buffer solution. A mixture of buffer and the organic solvents in different proportions were tested with isocratic pump mode, as variation in the mobile phase composition leads to substantial changes in the chromatographic conditions. Decreasing the organic modifier content resulted in the decrease in the retention time of the analyte with peak tailing and increased column pressure was observed. When experiments were performed with acetonitrile as the organic modifier in the mobile phase instead of methanol, the tailing and column pressure problem was resolved. Finally, the gradient program was initiated by using the mobile phase A (Buffer pH 3.0) and mobile phase B (Acetonitrile). In order to increase the selectivity and specificity of the developed method, the gradient program was finalized as; time (min) / % Mobile phase B: 0.01/50, 15/50, 20/75, 40/75, 45/50 and 60/50.

The column temperature was selected by taking many trails with different column oven temperature (20°C to 55°C).The analyte was well separated and the reproducible result was obtained at 30°C. The column temperature was selected by taking many trials with different column oven temperature (20°C to 55°C) in 5 °C steps. The analyte was well separated and the reproducible result was obtained at 30°C. The flow rate of the mobile phase was optimized from 0.5-1.5 ml/min for separation of analyte peak from blank and impurities peaks. It was found from the experiments that 1.0 ml/min flow rate was ideal for the successful elution of the compound. The standard solution was injected from 10 µl to 100 µl injection volume into HPLC system. Based on the response and shape of the peak 10 µl injection volume was selected.

As it can be seen in Table 4, the obtained concentrations were within the range except for Fe concentration which is less than the Libyan standard except for sample (WB). The two-way ANOVA test was carried out in the obtained concentration using AAS to clearly compare the difference between the different areas and different source of water whereas one-way ANOVA test was carried out to compare between the heavy metal levels as it can be seen in Table 5. P value of sample site is equal 0.998 (0.998 > 0.05) that mean we accept theory that there is no difference between metal concentrate from different site of collection. Moreover, P value of water type equal 0.955 (0.955 > 0.05) that mean we accept theory that there is no difference between metal concentrate from different type of water (source). P value of metal is equal 0.000 (0.000 <0.05) that mean we reject theory of the equalization between metal concentration. Additionally, the microbial evaluation of water sample using MPN test indicated that after 24hr of incubate there is no change indicate any bacterial growth in the media. After 48hr of incubation there is also no change in media color and there is no gas bubble in Durham tube, these indicate the absence of the bacteria in all water samples from different sources and areas. Absence of bacteria may be because the water from Made River Project is treated with chlorine, may also be the good deep wall effect on absence of bacteria (as deep of wall increase, the pollution will decrease because its distance from the sewer).

METHOD VALIDATION

The developed HPLC method has been validated for genotoxic impurity determination in the candesartan cilexetil sample according to ICH guideline [19]. The individual parameter of system suitability, specificity, limit of detection, limit of quantification, linearity, precision, accuracy, solution stability and robustness was experimentally evaluated by injecting standard and sample solution.

System suitability

According to USP [20], system suitability test is an integral part of liquid chromatographic methods to verify that the system is adequate for the analysis. The standard solution was prepared and 10 µL of six replicates was injected into HPLC system. The obtained peak was calculated for the theoretical plates, tailing factor and % RSD of six replicate areas. The result was found to comply with USP requirements and indicated that the chromatographic system is adequate for the intended analysis. The results are presented in table 1 and overlay chromatograms of replicate standard injection are presented in Figure 3.

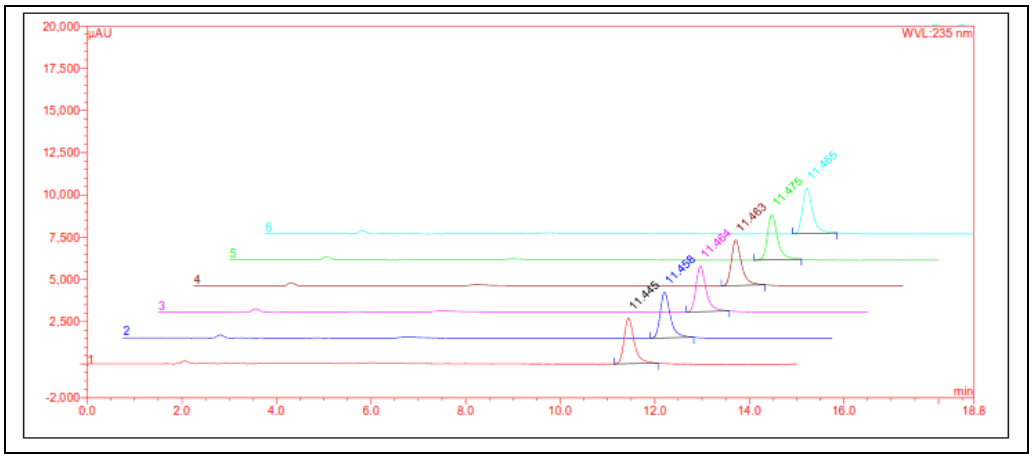


Figure 3: Overlay chromatograms of replicate standard injections

Table 1: Validation Results

Parameters	Acceptance Criteria			Results
System Suitability	The theoretical plates in standard solution NLT 2000			3245
	The tailing factor in standard solution NMT 2.0			1.28
	The % RSD in standard solution NMT 5.0 %			0.76
Specificity	No interference	Blank		No Peak
		Ethyl candesartan cilexetil	Retention time	14.20 min
		Desethyl candesartan cilexetil		21.51 min
		N-Ethyl candesartan cilexetil		42.17 min
		Candesartan		4.82 min
		Trityl Candesartan		23.68 min
		Methyl-2-amino-3-nitrobenzoate		11.45 min
Limit of Detection and Limit of Quantitation	LOD concentration (µg/g)			1.55
	S/N ratio LOD should be 3:1			4.14
	LOQ concentration (µg/g)			4.70
	S/N ratio LOD should be 10:1			14.26
	% RSD at LOQ level should NMT 10.0 %			3.46
Linearity	Slope (Record Results)			583.89
	Intercept (Record Results)			-79.43
	Correlation Coefficient (NLT 0.990)			0.9999
	Residual sum of square (Record Results)			448635.716
Precision	% RSD of repeatability study NMT 10.0 %			0.62 %
	% RSD of Intermediate precision study NMT 10.0 %			1.35 %
Solution stability	Absolute difference in impurity should be not more than 15 % of evaluation limit			Complies
Robustness	Deliberate changes in the developed condition should be not impact on system suitability			Complies

Specificity

For demonstrating the specificity of the method blank, USP listed known / process impurities, methyl-2-amino-3-nitrobenzoate standard, candesartan cilexetil sample were prepared individually at specification limit in the diluent and the solution of candesartan cilexetil spiked with methyl-2-amino-3-nitrobenzoate evaluation limit and injected into developed chromatographic condition. No chromatographic interference (Figure 4) from any of the blank, impurities and sample peak was found at the retention time of methyl-2-amino-3-nitrobenzoate. These results (Table 1) confirm the specificity of the method without any interfering peak around the retention time of methyl-2-amino-3-nitrobenzoate; also the base line did not show any significant noise.

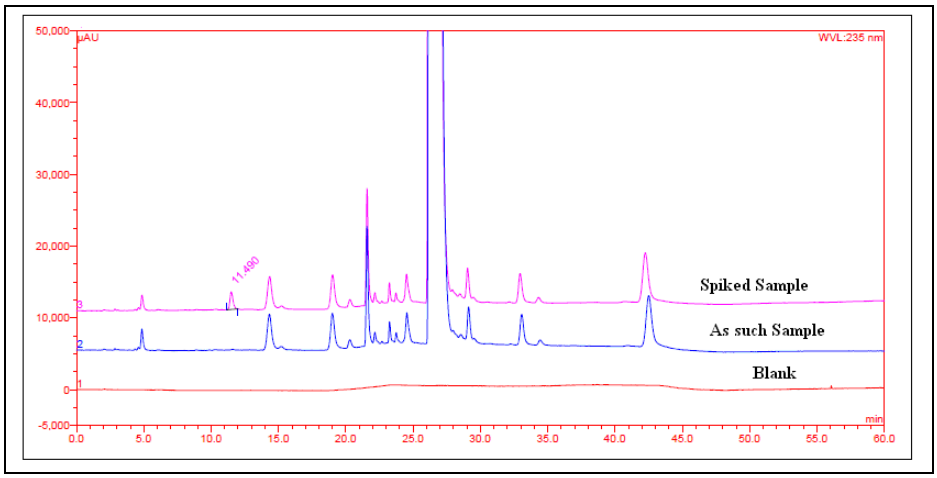


Figure 4: Overlay chromatogram of blank, sample and spiked sample

Limit of detection and Limit of quantitation

The LOD and LOQ for methyl-2-amino-3-nitrobenzoate were estimated through the signal-to- noise ratio of 3:1 and 10:1 respectively, by injecting a series of dilute solutions having known concentrations. LOD of the impurity is defined as the lowest concentration that can be detected. LOD was found to be 1.55 µg/g (Figure 5). LOQ is the lowest concentration that can be quantified with acceptable precision and accuracy. LOQ was found to be 4.70 µg/g (Figure 6). The low values of LOD and LOQ indicate the adequate sensitivity of the method. The precision study was also carried out at LOQ level by injecting six individual preparations and calculating the % RSD of the area. The results are present in table 1.

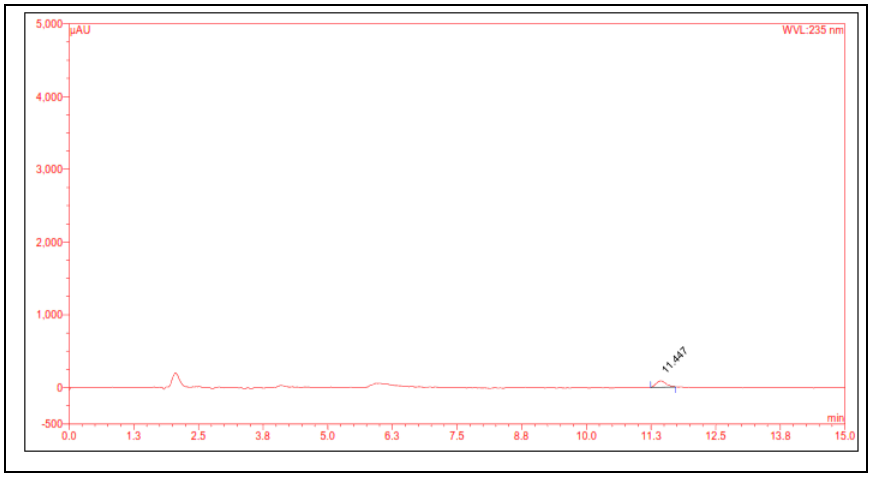


Figure 5: Chromatogram of Limit of detection

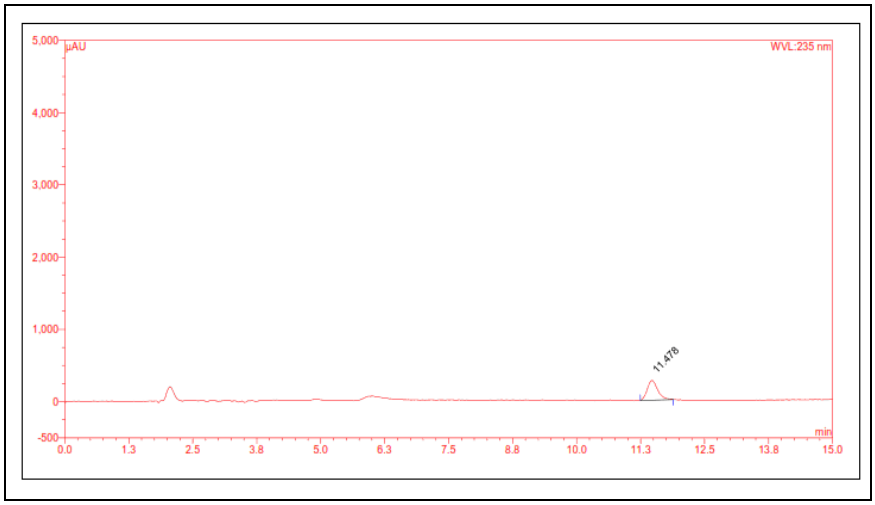


Figure 6: Chromatogram of Limit of quantitation

Linearity

To establish the linearity of the developed method, calibration solution were prepared by diluting the impurity stock solution to obtain solutions at LOQ, 50 %, 80 %, 100 %, 120 % and 150 % of the evaluation limits. Each solution was injected and area of responses was recorded at 235 nm.

The graph of peak area vs concentration in µg/g was plotted (Figure 7). The slope, intercept, correlation coefficient of the regression line and residual sum of square were calculated. The correlation coefficient obtained was greater than 0.990. The result shows that an excellent correlation existed between the peak area and the concentration of the impurity over the entire concentration.

The results are summarized in table 1 and overlay chromatograms of different linearity levels are presented in figure 8.

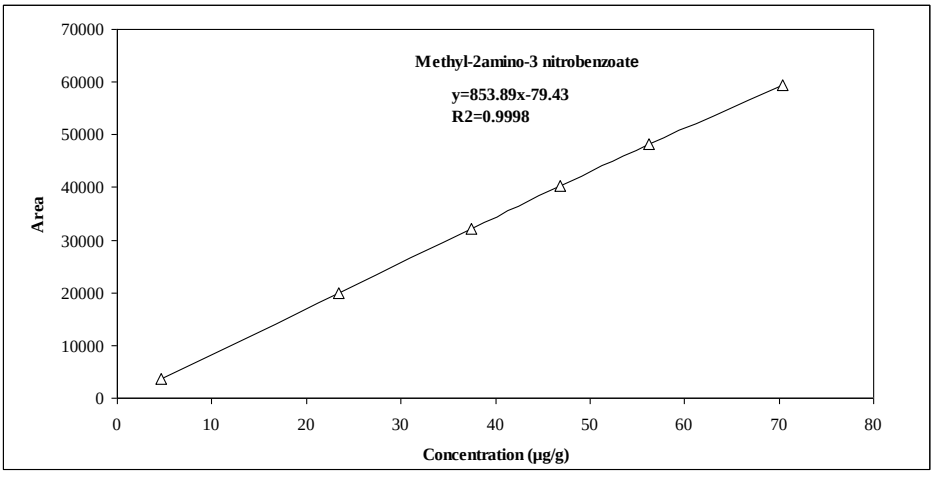


Figure 7: Linearity of Methyl-2amino-3 nitrobenzoate

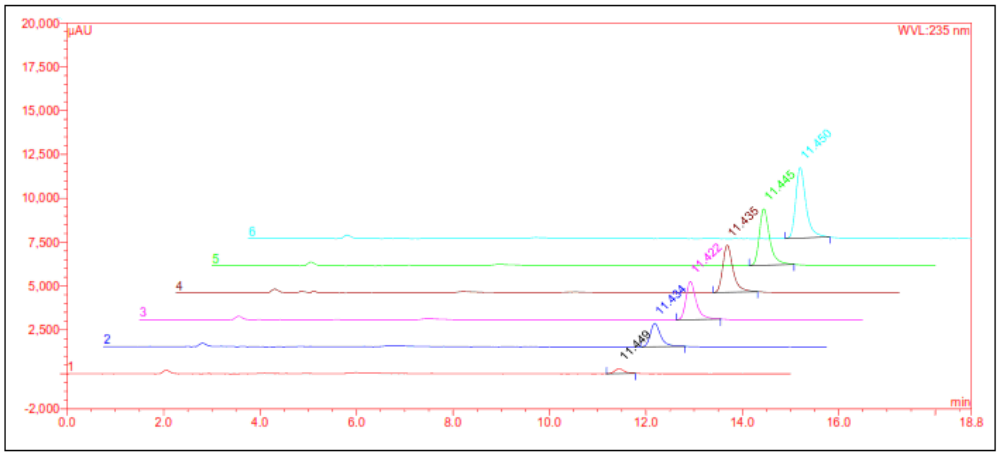


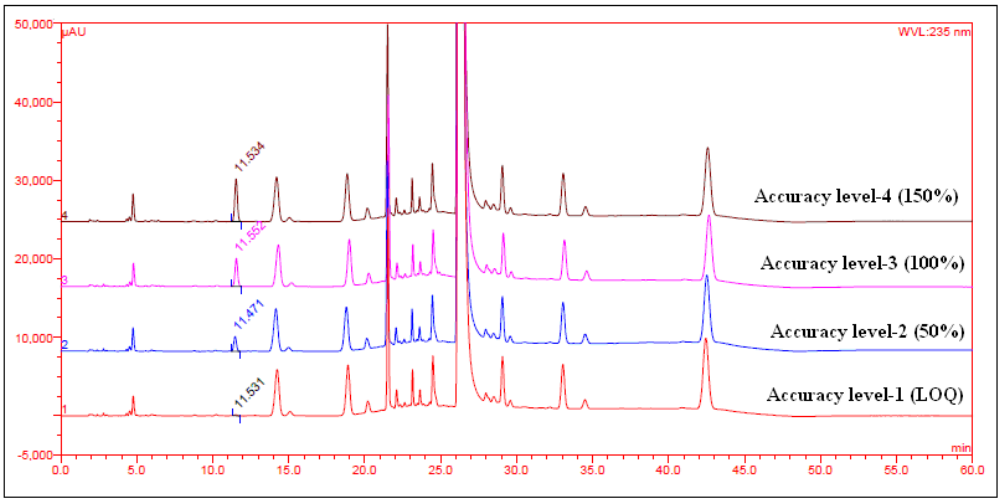
Figure 8: Overlay chromatograms of linearity study from LOQ to 150%

Precision

Precision was determined through repeatability and intermediate precision, Precision of the method was checked by injecting six individual preparation of candesartan cilexetil spiked with the impurity at evaluation limit. The percentage RSD of the content of impurity was calculated. Intermediate precision of the method was evaluated by injecting six individual preparation of the spiked sample at evaluation limit on a different day in the same laboratory. The % RSD for the content of methyl-2amino-3 nitrobenzoate impurity was very low, confirmed that the high precision of the method. The results are present in table 1.

Accuracy

The accuracy of the method was determined by analyzing the drug substances spiked with impurity. A known amount of impurity was spiked to the candesartan cilexetil sample at different concentration levels of LOQ, 50 %, 100 % and 150 % of the evaluation limit. Each concentration level was prepared in triplicate. The percentage recovery of impurity in the drug substances was calculated. The recovery of the methyl-2-amino-3-nitrobenzoate in candesartan cilexetil ranged from 99.02 to 102.68 % which is well within acceptance criteria 80% to 120%.The results are summarized in table 2 and it was observed that the method was accurate within a determined range. Overlay chromatograms of accuracy are presented in fig. 9.



.Figure 9: Overlay chromatograms of accuracy (at LOQ, 50 %, 100 %, and 150 %)

Table 2: Accuracy at different spiking concentration

Level	Amount in sample	Amount added (µg/g)	Amount found (µg/g)	Recovery (%)
LOQ	Nil	4.69	4.68	99.79
	Nil	4.67	4.71	100.86
	Nil	4.73	4.84	102.33
50%	Nil	23.54	23.59	100.21
	Nil	23.63	23.75	100.51
	Nil	23.51	24.14	102.68
100%	Nil	46.97	46.51	99.02
	Nil	47.04	47.15	100.23
	Nil	46.83	46.49	99.27
150%	Nil	70.41	71.57	101.65
	Nil	70.62	70.94	100.45
	Nil	70.54	71.12	100.82

Solution stability

The solution stability was established by spiking methyl-2-amino-3-nitrobenzoate impurity in candesartan cilexetil sample. The prepared solution was stored at room temperature for 24 h. The content of impurity was determined at 4 h interval for 24 h. The result was observed that no significant change in the content of the impurity.

Robustness

To determine the robustness of the method, the experimental conditions were deliberately altered and the system suitability result was evaluated. To study the effect of flow rate, it was changed by 0.1 units from 1.0 ml/min to 0.9 ml/min and 1.1 ml/min. The effect of column temperature was studied by changed 5 °C units from 30 °C to 25 °C and 35 °C. The results were found that the deliberate changes in the method, i.e. flow rate of mobile phase and column oven temperature have no impact on system suitability.

Batch analysis

The three production batches of candesartan cilexetil drug substance were analyzed in the validated method for determination of methyl-2-amino-3-nitrobenzoate and found the impurity was below the quantitation (1.63 µg/g) level in all three batches. Overlay chromatograms of three production batches are presented in figure 10.

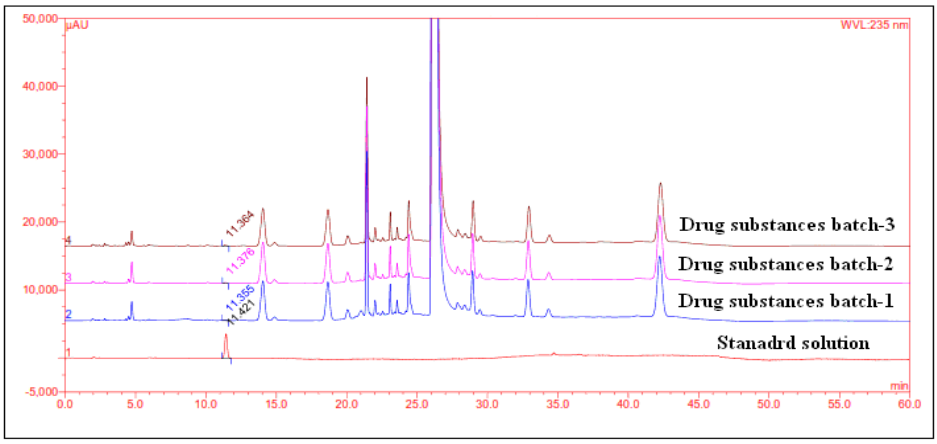


Figure 10: Overlap chromatogram of three production batches of candesartan cilexetil sample with standard solution

CONCLUSIONS

The gradient HPLC method developed for the trace level quantitative determination of genotoxic methyl-2-amino-3-nitrobenzoate in candesartan cilexetil is linear, precise, accurate, rugged and robust. Satisfactory results were obtained from validation of the method according to ICH guideline. This method exhibited an excellent performance in terms of sensitivity and specificity with no sample matrix and impurity interference observed. The sample prepared in analytical solution is found to be stable for 24 h. This method can be used for routine analysis of the trace level quantitative determination of methyl-2-amino-3-nitrobenzoate in candesartan cilexetil drug substances.

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REFERENCES

[1] J Patel; JB Dave; CN Patel; D patel, *J. Chem. Pharm. Res.*, **2010**, 2(3), 10-14.
[2] United States Pharmacopeia, USP39-NF-34, **2016**, 2894-2898.
[3] A Husain; MS Azim; M Mitra; PS Bhasin, *J. Appl. Pharm. Sci*., **2011**, 01(10), 12-17.
[4] H Buter; GY Navis; AJ Woittiez; D DeZeeuw; PE DeJong, *Eur. J. Clin. Pharmacol.*, **1999**, 54(12), 953-958.
[5] E Vaculikova; V Grunwaldova; V Kral; J Dohnal; J Jampilek, *Molecules*, **2012**, 17, 13221-13234.
[6] L Kumar; RK Meel; A Godara; V Singh; A Agarwal, *J. Chem. Pharm. Res.*, **2014**, 6(8), 421-428.
[7] S Zupanic**,** *US 20120029201 A1*, **2012.**
[8] S Venkataraman; PP Reddy; GS Reddy; SS Reddy; MH Narasimha, *WO 2006015134 A1*, **2006.**
[9] International conference on harmonization, Assessment and control of DNA reactive (Mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk M7, **2014**.
[10] European medicines agency evaluation of medicines for human use, guideline on the limits of genotoxic impurities, **2006**.
[11] B Raman; BA Sharma; G Mahale; D Singh; A Kumar, *J. Pharm. Biomed. Anal.*, **2011**, 56 (2), 256-263.
[12] S Mehta; RP Shah; R Priyadarshi, S Singh, *J. Pharm. Biomed. Anal.*, **2010**, 52(3), 345-354.
[13] R Raut; VB Narayanaswamy, *Int. J. Res. Pharm. Chem.*, **2015**, 5(3), 452-469.
[14] D Veeranjaneyulu; A Aneesha; N Agarwal, *Indian J. Res. Pharm. Biotechnol.*, **2013**, 1(5), 720-724.
[15] NDA Kumar; KS Babu; U Gosada; N Sharma, *Pharm. methods*, **2012,** 3(1) 31-39.
[16] B Raja; A R LA Rao A, *Indian J. Res. Pharm. Biotechnol.*, **2014,** 2(4), 1240-1245.
[17] MM Annapurnaa; A Narendra; KR Kumar, *J. Drug Deliv. Ther.*, **2012**, 2(2),48-54.
[18] AK Nalavade; K Ramakrishna; V Srinivasarao, *Int. J. Pharm. Pharm. Sci.*, **2014**, 6(11), 370-372.
[19] International conference on harmonization; Validation of analytical procedures: Text and methodology Q2; **2005**.
[20] United States Pharmacopoeia. General chapter <621> "Chromatography", USP 37, NF 32; **2014**.