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RESEARCH ARTICLE

Quantification and Validation of a HPLC-UV Method for Simultaneous Analysis of Nitrosoamine Impurities (NDMA, NDEA and NDIPA) in Losartan

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ABSTRACT

Impurity profiling is an important aspect in drug therapy for its safety and efficacy. The study of impurities of sartans, the first line antihypertensive drugs, has become critical due to presence of cancer causing N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA) and N-nitrosodiisopropylamine (NDIPA) in them, resulting from production and degradation process. These impurities have led to worldwide recall of products. Hence, a simple and accurate method has been developed and validated for simultaneous detection of NDMA, NDEA and NDIPA in Losartan using High Performance Liquid Chromatography - Ultra violet (HPLC - UV) system. The impurities were analyzed on Inertsil ODS 3V (250mm × 4.6mm, 5.0μm) analytical column by using water:methanol (60:40) as the mobile phase at a flow rate of 1.0 mL/min, with a run time of 30 mins. The method was developed for the acceptance limit of 0.64 ppm for NDMA, 0.177 ppm for NDEA and NDIPA respectively. On comparison with existing approaches, the developed method is fast, ideal for routine screening and is suitable for both laboratory and industrial uses.

Keywords: Losartan; HPLC - UV; N-nitrosodimethylamine; N-nitrosodiethylamine; N-nitrosodiisopropylamine

INTRODUCTION

Hypertension is one of the most prevalent cardiovascular diseases in the world, affecting majority of the adult population. Additionally, it is associated with an increased risk of stroke and coronary heart diseases[1]. Angiotensin II receptor type 1 antagonists like Valsartan and Losartan have been widely used in treatment of hypertension[2]. Angiotensin II is a major regulator of blood pressure, aldosterone secretion and fluid homeostatis, in addition to being an important etiological factor in hypertension and other cardiovascular diseases[3]. Sartans were developed as an additional curative agent for the rennin-angiotensin-aldosterone system[4,5].

In July 2018, the US FDA initiated recall of several drug products containing the active ingredient Valsartan, manufactured by Teva Pharmaceuticals Industries, Major Pharmaceuticals and Solco Healthcare[6,7]. The recall was due to presence of an impurity N-nitrosodimethylamine (NDMA)[8]. NDMA is classified as a probable human carcinogen. Subsequently, another cancer causing impurity, N-nitrosodiethylamine (NDEA) was found in Valsartan product manufactured by Torrent Pharmaceuticals leading to its recall in August 2018. The other sartans too were also found to be contaminated from NDMA, NDEA resulting in worldwide recalls[9]. Besides NDMA, NDEA, other impurities which are also present and needs to be controlled include: N-nitrosodiisopropylamine (NDIPA), N-nitrosoethylisopropylamine (NEIPEA), N-nitroso-N-methylamino butyric acid (NMBA) and N-nitrosodibuty amine (NDBA)[10].

Since the detection of these two impurities, a transition period of two years have been given to pharma industries for change in process to eliminate impurities[11].

The acceptable concentration for NDIPA was authorized by a European Medicines Agency on 20 August 2019 [12] to the level of 0.177 ppm, corresponding to its maximum daily dose of 26.5 ng/day. The acceptable concentrations for NDMA and NDEA were provided by European Medicines Agency on 14 February 2019 [13] to the level of 0.64 ppm and 0.177 ppm, corresponding to their maximum daily dose of 96.0 ng/day and 26.5 ng/day respectively.

Only a limited number of publications are available which report the analysis of NDMA, NDEA in API's or pharmaceutical preparation [14]. A majority of methods reported, utilizes LC-MS/MS or GC-HS/MS systems for determination of Nitrosamines (NDEA, NDMA) in sartans, either individually or in a group. Recently, as little as two methods have been reported for determination of NDMA in sartans using HPLC/UV system [15,16].

To the best of our knowledge, determination of NDIPA in sartans and concurrent estimation of NDMA, NDEA and NDIPA (Figure 1) in Losartan has not been reported previously and the present article discusses the method developed, quantified and validated in detail for parallel ascertainment of these Nitrosamine impurities in Losartan.

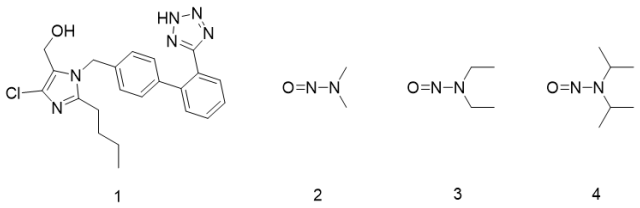

Fig. 1: Chemical structure of Losartan (1), NDMA (2), NDEA (3), NDIPA (4)

EXPERIMENTAL

Chemicals and reagents

The reference standards for NDMA and NDIPA were prepared in house and qualified by chromatographic and spectroscopic techniques. NDEA was purchased from Tokyo Chemical Industry Co., Ltd, Japan. Acetonitrile and methanol were purchased from Merck, India. Milli Q water was used during this study.

Instrumentation

The analysis was carried out using Waters Alliance HPLC system (e2695 separating module) (Waters Co., Milford, MA, USA) with a Ultraviolet - Visible detector (Waters 2489) with an auto sampler and column heater. Data were collected and processed using Empower^{TM} software (Version 3) from Waters.

Chromatographic Conditions

The method for NDMA, NDEA and NDIPA was developed, validated and applied to study the concurrent estimation of these impurities in Losartan. The mobile phase was filtered through 0.45μ filter (Millipore) and degassed using sonicator. Nitrosamine impurities were analyzed using Inertsil ODS 3V (250mm $\times$ 4.6mm, 5.0μm) column set at 30°C with water and methanol (60:40 v/v) as the mobile phase. A flow rate of 1.0 mL/min with an injection volume of 80μL and an absorption wavelength of 230 nm were used. The run time was 30 minutes and the retention time was 3.59, 6.73 and 17.13 minutes for NDMA, NDEA and NDIPA respectively.

HPLC Method development

Preparation of stock, mixed standards stock, working standard and sample solutions

All standard solutions were prepared using diluent containing water-methanol in the ratio of 60:40 (v/v). Stock standard solutions of NDMA, NDEA and NDIPA were prepared at a concentration of 10 μg/mL in water-methanol system and stored at 8°C. Mixed standard stock solutions of impurities were prepared, consisting of NDMA at a concentration of 1.5 μg/mL, NDEA and NDIPA at a concentration of 0.4 μg/mL in water-methanol diluent. Working standard solution was prepared by diluting the mixed standard stock solution by using water-methanol to yield concentration of 0.15 μg/mL for NDMA, 0.04 μg/mL for NDEA and NDIPA each.

Sample solution was prepared by dissolving approximately 1.173 g of Losartan in 5 mL of diluent (water-methanol, 60:40 v/v) to give a concentration of 234.6 mg/mL. The resultant solution obtained was milky. Further, the sample was centrifuged at 2500 rpm for 5 minutes and supernatant solution filtered through 0.45 μ syringe filter and transferred into an injection vial (Concentration: 234600 ppm).

Method validation

HPLC method was validated to ensure consistent and accurate results to determine the levels of three Nitrosamines impurities in Losartan sample. The HPLC method was validated in terms of system suitability, specificity, Linearity, LOD, LOQ, precision, accuracy and range.

Specificity

The specificity of each component was determined by comparing the chromatograms of blank solution (water-methanol, 60:40 v/v) with that of sample solution. Observations were made for any interfering peaks generated during the analysis.

Evaluation of Linearity

Standard solutions were evaluated for linearity within a concentration range of 0.021 - 0.976 μg/mL for NDMA, 0.025 - 0.251 μg/mL for NDEA and 0.028 - 0.277 μg/mL for NDIPA. The peak area was plotted against impurity concentration and the linearity was thus calculated by the linear regression equation y = mx + c, where y represents the peak area and x represents the impurity concentration in μg/mL. A correlation coefficient of approximately 0.9900 to 1.0000 was considered desirable for all calibration curves.

Determination of limit of detection (LOD) and limit of quantification (LOQ)

The LOD was determined by injecting lower concentrations of Nitrosamine impurities until a signal (peak) to noise ratio was obtained. The LOQ was also determined from the range of concentrations analyzed for the LOD determination.

Precision and Accuracy

The Precision was assessed by evaluating the % RSD of results obtained from six preparations of spiked samples. The validation was carried out with three determinations of four different concentrations (at LOQ, 80%, 100% and 150%). The accuracy and precision values were calculated using standard practice, as per ICH guidelines.

RESULTS

Method development and optimization

The most suitable isocratic condition to analyze the Nitrosamine impurities, after the chromatographic conditions were optimized for specificity, resolution and retention time, was a mobile phase consisting of water:methanol (60:40, v/v). The designing of mobile phase and diluents in the ratio of water:methanol (60:40, v/v) was crucial for extraction of nitrosamine impurities in this diluent and precluding Losartan. This discrimination was achieved by the difference in physico chemical properties of the analyte and Losartan especially solubility characteristics that has eliminated potential interference of Losartan that could arise due to its high concentration required for determination at below 0.1 ppm level. Such approaches of sample preparation can be extended for analysis of other sartans after optimization of diluents and sample quantity and analyzing the sample by this validated method.

Change in the concentration of organic solvent (higher) resulted in early elution of NDMA peak and organic solvent (lower) resulted in peak broadening of NDIPA peak. This method was developed and optimized for the current specified limits of Nitrosamine impurities i.e.; NDMA for NMT 0.64 ppm, NDEA & NDIPA for NMT 0.177 ppm each. Thus, based on the above parameters, NDMA, NDEA and NDIPA eluted at a retention time of 3.59, 6.73 and 17.13 minutes respectively, as shown in Figure 2. (Table 1) depicts the chromatographic parameters applied for the method.

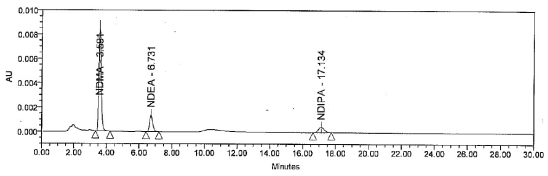

Fig. 2: Representative chromatogram of NDMA, NDEA and NDIPA in standard solution.

Table 1: HPLC isocratic method for NDMA, NDEA and NDIPA

Parameter	NDMA	NDEA	NDIPA
Column	Inertsil ODS 3V (250mm × 4.6mm, 5.0μ)		
Mobile phase	Water : Methanol (60:40 v/v)		
Flow rate (mL/min)	1.0		
Injection vol. (μL)	80		
Wavelength (nm)	230		
Retention time (min)	3.59	6.73	17.13
Relative Retention time	1.00	1.87	4.77

Method validation

The method was validated according to validation of analytical procedures provided in the ICH guidelines.

Specificity

Specificity was used to test the ability of the method to eliminate the effects of all interfering substances on NDMA, NDEA and NDIPA peaks, specifically by comparing the chromatograms to the blank samples. The validated method showed that there is no interference observed at the peak of interests and (Table 2 and Table 3) shows the summary of peak purity results.

Table 2: Summary of peak purity results for Individual impurity

Component	RT (min)	Purity Angle	Purity Threshold	Purity Flag
NDMA	3.65	0.619	0.693	Pass
NDEA	6.80	2.556	4.462	Pass
NDIPA	17.24	7.805	11.818	Pass

Linearity and range

A linear relationship was observed between the area response for all the three impurities and corresponding concentrations. The mean standard calibration curves are presented in Fig.3. The calibration curves exhibit a linearity over a range of 0.021 to 0.976 μg/mL for NDMA, 0.025 to 0.251 μg/mL for NDEA and 0.028 to 0.277 μg/mL

Table 3: Summary of peak purity results for spiked Individual impurity

Component	RT (min)	Purity Angle	Purity Threshold	Purity Flag
NDMA	3.66	0.903	2.431	Pass
NDEA	6.82	8.851	9.111	Pass
NDIPA	17.19	35.577	36.402	Pass

for NDIPA with regression coefficient values greater than 0.9900. The method provided a good correlation between the area response and component concentration.

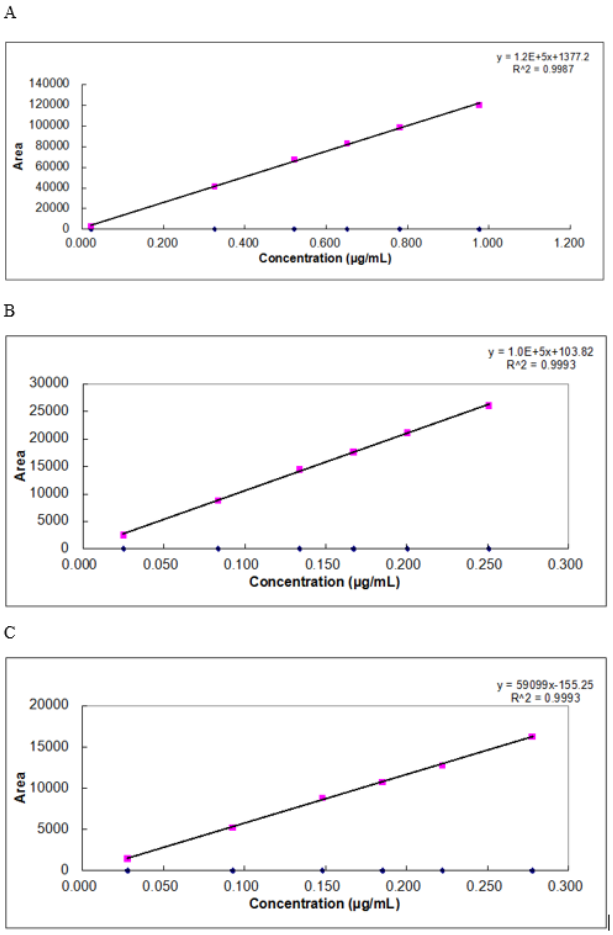

Fig. 3: Linearity of the HPLC method for analysis of (A) NDMA, (B) NDEA and (C) NDIPA

Sensitivity

The LOD was evaluated by determining the minimum levels of concentration of NDMA, NDEA and NDIPA that could be detected using this analytical method. The LOQ was studied by estimating the minimum concentration that could be quantified with acceptable accuracy and precision. The precision study was also carried out at LOQ level by injecting six preparations of all three impurities. LOD, LOQ and Percentage RSD for the area response of each impurity at LOQ level for NDMA, NDEA and NDIPA are shown in (Table 4).

Table 4: LOD and LOQ of Nitrosamine impurities

Parameter	NDMA (µg/mL)	NDEA (µg/mL)	NDIPA (µg/mL)
LOD	0.011	0.017	0.018
LOQ	0.021	0.025	0.028
Precision at LOQ %RSD (n=6)	6.25	0.60	4.21

Precision

The precision for the said nitrosamine impurities was evaluated by injecting six individual Losartan samples with 234.6 mg/mL each of concentration, spiked with 0.64 µg/mL of NDMA, 0.177 µg/mL NDEA and NDIPA each. Percentage relative standard deviation for actual recovered amount was calculated and were within 15% confirming the good precision of the developed analytical method (Table 5).

Table 5: Results of Precision

Parameter	NDMA	NDEA	NDIPA
Precision %RSD (n=6)	2.18	4.29	13.18

Accuracy

Accuracy was studied by analyzing three replicates at four different concentration levels: at LOQ, 80%, 100% and 150% (Tables 6, 7 and 8). The accuracy values were within a range of 93.2% to 100.1% for NDMA, 85.8% to 96.4% for NDEA and 83.3% to 101.8% for NDIPA. This indicates high accuracy of new method developed.

DISCUSSION

The criteria for the development of an isocratic reversed phase HPLC-UV method for the determination of three nitrosamine impurities was that it should be able to quantify all three impurities in a single run and should be accurate, precise, linear and free of interference's from blank for the use in routine for analytical development and quality control laboratories.

Generally, most of the reported methods for nitrosamine impurities analyses in scientific literature utilize Gas Chromatography separation and that too specifically for NDMA. Moderate to high polarity stationary phases are used in these separations with carbowax or analogous material[17–19]. Alternatively, High Performance Liquid Chromatography (HPLC) on reverse phase columns have been mentioned for NDMA determination by Ultraviolet (UV) using diode array detection (DAD) at wavelength of 230 - 233 nm[20–22].

Table 6: Accuracy of NDMA

% Level	Area in spiked sample	Area in sample	Difference in area	Recovered amount (µg/mL)	Added amount (µg/mL)	% Average Recovery
LOQ	3042	0	3042	0.02395	0.02170	99.7
	2725		2725	0.02152	0.02177	
	2478		2478	0.01955	0.02174	
80%	66171		66171	0.52258	0.52243	100.1
	66482		66482	0.52382	0.52123	
	66063		66063	0.52146	0.52216	
100%	72591		72591	0.57191	0.65148	95.7
	81712		81712	0.64273	0.65043	
	83132		83132	0.65423	0.65076	
150%	118298		118298	0.93058	0.97572	93.2
	114167		114167	0.89671	0.97423	
	114364		114364	0.89894	0.97497	

Table 7: Accuracy of NDEA

% Level	Area in spiked sample	Area in sample	Difference in area	Recovered amount (µg/mL)	Added amount (µg/mL)	% Average Recovery
LOQ	6990	4455	2535	0.02509	0.02507	85.8
	6341		1886	0.01873	0.02515	
	6551		2096	0.02079	0.02512	
80%	17050		12595	0.12507	0.13412	96.4
	17784		13329	0.13205	0.13381	
	17594		13139	0.13041	0.13405	
100%	18407		13952	0.13821	0.16725	95.5
	21703		17248	0.17059	0.16698	
	21607		17152	0.16973	0.16706	
150%	27395		22940	0.22690	0.25049	91.1
	27644		23189	0.22902	0.25011	
	27526		23071	0.22803	0.25030	

Table 8: Accuracy of NDIPA

% Level	Area in spiked sample	Area in sample	Difference in area	Recovered amount (µg/mL)	Added amount (µg/mL)	% Average Recovery
LOQ	2626	1483	1143	0.01972	0.02778	83.3
	2845		1362	0.02358	0.02787	
	3001		1518	0.02625	0.02783	
80%	9098		7615	0.13182	0.14860	84.8
	9066		7583	0.13096	0.14826	
	8135		6652	0.11509	0.14853	
100%	10929		9446	0.16312	0.18531	101.8
	13743		12260	0.21137	0.18501	
	12571		11088	0.19126	0.18510	
150%	15798		14315	0.24682	0.27754	88.8
	15525		14042	0.24174	0.27711	
	16029		14546	0.25061	0.27733	

Reproducible analyses of nitrosamines with pre column derivatization, ion chromatography and liquid chromatography using post column photolysis and chemiluminescence have also been utilized in past[23–27] 23-27], therein giving rise to need of a new method which is simple, free of any complexities with respect to column, system and sample preparation and can be replicated easily for concurrent determination of all three nitrosamine impurities at low concentrations.

In the present study, preliminary experiments were carried out until separation was achieved on Inertsil ODS 3V column using injection volume of 50µL and isocratic elution at a flow rate of 1.0 mL/min at 30°C (column oven) temperature. Low response and broad peak was observed when sample preparation was done by dissolving Losartan in methanol and further diluting with water. Peak merging were also frequent with high concentration of methanol in mobile phase. The peak shape and response improved when Losartan was converted into slurry in water, filtered and then diluted with methanol. Finally, satisfactory result was achieved in 30 minutes with flow rate of 1.0mL/min of mobile phase, water: methanol (60:40 v/v) and keeping injection volume of 80 µL.

Specificity is the ability of the method to measure the analyte response in the presence of diluents. Figure 2 shows that there is no interference at the R_t (retention time) for all the three nitrosamine impurities and Tables 2 and 3 shows the summary of peak purity results. To assess the ability of the method, mix standard solutions were prepared with known amounts of NDMA, NDEA and NDIPA. The sample solution was prepared as per methodology and chromatogram was recorded. The relative retention time for impurities is shown in Table 1. The specificity of the method was judged from the absence of interfering or false peaks at the analyte elution times for blank chromatograms.

Method precision was determined by six replicate injections of Losartan samples. Important characteristic including % RSD was measured. The % RSD of recovered amounts for six replicate injections were below 15.0%. This indicates that the method is precise and suitable for determination of all three Nitrosamine impurities (Table 5).

To confirm the accuracy of the proposed method, recovery was carried out by the standard technique. The accuracy of the method was determined by adding known amounts of each impurity corresponding to four concentration levels; LOQ, 80%, 100% and 150% of the target concentration to the sample examined in triplicate. The amount recovered was within ± 20% of the amount added, which indicates

that the method is accurate for determination of nitrosamine impurities (Tables 6, 7 and 8).

CONCLUSION

An isocratic reversed phase - HPLC method was successfully developed for the estimation of three nitrosamine impurities, namely NDMA, NDEA and NDIPA in single determination. The method developed is simple for the purpose of quantification for the current specified limits of Nitrosamine impurities i.e.; NDMA for NMT 0.64 ppm, NDEA & NDIPA for NMT 0.177 ppm. The method validation results proved that the method is specific, linear, precise and accurate and can be applied for estimation of NDMA, NDEA and NDIPA in Losartan for monitoring drug safety. Also, this method can be easily adopted for the determination of the mentioned N-Nitrosamine impurities in other drug substances like Valsartan, Olmesartan, Irbesartan and Candesartan etc. by using appropriate sample preparation technique based on solubility of individual molecule.

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