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Research Article

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Assessment and quantification of genotoxic impurities of Ziprasidone an antipsychotic drug

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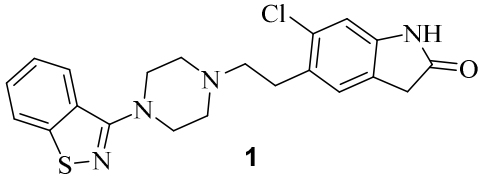
ABSTRACT

The objective of the analytical work is to assess the possible genotoxic impurities in common industrial synthetic route of famous atipycical antipsychotic drug Ziprasidone and to develop suitable analytical methods to quantify all the possible genotoxic impurities below the TTC limit.

Keywords: Genotoxic impurities, Ziprasidone, Antipsychotics, schizophrenia.

INTRODUCTION

Ziprasidone (**1**) [1-2] is an atypical antipsychotic drug. It is approved by the U.S. Food and Drug Administration in 2001 for the treatment of schizophrenia, and acute mania and mixed states associated with bipolar disorder. Ziprasidone is effective in the treatment of schizophrenia.



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Genotoxicity that refers to any deleterious change in the genetic material regardless of the mechanism by which the change is induced. Genotoxic impurities have also been defined as an impurity that has been demonstrated to be genotoxic in an appropriate genotoxicity test model. A potential genotoxic impurity (PGI) has been defined as an impurity that shows structural alerts for genotoxicity but that has not been tested in an experimental [3-7].

The European Medicines Agency (EMEA) issued guidelines for GTI limits and included the concept of threshold of toxicological concern (TTC) to define acceptable risk for new active substances. This guideline acknowledges that it is impossible to define a zero risk for genotoxic carcinogens without a threshold, and the realization that complete elimination is often unachievable. A TTC of 1.5 μg/day is given as a level at which exposure will not pose a significant carcinogenic risk. The EMEA guidance also indicates that the TTC may be raised for short-term exposures or for known impurities which have greater potential for exposure from other sources.

Maximum daily dose of Ziprasidone hydrochloride anhydrous after salt correction is 174 mg. As per European medical agency (EMEA) threshold of toxicological concern (TTC) for Ziprasidone hydrochloride is 1.5 µg/day (Exposure of genotoxic impurity in drugs that will be tested or dosed for longer than 12 months). Based on the TTC the concentration limits of genotoxic impurities in Ziprasidone hydrochloride is 8.62 ppm [1.5 µg/day)]/[0.174 g (dose)].

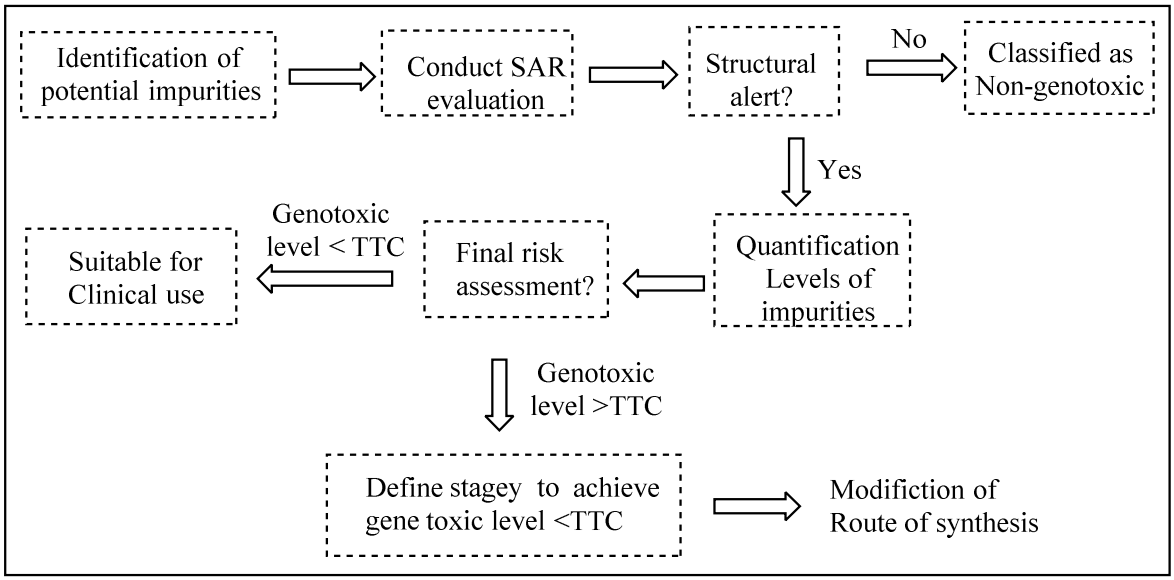


Figure.1. Assessment flow of Genotoxic impurities in Ziprasidone synthetic route

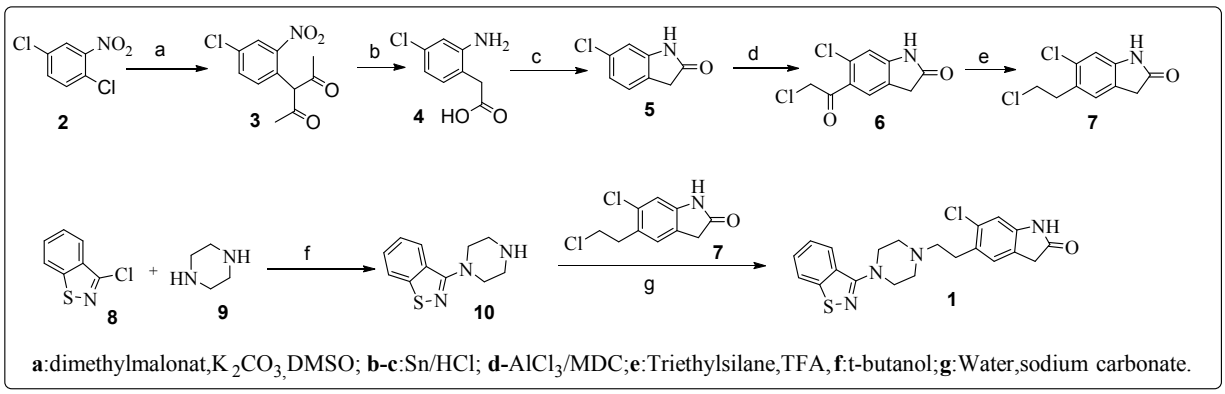
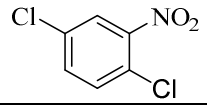
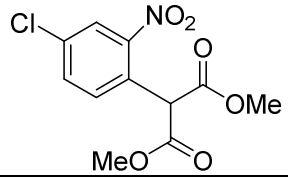
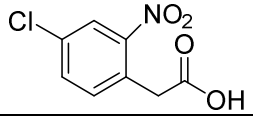
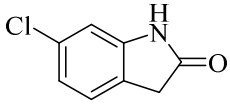
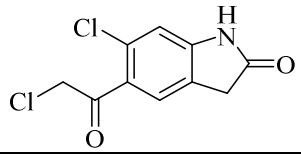
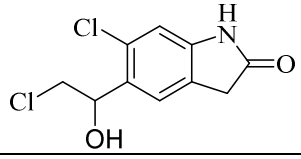
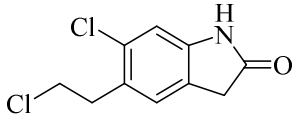
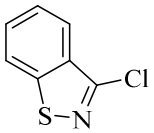
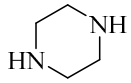


Figure.2. General Industrial route for synthesis Ziprasidone

Based on the encountered search results obtained through available references Ames ,Derek and Toxnet it appears that the following compounds may be categorized as the possible genotoxic impurities/alerts, which may be controlled at below 8.62 ppm (using 174 mg max. daily dose of Ziprasidone hydrochloride anhydrous after salt correction) based on Threshold of Toxicology Concern (TTC) calculation.

Table 1: Assessment of Genotoxic impurities in Ziprasidone

S. No.	Structural Alert	Chemical Name	Genotoxic (Yes/No)	Ref Source (Ames/Derek/Toxnet)
01		1,4-dichloro-2-nitrobenzene	Yes	Toxnet
02		Dimethyl-2-(4-chloro-2-nitrophenyl)malonate	Yes	Derek
03		2-(4-chloro-2-nitrophenyl)acetic acid	Yes	Derek
04		6-chloroindolin-2-one	No	Derek Toxnet
05		5-(2-Chloro acetyl)-6-chloro 2-Oxindole	Yes	Derek
06		6-chloro-5-(2-chloro-1-hydroxyethyl) indolin-2-one	Yes	Derek
07		6-chloro-5-(2-chloroethyl)indolin-2-one	No	Ames Derek Toxnet
08		3-chlorobenzo[d]isozole	No	Ames Derek Toxnet
09		Piperazine	No	Ames Derek Toxnet

The objective of the Method development was to develop quantification methods by HPLC with shorter run times for all the five genotoxic impurities from the above table. As of today in literature there are no methods found to quantify any of these impurities in Ziprasidone. The methodologies described in the literature and in the USP Pharmacopeia are not suitable for the quantification of these impurities. It is therefore, necessary to develop quantification methods for the determination of possible genotoxic impurities in Ziprasidone.

Solubility of ziprasidone is crucial to achieve desired LOD, LOQ values. Initially we tried to improve the solubility in different ratios of water, methanol and acetonitrile found that the solubility is less than 0.5 mg/ml. Then studied solubility in ortho phosphoric acid (0.25%) and acetonitrile at different ratios and found that solubility is improved up to 3.0 mg/ml in the ratio of 50:50. As per regulatory requirement we need to show these impurities at very low level (less than 8.62 ppm) which causes the interference of related impurities as well as unknown impurities present in the drug substance. Method development has been done to separate the target genotoxic impurities from the rest

of all impurities including known and un-known present in the ziprasidone. Development has been done on different reverse phase stationary phases (c18, c8, cyano and phenyl) and different manufacturer (Inertsil, Kromasil, waters symmetry). During development mobile phase Ph played typical role in the separation. Studies continued at different mobile phase Ph to achieve target separation. Finally found adequate separation of Impurity-1 and Impurity-4 at Ph-3.0 and Impurity-2, Impurity-3 and Impurity-5 at Ph 6.5. Potassium phosphate used as buffer during entire development because of its sustainability at both acetic and basic Ph. Based on the above developments finalized chromatographic conditions were mentioned in the experimental section.

Chemical and reagents

Samples of Ziprasidone HCl anhydrous and impurities-1, 2, 3, 4 and 5 were prepared from available route [8-11].HPLC grade Acetonitrile, Potassium dihydrgen phosphate and sodium hydroxide was purchased from Rankem, Mumbai, India. Ortho phosphoric acid was purchased from Sigma Aldrich, Mumbai, India. High pure water was prepared by using Millipore Milli Q plus purification system (Millipore, USA).

EXPERIMENTAL SECTION

Quantification of Dimethyl (4-chloro-2-nitrophenyl) malonate (Impirity-1)

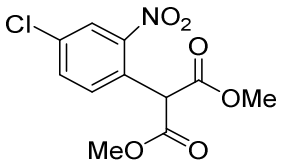


Figure.3. Structure of Dimethyl (4-chloro-2-nitrophenyl) malonate

Equipment

The HPLC method development done using Waters e 2695 separation module connected Waters 2489 UV/Visible detector and waters 2998 photodiode array detector and integrator. The data were collected using empower software.

Chromatographic conditions

A new HPLC method is developed for separating Dimethyl (4-chloro-2-nitrophenyl) malonate from Ziprasidone and its impurities. The LC chromatographic separations were achieved on Kromasil C18 column 250 mm length x 4.6 mm ID with 5µm particle size using isocratic mobile phase of mixture of 0.02 M potassium di-hydrogen orthophosphate adjusted Ph to 3.0 with ortho phosphoric acid and Acetonitrile in the ratio of 400: 600 (v/v) at a flow rate of 1.0 ml/min. UV detector was operated at 220 nm and the column temperature was set to 40°C. The test concentration was about 3.0 mg/ml and the injection volume was 50 µL. 0.25% phosphoric acid and Acetonitrile in the ratio of 50:50 was used as diluent during the standard and test sample preparation.

Preparation of impurity standard and test sample solution:

The impurity stock solution prepared at 0.5 mgmL-1 in diluent. For LOD and LOQ establishment, the impurity stock solution was diluted using diluent to give standards at 2.0, 6.0 ppm with respect to test concentration. The test samples of API were typically prepared at approximately 3.0 mg/mL in diluent and sonicated about 5 minutes.

Method validation

Limit of Detection (LOD) and Limit of Quantification

The LOD and LOQ values for Dimethyl (4-chloro-2-nitrophenyl) malonate were established by injecting series of dilutions from standard preparation to get the Signal to noise ratio 2 to 3 for LOD and 9.5 to 10.4 for LOQ.

Table 2: LOD, LOQ results

Dimethyl(4-chloro-2-nitrophenyl) malonate	Concentration (ppm)	S/N ratio
LOD	2.0	2.9
LOQ	6.0	10.2

Precision:
Precision is evaluated at LOQ level by carrying out six individual preparations of 6 ppm Dimethyl (4-chloro-2-nitrophenyl) malonate in to the chromatographic system and checked % relative standard deviation (RSD). The % relative standard deviation (RSD) of the area at LOQ level is 3.96%.

Table 3: Precision results

Injection	1	2	3	4	5	6	Average	SD	%RSD
Area	2922	3214	3088	3057	2949	3198	3071.33	121.78	3.96

SD: Standard deviation, RSD: Relative standard deviation.

Accuracy:
The accuracy of the method is evaluated at LOQ level by preparing sample solutions in triplicate by spiking Dimethyl (4-chloro-2-nitrophenyl) malonate at LOQ level, with Ziprasidone hydrochloride anhydrous and injected each solution in to HPLC as per methodology. The percentage of recovery for the impurity was calculated and the value is 98.1%. At such low levels these recoveries and % relative standard deviation (RSD) were satisfactory.

Table 4: Accuracy results

Level	Amount spiked (ppm)	Amount recovered (ppm)	% Recovery	Mean	SD	% RSD
LOQ spiked-1	6	5.63	93.8	98.1	3.8	3.9
LOQ spiked-2		6.05	100.8			
LOQ spiked-3		5.99	99.8			

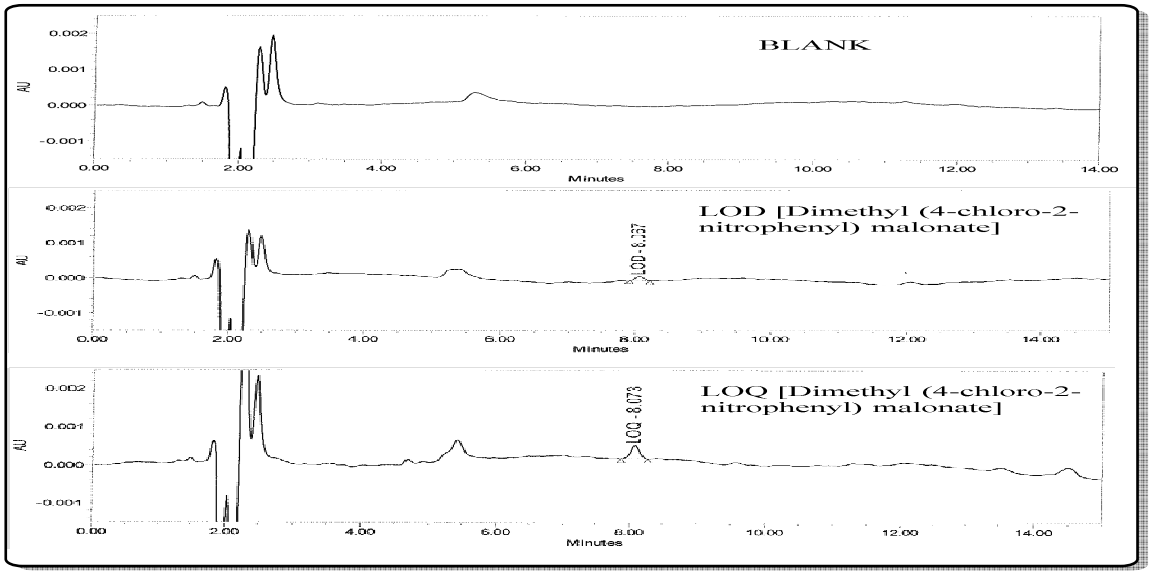


Figure.4.Typical chromatograms of Dimethyl (4-chloro-2-nitrophenyl) malonate BLANK, LOD and LOQ

Quantification of 2-(4-chloro-2-nitrophenyl) acetic acid & 6-chloro-5-(2-chloro-1-hydroxy ethyl) indolin-2-one (Impirity-2&3)

Equipment
The HPLC method development done using Waters e 2695 separation module connected waters 2489 UV/Visible detector and waters 2998 photodiode array detector and integrator. The data were collected using empower software.

Chromatographic conditions
A new gradient method is developed for separating 2-(4-chloro-2-notrophenyl) acetic acid from Ziprasidone and its impurities. The method was developed by using Inertsil ODS-3V (250 mm x 4.6 mm, 5 µm) column with mobile phase containing a gradient mixture of solvent A (A Mixture of 0.02 M potassium phosphate, pH adjusted to 6.5 with 1N Sodium hydroxide and Acetonitrile in the ratio of 80:20) and B (A Mixture of 0.02 M potassium phosphate, pH adjusted to 6.5 with 1N Sodium hydroxide and Acetonitrile in the ratio of 20:80). The separation was achieved by gradient elution (T/%B) set as 0/30, 5/30, 20/60, 35/60, 37/30, 45/30. The flow rate of mobile phase was 1.0

mL/min with column temperature of 40°C and detection wavelength at 215nm. The test concentration was about 3.0 mg/ml and the injection volume was 100 µL. 0.25% phosphoric acid and Acetonitrile in the ratio of 50:50 was used as diluent during the standard and test sample preparation.

2-(4-chloro-2-nitrophenyl) acetic acid (Impurity-2)

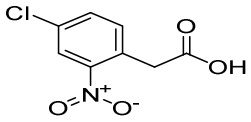


Figure.5. 2-(4-chloro-2-nitrophenyl) acetic acid

Preparation of impurity standard and test sample solution:
The impurity stock solution prepared at 0.5 mgmL-1 in diluent. For LOD and LOQ establishment, the impurity stock solution was diluted using diluent to give standards at 2.2, 6.7 ppm with respect to test concentration. The testing API samples were typically prepared at approximately 3.0 mg/mL in diluent and sonicated about 5 minutes.

Method validation
Limit of Detection (LOD) and Limit of Quantification:
The LOD and LOQ values for 2-(4-chloro-2-nitrophenyl) acetic acid were established by injecting series of dilutions from standard preparation to get the Signal to noise ratio 2 to 3 for LOD and 9.5 to 10.4 for LOQ.

Table 5: LOD, LOQ results

2-(4-chloro-2-nitrophenyl) acetic acid	Concentration (ppm)	S/N ratio
LOD	2.2	2.5
LOQ	6.7	10.2

Precision:
Precision is evaluated at LOQ level by carrying out six individual preparations of 6.7 ppm solution of 2-(4-chloro-2-nitrophenyl) acetic acid in to the chromatographic system and checked % relative standard deviation (RSD). The % relative standard deviation (RSD) of the area at LOQ level is 3.53%.

Table 6: Precision results

Injection	1	2	3	4	5	6	Average	SD	%RSD
Area	7775	7705	7803	7146	7320	7584	7555.50	266.74	3.53

SD: Standard deviation, RSD: Relative standard deviation.

Accuracy:
The accuracy of the method is evaluated at LOQ level by preparing sample solutions in triplicate by spiking 2-(4-chloro-2-nitrophenyl) acetic acid at LOQ level and injected each solution in to HPLC as per methodology. The average percentage of recovery for the impurity was calculated and the value is 99.3%. At such low levels these recoveries and % relative standard deviation (RSD) were satisfactory.

Table 7: Accuracy results

Level	Amount spiked (ppm)	Amount recovered (ppm)	% Recovery	Mean	SD	% RSD
LOQ spiked-1	6.7	6.72	100.3	99.3	0.95	0.96
LOQ spiked-2		6.59	98.4			
LOQ spiked-3		6.65	99.3			

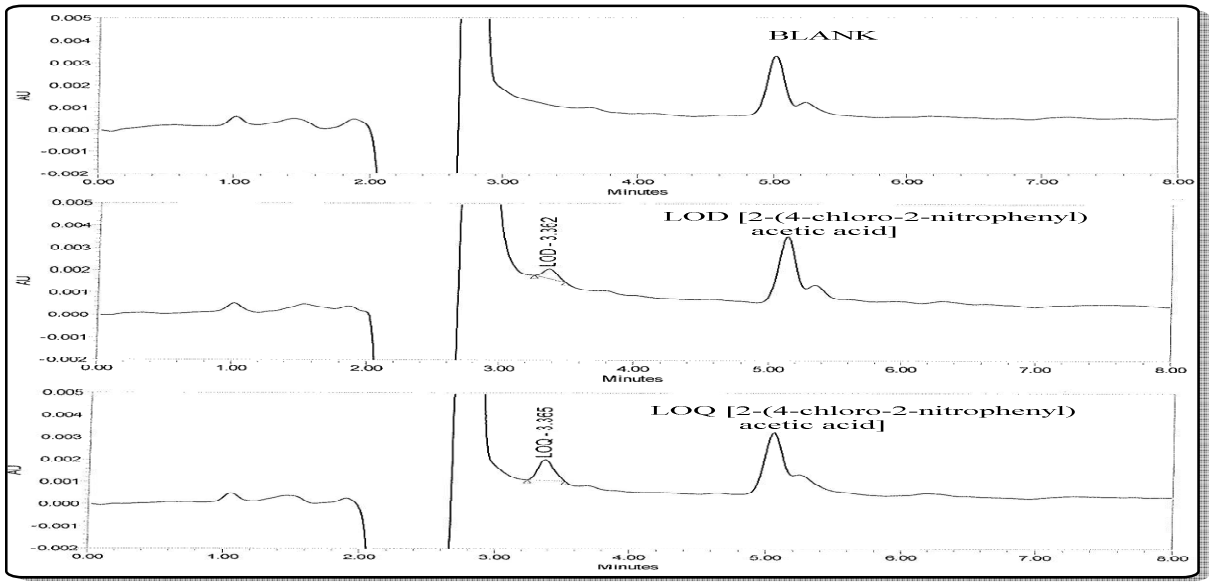


Figure.6.Typical chromatograms of 2-(4-chloro-2-nitrophenyl) acetic acid BLANK, LOD & LOQ

6-chloro-5-(2-chloro-1-hydroxy ethyl) indolin-2-one (Impurity-3)

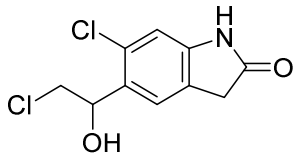


Figure.7. 6-chloro-5-(2-chloro-1-hydroxy ethyl) indolin-2-one

Preparation of impurity standard and test sample solution:

The impurity stock solution prepared at 0.5 mgmL-1 in diluent. For LOD and LOQ establishment, the impurity stock solution was diluted using diluent to give standards at 1.3, 3.9 ppm with respect to test concentration. The testing API samples were typically prepared at approximately 3.0 mg/mL in diluent and sonicated about 5 minutes.

Method validation

Limit of Detection (LOD) and Limit of Quantification

The LOD and LOQ values for 6-chloro-5-(2-chloro-1-hydroxy ethyl) indolin-2-one were established by injecting series of dilutions from standard preparation to get the Signal to noise ratio 2 to 3 for LOD and 9.5 to 10.4 for LOQ.

Table 8: LOD, LOQ results

6-chloro-5-(2-chloro-1-hydroxy ethyl) indolin-2-one	Concentration (ppm)	S/N ratio
LOD	1.3	3.0
LOQ	3.9	10.2

Precision

Precision is evaluated at LOQ level by carrying out six individual preparations 6.7 ppm solution of 6-chloro-5-(2-chloro-1-hydroxy ethyl) indolin-2-one in to the chromatographic system and checked % relative standard deviation (RSD). The % relative standard deviation (RSD) of the area at LOQ level is 3.82%.

Table 9: Precision results

Injection	1	2	3	4	5	6	Average	SD	%RSD
Area	10333	9734	10389	9830	9591	10478	10059.17	383.83	3.82

SD: Standard deviation, RSD: Relative standard deviation.

Accuracy:
The accuracy of the method is evaluated at LOQ level by preparing sample solutions in triplicate by spiking 6-chloro-5-(2-chloro-1-hydroxy ethyl) indolin-2-one at LOQ level and injected each solution in to HPLC as per methodology. The percentage of recovery for the impurity was calculated and the value is 96.6%. At such low levels these recoveries and % relative standard deviation (RSD) were satisfactory.

Table 10: Accuracy results

Level	Amount spiked (ppm)	Amount recovered (ppm)	% Recovery	Mean	SD	% RSD
LOQ spiked-1	3.9	4.1	105.1	96.6	7.8	8.1
LOQ spiked-2		3.5	89.7			
LOQ spiked-3		3.7	94.9			

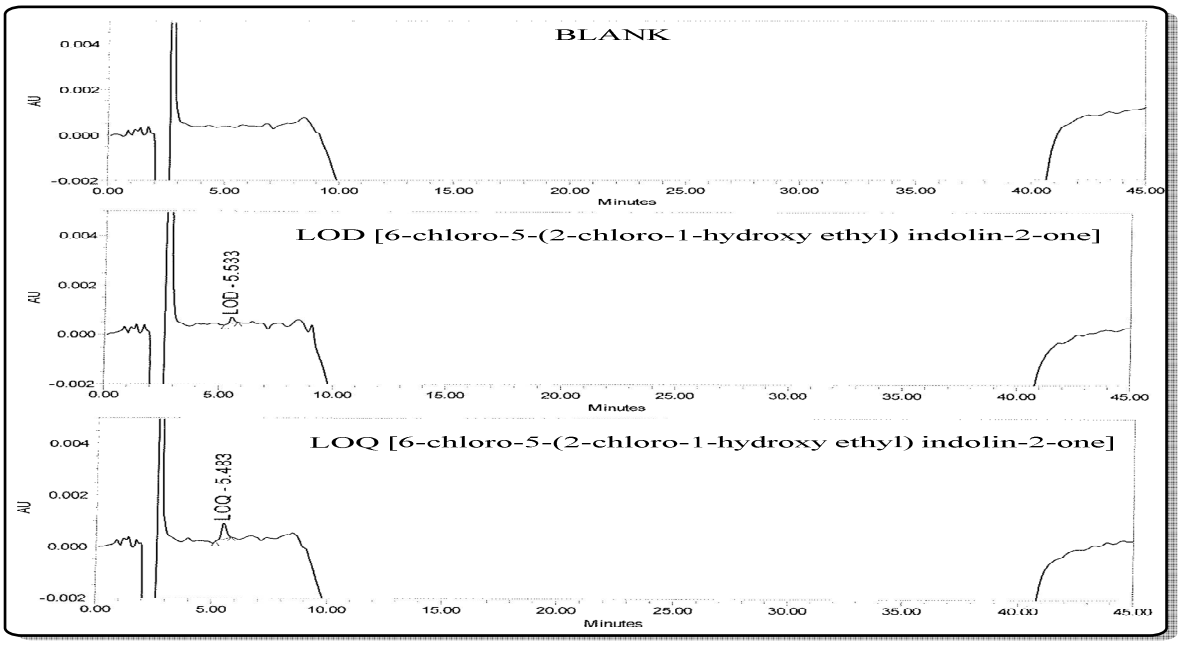


Figure.8. Typical chromatograms of 6-chloro-5-(2-chloro-1-hydroxy ethyl) indolin-2-one BLANK, LOD and LOQ

Quantification of 2, 5-Dichloro nitrobenzene (Impirity-4)

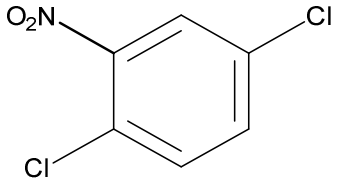


Figure.9. 2, 5-Dichloro nitrobenzene

Equipment
The HPLC method development done using Waters e 2695 separation module connected Waters 2489 UV/Visible detector and waters 2998 photodiode array detector and integrator. The data were collected using empower software.

Chromatographic conditions
A new gradient HPLC method is developed for separating 2, 5-Dichloro nitrobenzene from Ziprasidone and its impurities using Kromasil C18 (250 mm x 4.6 mm) 5 µm column with mobile phase containing a gradient mixture of solvent A (A Mixture of 0.02 M potassium phosphate, pH adjusted to 3.0 with 1N Sodium hydroxide and Acetonitrile in the ratio of 80:20) and B (A Mixture of 0.02 M potassium phosphate, pH adjusted to 3.0 with ortho phosphoric acid and Acetonitrile in the ratio of 20:80).). The separation was achieved by gradient elution (T/%B) set as 0/50, 5/50, 15/70, 30/70, 35/50, 45/50. The flow rate of mobile phase was 1.0 mL/min with column

temperature of 40°C and detection wavelength at 220nm. The test concentration was about 3.0 mg/ml and the injection volume was 50 µL. 0.25% phosphoric acid and Acetonitrile in the ratio of 50:50 was used as diluent during the standard and test sample preparation.

Preparation of impurity standard and test sample solution:
The impurity stock solution prepared at 0.5 mgmL-1 in diluent. For LOD and LOQ establishment, the impurity stock solution was diluted using diluent to give standards at 2.0, 5.9 ppm with respect to test concentration. The test samples of API were prepared at approximately 3.0 mg/mL in diluent and sonicated about 5 minutes.

Method validation
Limit of Detection (LOD) and Limit of Quantification
The LOD and LOQ values for 2, 5-Dichloro nitrobenzene were established by injecting series of dilutions from standard preparation to get the Signal to noise ratio 2 to 3 for LOD and 9.5 to 10.4 for LOQ.

Table 11: LOD, LOQ results

2, 5-Dichloro nitrobenzene	Concentration (ppm)	S/N ratio
LOD	2.0	2.5
LOQ	5.9	9.9

Precision
Precision is evaluated at LOQ level by carrying out six individual preparations of 6 ppm 2, 5-Dichloro nitrobenzene in to the chromatographic system and checked % relative standard deviation (RSD). The % relative standard deviation (RSD) of the area at LOQ level is 3.11%.

Table 12: Precision results

Injection	1	2	3	4	5	6	Average	SD	%RSD
Area	4333	4328	4314	4352	4515	4651	4415.50	137.23	3.11

SD: Standard deviation, RSD: Relative standard deviation.

Accuracy
The accuracy of the method is evaluated at LOQ level by preparing sample solutions in triplicate by spiking 2, 5-Dichloro nitrobenzene at LOQ level and injected each solution in to HPLC as per methodology. The percentage of recovery for the impurity was calculated and the value is 99.6%. At such low levels these recoveries and % relative standard deviation (RSD) were satisfactory.

Table 13: Accuracy results

Level	Amount spiked (ppm)	Amount recovered (ppm)	% Recovery	Mean	SD	% RSD
LOQ spiked-1	5.9	5.79	98.1	99.6	1.27	1.28
LOQ spiked-2		5.92	100.3			
LOQ spiked-3		5.92	100.3			

Quantification of 5-(2-Chloro acetyl)-6-chloro 2-oxindole (Impirity-5)

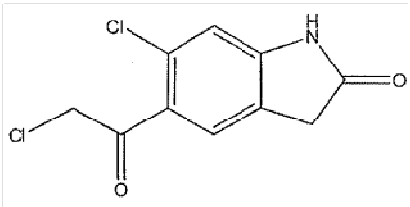


Figure.10. Structure of 5-(2-Chloro acetyl)-6-chloro 2-oxindole

Equipment
The HPLC method development done using Waters e 2695 separation module connected Waters 2489 UV/Visible detector and waters 2998 photodiode array detector and integrator. The data were collected using empower software.

Chromatographic conditions

A new HPLC method is developed for separating 5-(2-Chloro acetyl)-6-chloro 2-oxindole from Ziprasidone and its impurities using Symmetry shield RP C18 column 150 mm length x 4.6 mm ID with 3.5µm particle size using isocratic mobile phase of mixture of 0.02 M potassium di-hydrogen orthophosphate adjusted Ph to 6.5 with 1N sodium hydroxide and Acetonitrile in the ratio of 600: 400 (v/v) at a flow rate of 1.0 ml/min. UV detector was operated at 290 nm and the column temperature was set to 40°C. The test concentration was about 3.0 mg/ml and the injection volume was 50 µL. 0.25% phosphoric acid and Acetonitrile in the ratio of 50:50 was used as diluent during the standard and test sample preparation.

Preparation of impurity standard and test sample solution:

The impurity stock solution prepared at 0.5 mgmL-1 in diluent. For LOD and LOQ establishment, the impurity stock solution was diluted using diluent to give standards at 2.2, 6.7 ppm with respect to test concentration. The testing API samples were typically prepared at approximately 3.0 mg/mL in diluent and sonicated about 5 minutes.

Method validation

Limit of Detection (LOD) and Limit of Quantification

The LOD and LOQ values for 5-(2-Chloro acetyl)-6-chloro 2-oxindole were established by injecting series of dilutions from standard preparation to get the Signal to noise ratio 2 to 3 for LOD and 9.5 to 10.4 for LOQ.

Table 14: LOD, LOQ results

5-(2-Chloro acetyl)-6-chloro 2-oxindole	Concentration (ppm)	S/N ratio
LOD	2.2	2.5
LOQ	6.7	10.1

Precision

Precision is evaluated at LOQ level by carrying out six individual preparations of 6.7 ppm solution of 5-(2-Chloro acetyl)-6-chloro 2-oxindole in to the chromatographic system and checked % relative standard deviation (RSD). The % relative standard deviation (RSD) of the area at LOQ level is 7.92%.

Table 15: Precision results

Injection	1	2	3	4	5	6	Average	SD	%RSD
Area	2368	2881	2899	2523	2739	2579	2664.83	211.02	7.92

SD: Standard deviation, RSD: Relative standard deviation.

Accuracy

The accuracy of the method is evaluated at LOQ level by preparing sample solutions in triplicate by spiking 5-(2-Chloro acetyl)-6-chloro 2-oxindole at LOQ level and injected each solution in to HPLC as per methodology. The percentage of recovery for the impurity was calculated and the value is 91.2%. At such low levels these recoveries and % relative standard deviation (RSD) were satisfactory.

Table 16: Accuracy results

Level	Amount spiked (ppm)	Amount recovered (ppm)	% Recovery	Mean	SD	% RSD
LOQ spiked-1	6.7	6.5	97.0	91.1	5.4	5.9
LOQ spiked-2		6.0	89.6			
LOQ spiked-3		5.8	86.6			

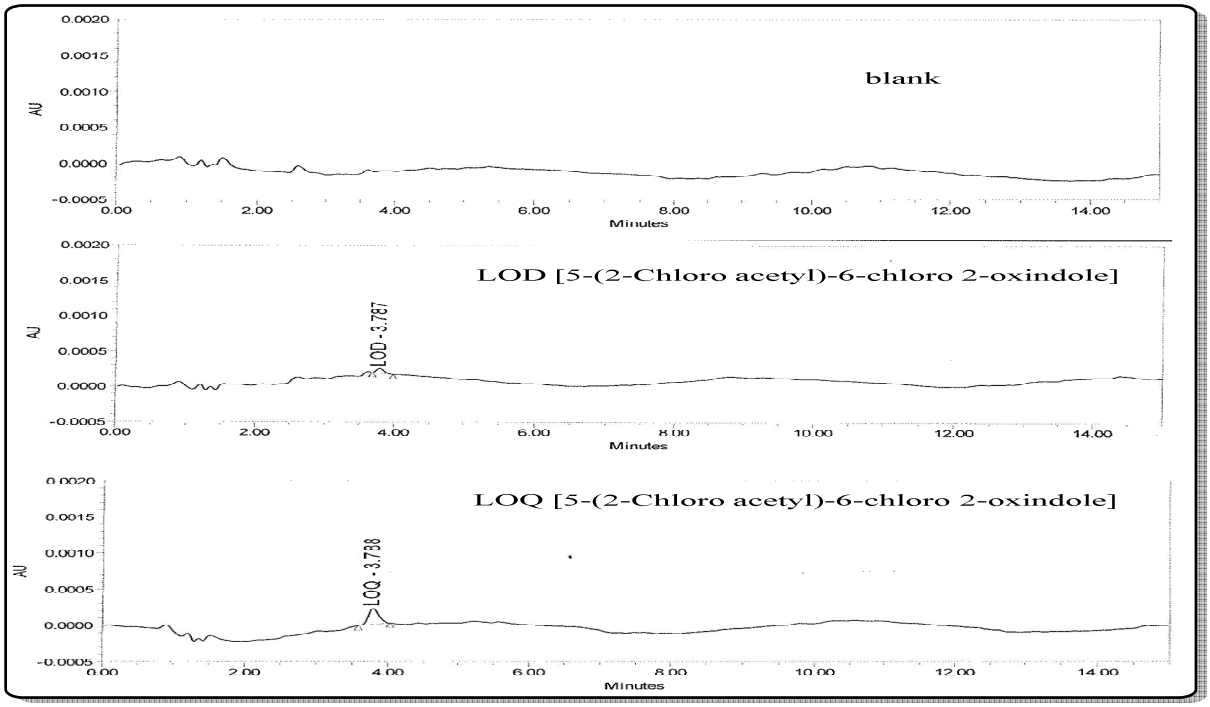


Figure-11: Typical chromatograms of 5-(2-Chloro acetyl)-6-chloro 2-oxindole BLANK, LOD and LOQ

RESULTS AND DISCUSSION

Table 16: Summary of results

S.no	Name of impurity	LOD (ppm)	LOQ (ppm)	Precision (%RSD)	Accuracy (%)
1	Dimethyl (4-chloro-2-nitrophenyl) malonate	2.0	6.0	3.96	98.1
2	2-(4-chloro-2-nitrophenyl) acetic acid	2.2	6.7	3.53	99.3
3	6-chloro-5-(2-chloro-1-hydroxy ethyl) indolin-2-one	1.3	3.9	3.82	96.6
4	2, 5-Dichloro nitrobenzene	2.0	5.9	3.11	99.6
5	5-(2-Chloro acetyl)-6-chloro 2-oxindole	2.2	6.7	7.92	91.1

Through evolution described the Assessment Quantification of Genotoxic impurities of Ziprasidone an antipsychotic drug.

CONCLUSION

In summary, we have developed a superior methodology for Assessment and Quantification of genotoxic impurities of Ziprasidone an antipsychotic drug which is comparatively better approach with respect to safety and quality for good health of patients. Moreover this sequence was also applied to the Assessment, synthesis and Quantification of Genotoxic impurities of other active pharmaceutical ingredients.

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