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Research Article

Application of 2, 4-Dinitro Phenyl Hydrazine as a Derivatizing Reagent for the Trace Level Quantification of Toxic Impurity Formaldehyde in Entecavir – An Antiviral Drug for Treatment of Hepatitis B by High Performance Liquid Chromatography

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ABSTRACT

The objective of present research work was to develop a suitable and sensitive analytical method for the quantification of trace level of toxic impurity Formaldehyde in the active pharmaceutical ingredient Entecavir. Formaldehyde was quantified through pre-column derivatization using 2,4-Dinitro Phenyl Hydrazine (2,4-DNPH) by gradient elution on HPLC. The analysis was accomplished on an Inertsil ODS C18 column (50mm x 4.6mm, 3µ) using 0.1% Triethyl amine in water and pH was adjusted to 3.0 with Trifluoro acetic acid as buffer and Acetonitrile as the organic modifier. The flow rate was set at 1.5 ml/minute with an analysis time of about 15 minutes. The method was validated for the analytical parameters such as System Suitability, Specificity, Linearity and Range, Accuracy, Limit of Detection (LOD), Limit of Quantification (LOQ), Precision, and Solution Stability. The limit of detection and limit of quantification of Formaldehyde was found to be 0.2µg/ml and 0.6µg/ml respectively with respect to Entecavir sample concentration (1mg/ml).

Keywords: Toxic, Entecavir, Mutagenic, Carcinogenic, HPLC and 2, 4-DNPH.

1. INTRODUCTION

Aldehydes are the major by-products of lipid-oxidation and are also extremely biologically reactive.[1] Formaldehyde is the major by-product of lipid oxidation, which has clearly shown to be carcinogenic via inhalation route.[2,3] Acetaldehyde has shown to be rodent carcinogenic via inhalation route.[4] Formaldehyde, monoaldehyde, acrolein, glyoxal and aldehydes are not considered as carcinogenic via oral route.[5-9]Quantitative analysis of trace level carbonyl compounds (viz., aldehydes and ketones) from the pharmaceutical drug substances is especially complex and time-consuming. The Complexities involved with the analysis of these compounds include volatility and instability at higher temperatures and sample matrices in acidic environments. Consequently, carbonyl compounds are generally derivatized using 2, 4-dinitrophenylhydrazine (DNPH) to improve stability (Fig.1). DNPH derivatives are relatively more stable thermally, allowing for analysis by Gas chromatography (GC). Quantitative analysis by GC coupled with Mass spectrometry (MS) for many carbonyl-DNPH derivatives in different samples have been reported previously.[10] DNPH derivatization also improves chromatographic properties and increases UV absorptivity for analysis by High-Performance Liquid Chromatography (HPLC–UV), in addition to increased thermal stability. However, most methods for the quantification of multiple carbonyl groups using DNPH derivatizing reagent requires a long analysis time and have limited selectivity, especially when the sample matrices comes into the effect. For

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instance, lengthy chromatographic separations using HPLC–UV, which can be as long as 40-60 minutes, are described for the separation of DNPH derivatives of commonly occurring carbonyls (especially aldehydes and ketones) in air and water.[11,12,13,14]

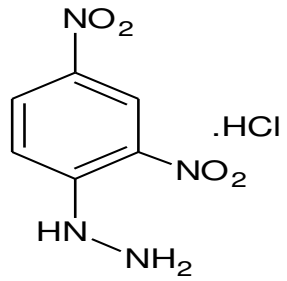

Fig 1: Structure of 2,4-dinitro Phenyl Hydrazine Hydrochloride

Of all the earlier methods reported for the detection and quantification of carbonyls in environmental samples (viz., smoke) at trace levels, many issues still remain unresolved.[15] Smoke is considered to be an extremely complex matrix and reportedly contains over 4000 compounds.[16,17] This also implies that the complexity of the sample matrix may cause interferences that cannot be resolved by HPLC–UV.

The present work demonstrates the application of HPLC for the analysis of formaldehyde in the active pharmaceutical ingredient, Entecavir, through pre-column derivatization of the aldehyde with 2,4 - DNPH. Although the paper only emphasizes on the detection of the toxic compound, Formaldehyde, the chromatographic separation and detection techniques employed here could also be applied to the analysis of carbonyls ranging from pharmaceutical products/substances to environmental samples.

2. MATERIALS AND METHODS

2.1 Chemicals

Formaldehyde was sourced from Teckbond Laboratories. 2,4–Dinitro Phenyl Hydrazine was procured from S.D. Fine Laboratories which was 97 % pure. Acetonitrile used was purchased from Fisher-Scientific. Sulfuric acid used was of Merck (A.R.Grade) and Triethyl amine and Trifluoro acetic acid used was purchased from Fluka Analytical. The Water used for analysis was purified using Merck Millipore TKA Water purifier.

2.2 Preparation of the mobile phase and the solutions for derivatization

2.2.1 Buffer solution

Solvent A - The aqueous buffer solution was prepared by adding 1.0 ml of Triethylamine to 1000 ml of purified water. The pH of the buffer solution was adjusted to 3.0 using Trifluoro acetic acid.

Solvent B – Acetonitrile

2.2.2 Catalyst solution

The catalyst solution 0.5M Sulfuric acid was prepared in Acetonitrile.

2.2.3 Derivatization reagent

The Derivatizing reagent 2,4-DNPH was prepared by dissolving 10mg of reagent in 50ml of Acetonitrile.

2.3 Instrumentation

The analysis was performed on Agilent 1200 High Performance Liquid Chromatography (HPLC) system (Milford, MA) with an auto sampler and binary solvent system interfaced to an Agilent DAD detector and Chromeleon Software. The detection was carried out at 360nm using an Inertsil ODS-3 C18 column (50mm x 4.6mm, 3µ) and the gradient program as given in Table 1.

Table 1: Gradient Program

Time (Min)	Solvent A (%)	Solvent B (%)
0.00	75	25
4.00	75	25
10.00	20	80
12.00	20	80
12.01	75	25
15.00	75	25

2.4 Chromatographic conditions

Agilent HPLC 1200 series instrument equipped with column oven, PDA detector and the data was processed using computer program Chromeleon software. The chromatographic condition was optimized using a C18 Stationary, Inertsil ODS (50mm x 4.6mm, 3µ). The Gradient HPLC method was developed with mobile phase 0.1% Triethyl amine in water (pH adjusted to 3.0 with Trifluoro acetic acid) i.e Solvent A and Solvent B is acetonitrile, which was pumped at a flow rate of 1.5mL/min.

The temperature of the column was maintained at 45°C and the wavelength selected was 360nm. The injection volume was 5µL.

2.5 Formaldehyde standard and Entecavir Sample Preparations

The blank solution was prepared by transferring 1.0ml of the Catalyst Solution to a 10ml volumetric flask containing 1.0ml of DMSO. This solution was swirled for proper mixing and then diluted up to the mark with the Derivatizing Reagent. Formaldehyde Stock Solution was prepared by adding about 260mg of Formaldehyde in a 100ml volumetric flask containing sufficient Acetonitrile and was further diluted to the mark using Acetonitrile. The resultant solution was further diluted using Acetonitrile to obtain the 10ppm Formaldehyde diluted solution. Further, transfer 1.0mlof the 10ppm Formaldehyde diluted solution into a 10ml volumetric flask containing 1.0ml each of DMSO, Catalyst Solution and Derivatizing Reagent. Dilute this solution up to the mark with Acetonitrile and mix well. This solution is called as Formaldehyde Standard Solution. The Entecavir Sample Solution was prepared by transferring 10mg of sample into 10ml volumetric flask followed by addition of 1ml each of DMSO, Catalyst Solution and Derivatizing Reagent. This solution is then diluted upto with mark with Acetonitrile.

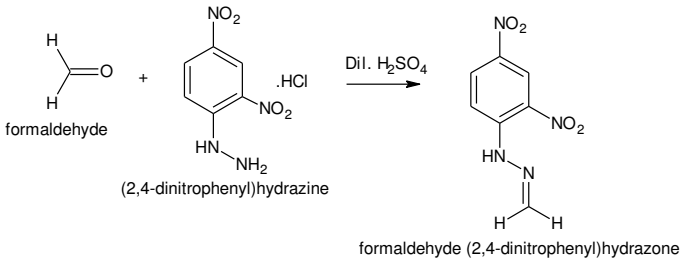

Fig. 2: Reaction scheme for Formaldehyde Derivative

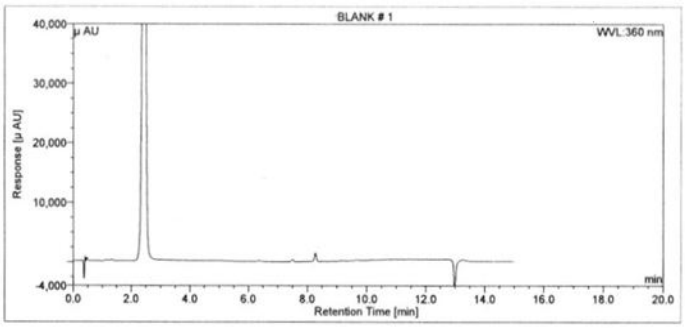

Fig.3: Blank Chromatogram

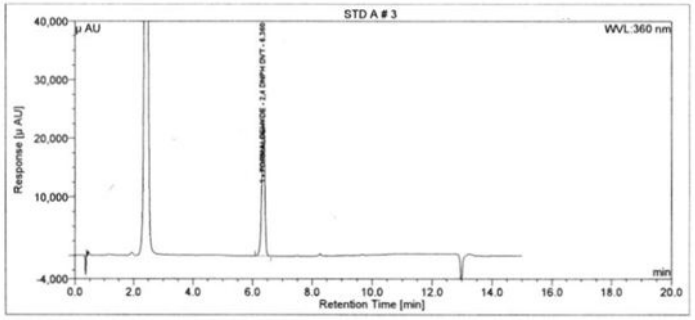

Fig.4: Standard Chromatogram for Formaldehyde Derivative

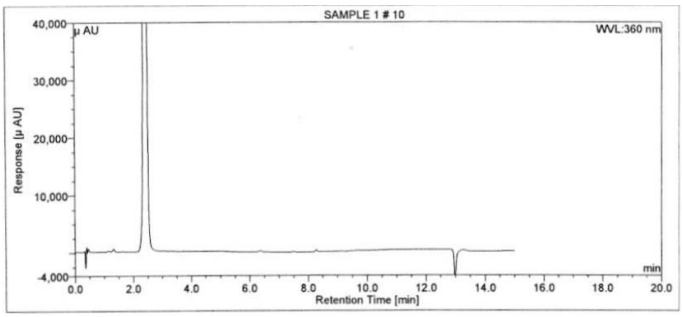

Fig.5: Chromatogram of Entecavir Sample

3. RESULTS AND DISCUSSION

3.1 Method development and column selection

Formaldehyde is a toxic impurity in Entecavir. The main purpose was to develop a method for the quantification of Formaldehyde at trace levels using pre-column derivatization technique in the Antiviral drug Entecavir.

Formaldehyde reacts rapidly with 2,4-DNPH in acidic condition to form Formaldehyde-2,4-Dinitro Phenyl Hydrazone. Selection of the HPLC column has played a critical role in achieving the separation of the Formaldehyde derivative with an unknown peak from Entecavir. Method development was initiated by using water and acetonitrile (50:50v/v) at a flow rate of 1.0ml/min. The column used was Inertsil ODS C18, 150mm in length, internal diameter 4.6mm and 5µm particle size Stationary phase. The response of Formaldehyde derivative was not appreciable. To improve the peak shape and sensitivity of the method, water was replaced with 0.1% TEA buffer solution and Formaldehyde derivative was injected into the HPLC system. The sensitivity of the peak was slightly improved, but the unknown peak was still co-eluting with the analyte peak.

In the next trial, the column was replaced with Inertsil ODS C18 50mm in length with an internal diameter 4.6 and 3µm particle size Stationary phase. An Isocratic method comprising of mobile phase 0.1% TEA (pH adjusted to 3.0 with orthophosphoric acid) and acetonitrile (70:30v/v) was

considered for improving the resolution. The flow rate was set at 1.5ml/min and wavelength selected was 360nm. The unknown peak was separating from the Formaldehyde peak, but no significant improvement in the response was achieved.

The desired chromatographic separation and sensitivity was successfully achieved on the Inertsil ODS C18 column (50mm x 4.6mm x 3µm) and the mobile phase system comprising of 0.1% TEA (pH adjusted to 3.0 with trifluoro acetic acid) and acetonitrile using gradient elution program as given in Table 1, at a flow rate of 1.5mL/min. The column temperature was maintained at 45°C and the detection wavelength was set at 360 nm. The injection volume was 5µl, with a sample loading of 1mg/ml. In the optimized conditions, Formaldehyde derivative was well-separated from the unknown peak and its detection sensitivity was also enhanced. The typical chromatogram of Formaldehyde derivative with Entecavir is presented in (Fig.4). The method was specific to separate toxic impurity Formaldehyde from Entecavir. The system suitability data was shown in (Table 2).

Table 2: System Suitability

Parameter	Limit	Value
Tailing Factor	Between 0.8 to 2.0	0.9
Theoretical plates	Not less than 2000	18117

3.2 Specificity

Specificity of the method is its ability to detect and separate all the impurities present in the drug. Specificity of the method is demonstrated in terms of spectral as well as peak purity data of the drug and impurities present in drug. Identification solutions of Formaldehyde derivatives was injected at limit level concentration along with Entecavir 1mg/ml. Spiked sample of Entecavir with Formaldehyde derivative was also injected (Fig.6). Peak purity data was confirmed to show, that there were no interferences at the retention time of Formaldehyde derivative and Entecavir.

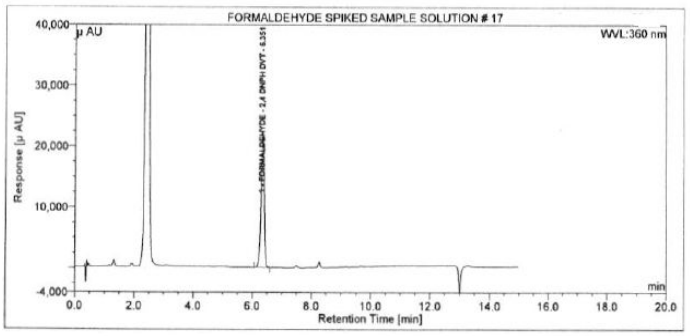

Fig. 6: Spiked Sample of Entecavir with Formaldehyde Derivative

3.3 Linearity

Linearity of the method was checked by preparing derivatives of four concentration levels of 0.012% (Level 1), 0.06% (Level 2), 0.15% (Level 3) and 0.225% (Level 4). Level 1 and level 4 was injected three times, whereas level 2 and level 3 was injected two times. The mean responses recorded for Formaldehyde derivative was plotted against Concentration. The Correlation Coefficient was found to be 1.000, which indicates good linearity. (Fig. 7) (Table no. 3)

Table 3: Linearity data

Peak Name	Slope	% y intercept	Correlation Coefficient	R Square
Formaldehyde 2,4 DNPH	91689.812	4.2	1.000	0.99997

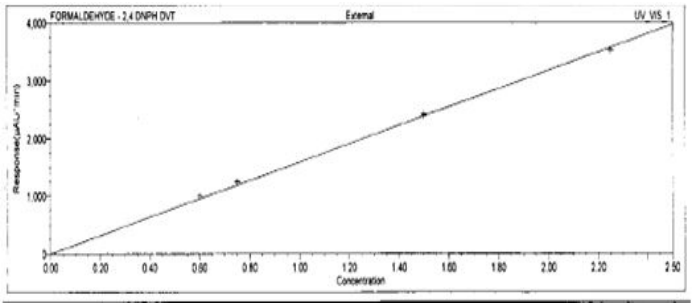

Fig. 7: Linearity of Formaldehyde Derivative

3.4 Accuracy

Entecavir sample solutions of 1mg/ml were spiked with Formaldehyde at different concentration levels of 0.06% (0.6µg/ml), 0.075% (0.75µg/ml), 0.15% (1.5µg/ml) and 0.23% (2.3µg/ml). Each Level of solution was prepared in duplicate and injected. The recovery percentage and %R.S.D was calculated for Formaldehyde derivative. Recoveries of results are shown in Table 4 respectively. The acceptance criterion for recovery of an impurity at a concentration level of 0.6µg/ml was

between 85% and 115% and at 0.15% and 0.23% was 90% to 110%.

Table 4: Accuracy results for Formaldehyde Derivative

Amount added (µg/ml)	Amount obtained (µg/ml)	Recovery (%)
0.6	0.59	98.33
0.75	0.66	88.00
1.5	1.42	94.66
2.3	2.31	100.40

3.5 Limit of Detection

The sensitivity for detection can be demonstrated by determining the Limit of Detection (LOD). A signal to noise (S/N) ratio between 3 to 10 is generally considered to be acceptable for estimating the detection limit. S/N ratio of the individual peak was determined at different concentrations to estimate LOD and respective %RSD was calculated for replicate injection (n=3). The LOD was found to be 0.2µg/ml for Formaldehyde derivative. The results are shown in the Table 5.

Table 5: LOD and LOQ results of Formaldehyde Derivative

Limit of detection	0.2 µg/ml
Signal to Noise ratio	33
Limit of Quantification	0.6 µg/ml
Signal to Noise ratio	92

3.6 Limit of Quantification

The quantification limit is the lowest concentration of a substance that can be quantified with acceptable precision and accuracy. A typical S/N ratio of 10-30 is generally considered to be acceptable for estimating the limit of quantification. The LOQ was determined to be 0.6µg/ml for Formaldehyde derivative. The results were shown in Table 5.

3.7. Precision

The system for formaldehyde impurity was checked for repeatability. The sample was prepared by spiking Entecavir with the formaldehyde a concentration of 0.15% of target analyte concentration and injected six times. The %RSD was found to be less than 5.0% for system precision.

To determine the method precision six independent solutions were prepared by spiking Entecavir with the impurities at a concentration of 0.15% with respect to target analyte concentration. Each solution was injected once. The variation in the results for the Formaldehyde were expressed in terms of % RSD. The values calculated were found to be below 15.0% RSD for impurities, indicating satisfactory method precision.

3.8. Solution stability

A solution of Entecavir containing impurities was prepared and kept at room temperature. This solution was injected at intervals of 0, 4, 8, 12, 16, 20 and 24hr. Area of Formaldehyde were nearly identical to that obtained at 0h and additional peaks were not observed which indicate solution stability.

4. CONCLUSION

Due to the absence of Chromophore, Formaldehyde does not exhibit absorption in the UV range and hence cannot be detected by HPLC equipped with UV detector. However, through pre-column Derivatization using chromatographic reagents, its detection can be significantly increased. Different derivatizing reagents have been reported in the literature for detection of Formaldehyde. In the present work we have used 2,4-DNPH since it provides stable derivative with absorption maximum at 360nm. The proposed HPLC method is selective and sensitive for the quantification of Formaldehyde in Entecavir. The method is validated for all the analytical parameters, which can be used for routine analysis in quality control.

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