QUANTIFICATION OF GENOTOXIC IMPURITY 3-CHLORO ACETANILIDE IN ACETAMINOPHEN DRUG SUBSTANCE USING RP-HPLC

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ABSTRACT

Highly sensitive method for the determination of genotoxic impurity such as 3-Chloro acetanilide impurity in acetaminophen drug substance using RP-HPLC method has been presented in the paper. Quantification of 3-chloro acetanilide content in acetaminophen samples by HPLC with UV Detector (254 nm). 3-chloro acetanilide was determined by RP-HPLC method using Zorbax SB-C8 (250x4.6mm, 5µm) column as stationary phase. Flow rate was 0.8 mL/min, column temperature maintained 35°C, sample cooler temperature 25°C, injection volume 10µL and run time was 73 minutes. Mobile phase-A was used as 0.1% formic acid in water and methanol in the ratio of (950:50 v/v) and Mobile phase-B was used as 0.1% formic acid in water and methanol in the ratio of (500:500 v/v). The method validation has been carried as per international conference on harmonization guidelines (ICH). Limit of quantitation (LOQ) and Limit of detection (LOD) was found 3.219 ppm and 1.062 ppm for 3-chloro acetanilide.

Key words: Genotoxic impurity, 3-chloro acetanilide, Acetaminophen, RP-HPLC method, Validation and Limit of quantitation and Limit of detection .

1.0 Introduction

Synthesis of drug substances often involves the use of reactive reagents and hence, these reagents may be present in the final drug substances as impurities. Such chemically reactive impurities may have unwanted toxicities, including genotoxicity and carcinogenicity and are to be controlled based on the maximum daily dose [1]. These limits generally fall at low µg/mL levels. HPLC, GC methods (or final drug substance methods) are suitable for their determination. Their applications are oriented towards the potential identification and quantitation of trace level of impurities in drug substances [2].

The chemical name of N-(4-hydroxyphenyl)acetamide Corresponding to the molecular formula $C_8H_9NO_2$. It has a relative molecular mass of 151.165 g/mol. Acetaminophen (n-acetyl-p-aminophenol, APAP) is a valuable non-steroidal anti-inflammatory drug in widespread use for

the management of pain and fever and in a variety of patients, including children, pregnant women, the elderly; and those with ostheoartheritis, simple headaches, and non-inflammatory musculoskeletal conditions [3-4].

Acetaminophen is a white to off-white hygroscopic powder. Its solubility in organic solvents freely soluble in alcohol, soluble in methanol, ethanol, dimethylformamide, ethylene dichloride, acetone, ethyl acetate, slightly soluble in ether, practically insoluble in petroleum ether, pentane, benzene. Its pKa has been found to be 9.5 and its partition coefficient was found to be 0.91. In the manufacturing process of Acetaminophen, 3-Chloro acetanilide is used as reagent and hence genotoxic 3-chloro acetanilide may exist as impurity in Acetaminophen drug substance.

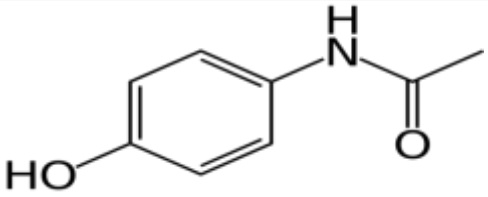

Figure 1.1: Chemical structure of acetaminophen

1.1 Impurity structure:

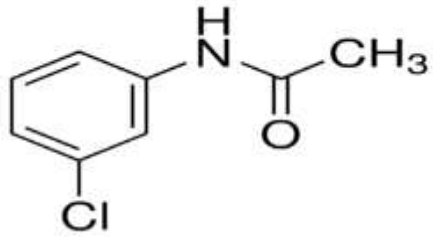

Figure 1.2: Chemical structure of 3-chloro acetanilide

Literature survey reveals that various analytical techniques like, UV spectrophotometry [5-9] spectrofluorimetric [10], high performance liquid chromatography HPLC [11-17] and high performance thin layer chromatography HPTLC [18-19] were reported for the analysis of acetaminophen in pharmaceuticals.

In literature, no analytical method was reported for the determination of 3-chloro acetanilide in acetaminophen drug substance. Hence the author was aimed towards the development of rapid, specific and robust methods for the determination of 3-chloro acetanilide in acetaminophen drug substance at trace level concentration. The method validation has been carried as per International Conference on Harmonization guidelines (ICH) [20].

2.0 Experimental

Chemicals and reagents:

Acetaminophen, 3-chloro acetanilide purchased from Sigma-Aldrich., Mumbai, India. Methanol and formic acid were procured from Merck, India.

Preparation solutions:

Preparation of 0.1% formic acid buffer:

Transferred 1.0 mL of formic acid solution into a 1000 mL water and mixed well.

Mobile phase-A:

Mixed accurately 0.1% formic acid buffer and methanol in the ratio of 950:50 v/v and mixed well.

Mobile phase-B:

Mixed accurately 0.1% formic acid buffer and methanol in the ratio of 500:500 v/v and mixed well.

Preparation of diluent:

Methanol was used diluent.

Preparation of 3-chloro acetanilide stock solution:

Weighed and transferred 10.165 mg of 3-chloro acetanilide impurity into a 100mL volumetric flask. Added 20 mL of diluent, dissolved and made up to volume with diluent and mixed well.

Preparation of standard solution:

Transferred 125 µL of 3-chloro acetanilide impurity stock solution into a 50 mL volumetric flask containing about 25 mL of diluent. Mixed well and made up to the mark with diluent. This solution is equivalent to 10 ppm of 3-chloro acetanilide with respect to 25.0 mg/mL of sample solution.

Preparation of sample solution:

Weighed 250 mg of the sample into a 10 mL volumetric flask. Dissolved in 5 mL of diluent and mixed well and then made up to the mark with diluent.

Preparation of spiked sample solution:

Weighed 250 mg of the sample into a 10 mL volumetric flask. Dissolved in 5 mL of diluent and added 25 µL of 3-chloro acetanilide impurity stock solution. Mixed well and then made up to the mark with diluent.

Chromatographic conditions:

RP-HPLC analysis was carried out on Agilent-1200 (Agilent Corporation, USA) wavelength 254 nm. Zorbax SB-C8 (250 x 4.6mm, 5µm)column was used as stationary phase. Mobile phase-A was used as 0.1% formic acid in water and methanol in the ratio of (950:50 v/v) and Mobile phase-B was used as 0.1% formic acid in water and methanol in the ratio of (500:500 v/v). The flow rate of the mobile phase was kept at 0.8 mL/min. The injection volume was set as 10 µL. Column oven temperature and auto sampler temperature were set as 35°C and 25°C respectively.

3.0 Method development

A blend solution containing 3-chloro acetanilide impurity and acetaminophen was run in 1.0 mL/min flow rate. The resolution between acetaminophen related compound-J and 3-chloro acetanilide impurities are found very less and hence the flow rate of the mobile phase was decreased from 1.0 mL/min to 0.8 mL/ min. In this condition the resolution between acetaminophen related compound-J and 3-chloro acetanilide impurities are found satisfactory, but the retention time of 3-chloro acetanilide impurity was drastically increased. Hence, the elution order was observed from the chromatogram **(Figure 1.3-1.6)** acetaminophen solution spiked with 3-chloro acetanilide impurity (10 µg/mL).

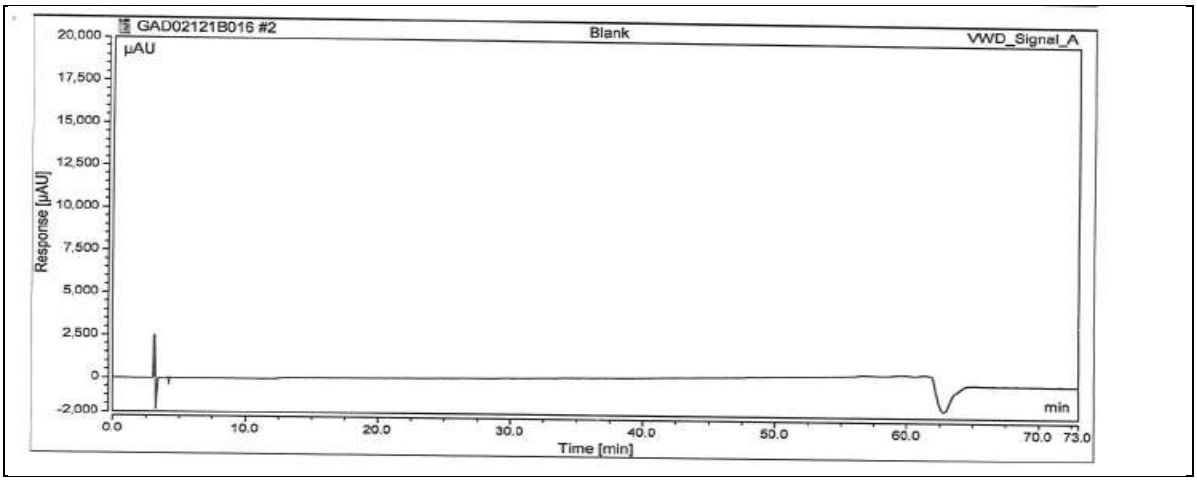

Figure 1.3: typical chromatogram of blank

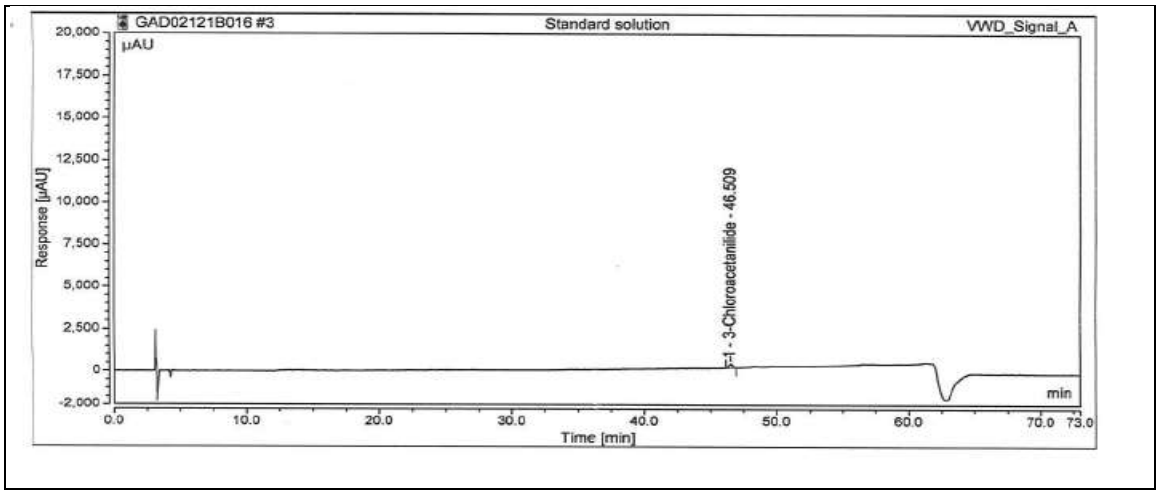

Figure 1.4: typical chromatogram of standard

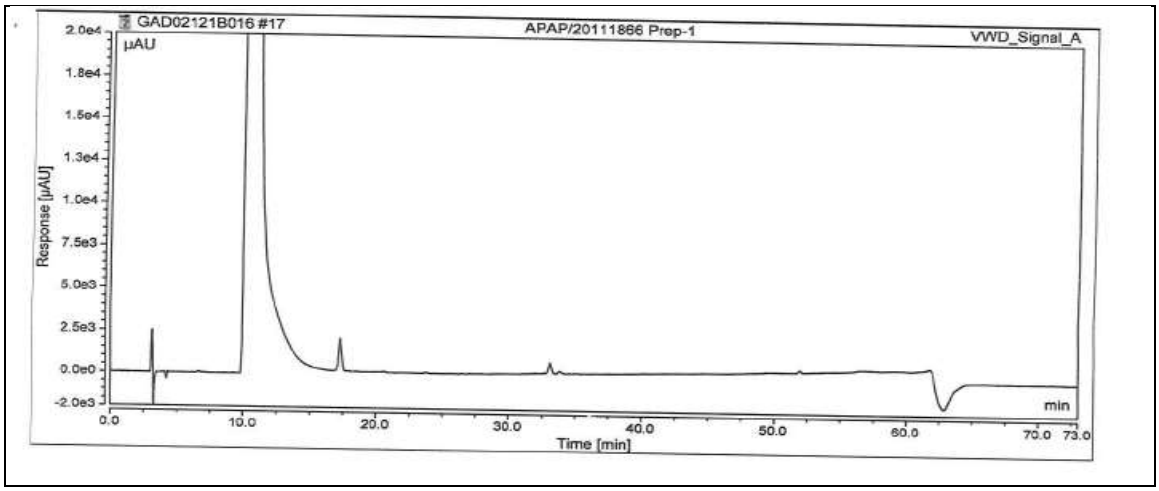

Figure 1.5: typical chromatogram of acetaminophen sample

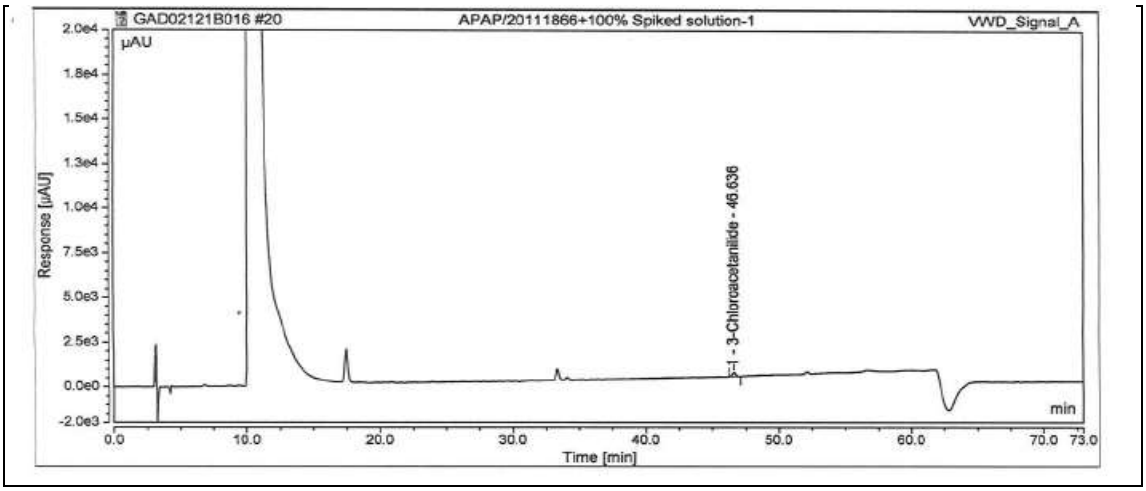

Figure 1.6: Spiked 3-Chloro acetanilide chromatogram of acetaminophen

3.2 Method validation

Results and Discussion

The developed method was validated as per ICH guidelines [20] in terms of specificity, limit of detection (LOD), limit of quantitation (LOQ), precision, linearity, accuracy and system suitability and the data are presented in **Table 1.0.**

The specificity of the developed LC method was indicated by 3-chloro acetanilide impurity solution (10 µg/mL) with respect to 25 mg/mL of acetaminophen was injected separately and S/N ratios were recorded. These solutions were further diluted to achieve the signal-to-noise (S/N) ratios at 3 and 10 for determining LOD and LOQ, respectively for both the methods. The precision of the methods was checked by injecting LOQ solutions for six times. The value of RSD for area of 3-Chloro acetanilide impurity was calculated.

Table 1.0 Validation data of acetaminophen for the determination of 3-chloro acetanilide impurity

Parameter	3-chloro acetanilide impurity
LOD (µg/mL)	1.062
LOQ (µg/mL)	3.219
Precision at LOQ level (RSD, %)	7.06
Precision at sixth level (RSD, %)	2.04
Linearity (µg/mL)	3.22-15.9
Correlation coefficient	0.9990
Slope	407.98
Intercept	184.75
% of y-intercept	4.1
Accuracy at LOQ (recovery, %)	104.3
Preparation-1	109.6
Preparation-2	101
Preparation-3	102.4
Accuracy at 150 (recovery, %)	96.4
Preparation-1	97.4
Preparation-2	95.4
Preparation-3	96.4

The intermediate precision of the method was also verified on six different days in the same laboratory using the LOQ level solutions. The low RSD values ensured the precision of the developed method. Linearity test solution for 3-chloro acetanilide impurity was prepared

individually at six concentration levels in the range of LOQ to 150% of the specification level 10 µg/mL. LOQ and sixth levels were injected six times and other four levels were injected thrice. The average peak areas versus concentrations were subjected to least-squares linear regression analysis. The derived correlation coefficients were above 0.9990 indicating the best fitness of the linearity curves of the developed method.

Standard addition experiments were conducted in triplicate preparations to determine accuracy of the methods at LOQ and 150% level and recoveries of all the genotoxins were determined. The recoveries were found to be in the accepted range. The system suitability of the method was ensure by getting the % RSD less than 10.0 for six injections of the 3-chloro acetanilide impurity in RP-HPLC method at specification level. Acetaminophen at trace level concentration have been developed and validated as per ICH guidelines.

4.0 Conclusion

The proposed RP-LC method that can quantify genotoxic 3-chloro acetanilide impurity in acetaminophen at trace level concentration have been developed and validated as per ICH guidelines. The effectiveness of the method was ensure by the specificity, precision and accuracy. Hence, the method well suit for their intended purposes and can be successfully applied for the release testing of acetaminophen into the market.

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Conflict of interests

The authors claim that there is no conflict of interest.

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