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Indian
Journal of Advances in
Chemical Science

Indian Journal of Advances in Chemical Science 3(3) (2015) 267-273

Method Development and Validation for Quantitative Determination of Para Chloroaniline, an Impurity, in Efavirenz Active Pharmaceutical Ingredient by Liquid Chromatography - Electrospray Ionization - Mass Spectrometry/Mass Spectrometry

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Received 11th April 2015; Revised 28th April 2015; Accepted 30th April 2015

ABSTRACT

A simple, sensitive and rapid liquid chromatography (LC) - electrospray ionization (ESI) - mass spectrometry (MS)/MS method has been developed and validated for the trace analysis (>1 ppm level) of para chloroaniline, a genotoxic impurity, in Efavirenz drug. The chromatographic separation was achieved on a hypersil BDS (150 × 4.6 mm, 5 µm) column using a mobile phase consisting of 5 mM ammonium acetate buffer (pH 4) and acetonitrile (60:40, v/v) at flow rate of 0.7 mL/min and elution was monitored at 305 nm. The active pharmaceutical ingredient-4000 LC-MS/MS was operated on an ESI equipped with an ESI interface operated in positive ionization (single reaction monitoring) mode and it is able to quantitate up to 0.3 ppm of para chloroaniline. The newly developed method was validated as per ICH guidelines.

Key words: *Efavirenz, Para chloroaniline method validation, Liquid chromatography-electrospray ionization-Mass Spectrometry/Mass Spectrometry, Trace analysis.*

1. INTRODUCTION

Efavirenz (EFV, Sustiva®) is a non-nucleoside reverse transcriptase inhibitor (NNRTI) that was Food and Drug Administration (FDA) - Approved in 1998 for the treatment of HIV as part of highly active antiretroviral (ARV) therapy. EFV is currently used in combination with lamuvudine and zidovudine or tenofovir and emtricitabine as the preferred NNRTI-based combination regimen for treatment-native HIV patients [1]. ARV therapy has shown to effectively reduce viral replication in patients and is the main course of treatment. Many ARV drugs, however, inadequately penetrate into the male genital tract (MGT). Limited drug access to the MGT would permit viral replication and could potentially engender resistance, creating a "pharmacological sanctuary." The mechanism of ARV drug penetration and distribution into the MGT is largely unknown [2]. Many ARV's have large blood plasma: Seminal plasma ratios of total drug concentrations [3]. Protein binding has been suggested as an explanation for these large observed ratios. The extent of protein binding of an anti-retroviral drug in the blood correlates well with the observed blood: Semen drug concentration ratios and may be the dominant factor influencing these ratios. It is generally assumed that only the unbound "free" drug is able to cross into the MGT is active against viral replication. EFV is one of the most highly protein bound ARV drugs existing at >99% protein bound, predominantly to albumin [4]. EFV's very high protein binding in blood makes it a unique and optimum candidate to study the distribution and penetration into the MGT. The availability of a method for reliable detection and quantitative measurement of protein-free EFV in human seminal plasma would enable investigation of the MGT as a "pharmacological sanctuary" for EFV.

Impurity profiling is now receiving important critical attention from regulatory authorities. Different compendia such as the British, the United States and the Brazilian pharmacopoeias have been specifying limits to allowable levels of impurities present in drug substances or products. Besides, ICH and FDA have published guidelines on residual solvents and impurities in new drug substances and products [5-8].

The control of drug chemical impurities is a very difficult issue to the pharmaceutical industries.

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The contain of very small amount of unwanted chemicals may affect the safety and efficiency of the pharmaceutical products [9]. The monograph was adopted at the Fortieth WHO Expert Committee on the specification for pharmaceutical preparation in October 2005 for addition to the 4th edition of the international pharmacopoeia. EFV is white to slightly pink powder and practically insoluble in water, but freely soluble in methanol. EFV should be kept in a well-closed container, protected from light. Chemical name of EFV is (4S)- 6-Chloro-4- (cyclopropylethynyl)-1,4- dihydro-4-(trifluoromethyl)-2H-3,1- benzoxazin-2-one of CAS No. is 154598-52-4. The molecular formula is C14H9ClF3NO2 and the molecular mass is 315.7. The molecular structure is given in the Figure 1.

Pharmaceutical genotoxic impurities (PGIs) may induce genetic mutations, chromosomal breaks (rearrangements) and they have potential to cause cancer in human [10,11]. Therefore exposure to even low levels of such impurities present in final active pharmaceutical ingredient (API) may be of significant toxicol importance [12]. Hence, it is significant for process chemists to avoid such genotoxic impurities in the manufacturing process [13]. However, it would be difficult or impossible to eliminate PGIs completely from the synthetic scheme. Therefore it is a great challenge to analytical chemists to develop an appropriate analytical method to quantify the impurity accurately and control their levels in APIs. According to the European medicines evaluation agency and feedback from US FDA the proposed use of a threshold of toxicological concern, it is accepted that genotoxic impurities will be limited to a daily dose of 1.0-1.5 µg/day [14,15].

Several methods are described in literature for the quantification of EFV in biological fluids by high performance liquid chromatography (HPLC) with ultraviolet detection [16-20], HPLC with fluoresecence detector [21], liquid chromatography with mass spectrometry detection (LC-MS), [22] LC with MS in tandem detection (LC-MS/MS) [23,24] and matrix assisted laser desorption/ionization with time-of-flight in tandem detection [25], UPLC-MS/MS methods are reported [26].

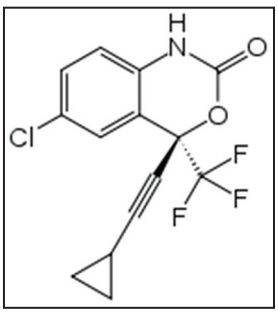

Figure 1: Efavirenz structure.

Although there are different methods are available in the literature there is no single method to the determination of para chloroanillin in EFV (API). In the present paper, a simple, sensitive and rapid LC-MS/MS validated method has been proposed for determination of para chloroanillin in EFV API.

2. EXPERIMENTAL

2.1. Chemicals and Reagents

Methanol and acetonitrile of HPLC grade were purchased from J.T Baker (Phillipsburg, USA). Analytical grade ammonium acetate, formic acid and HPLC grade water were purchased from Merck, (Mumbai, India). Water used for the LC-MS/MS analysis was prepared from Milli Q water purification system procured from Millipore (Bangalore, India). Reference substance of para chloroaniline was obtained from Sigma-Aldrich (St. Louis, USA).

2.2. Preparation of Stock and Standard Solutions

Primary stock solutions of para chloroaniline and EFV were prepared in methanol (1 mg/mL). Another set of working stock standard solution of 0.001 mg/mL was achieved on further dilution with mobile phase. The stock solutions stored at 2-8°C were found to be long-term stability for 20 days (data not shown), consecutively diluted with methanol to final concentration (7.5 ng/mL) to get working solutions for obtaining calibration curve.

2.3. HPLC Operating Conditions

A Shimadzu LC-20 AD Series HPLC system (Shimadzu Corporation, Kyoto, Japan) was used to inject 20 µL aliquots of the processed samples on a Hypersil BDS column (150×4.6 mm, 5 µm), which was kept at 40±2°C temperature. The isocratic mobile phase, a mixture of 5 mM ammonium acetate (pH 4):acetonitrile (60:40, v/v) was filtered through a 0.45 µm membrane filter (XI5522050) (Millipore, USA or equivalent), then degassed ultrasonically for 5 min and delivered at a flow rate of 0.7 mL/min into the MS electrospray ionization chamber.

2.4. MS Operating Conditions

Quantitation was achieved with MS-MS detection using a MDS Sciex API-4000 MS (Foster City, CA, USA) equipped with Turboionspray™ interface at 400°C. The MS/MS method consists of positive ionization mode. The ion spray voltage was set at 5000 V. The source parameters *viz*., the ion source gases GS1, GS2 and curtain gas were set at 30, 25, and 12 psi, respectively. The compound parameter *viz*. the declustering potential was set at 52. Detection of the ions was carried out in the selective ion monitoring mode (SIM) was considered to get better selectivity, by monitoring the para chloroaniline of $[M+H]^+$ *m/z* 127.3 precursor ion and the $[M+H]^+$ *m/z*

315.6 precursor ion for EFV. The analytical data obtained were processed by Analyst software™ (version 1.4.2).

3. RESULTS AND DISCUSSION

3.1. Method Development and Optimization

Optimization of chromatographic conditions was performed, particularly the composition of mobile phase, through several trials to achieve symmetric peak shapes of the analytes peaks, as well as short run time. Resolution positive mode EFV was achieved by using acetonitrile as an organic content in the mobile phase. Separation was attempted using various combinations of acetonitrile and buffer with varying contents of each component on different columns like C_8 and C_{18} of different makes such as chromolith, hypersil, hypurity advance, zorbax, kromasil and inertsil. Hypersil BDS column was found to give the best chromatographic resolution with a flow rate of 0.7 mL/min and total run time of 15 min. The para chloroaniline and EFV were eluted at 6.9 min and 9.1 min with SIM mode. The inclusion of 5 mM ammonium acetate instead of pure water enhanced the response and improved the reproducibility.

3.2. Method Validation

3.2.1. Specificity and selectivity

Specificity is the ability of the method to assess unequivocally the analyte response in the presence of components that may be expected to be present in the sample. EFV and para chloroaniline solutions were prepared individually at a concentration of about 0.01 mg/mL in the diluents and a solution of EFV spiked with para chloroaniline was also prepared. Specificity was established by injecting EFV spiked with its impurity where in no interference was observed. Blank and specificity chromatograms are shown in Figure 1.

3.2.2. Robustness

The robustness of the developed method was studied with slight and deliberate changes in experimental conditions. The effect of changes in flow rate of mobile phase (−10% to +10%), percentage of organic modifier in mobile phase (−2% to +2%) while the amounts of the other mobile phase components were held constant, column oven temperature (−2°C to +2°C) i.e. at 38°C and 42°C and pH of the buffer (−0.2 units to +0.2 units) was studied. For all the above deliberately varied experimental conditions,

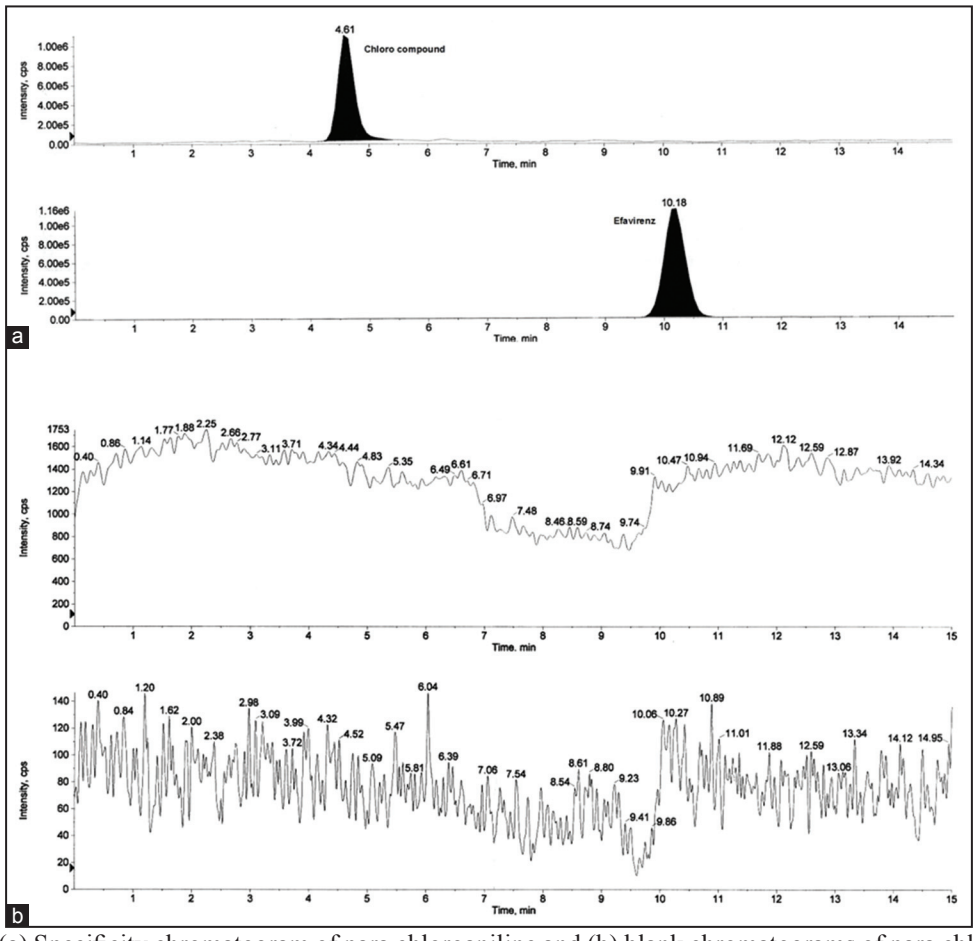

Figure 2: (a) Specificity chromatogram of para chloroaniline and (b) blank chromatograms of para chloroaniline.

that these changes do not impact on chromatographic performance.

3.2.3. Determination of limits of detection (LOD) and limits of quantitation (LOQ)

The LOD and LOQ, as a measure of method sensitivity, were calculated from signal to noise (S/N) ratios. To determine LOD and LOQ values for a para chloroaniline concentrations were reduced sequentially such that they yield S/N ratio as 3.2 and 10.1 respectively. The determined LOD and LOQ chromatograms were shown in Figure 2. Data generated from six injections of para chloroaniline (without API) containing 0.3 ppm of each para chloroaniline with respect to an API sample concentration 10 mg/mL. The LOQ of 0.3 ppm is typical for the para chloroaniline, with a LOD

Table 1: Accuracy/recovery of para chloroaniline at 0.3 ppm concentration.

Sample area	Standard area	Spiked area	Theoretical concentration	Measured concentration	% recovery
0	32,300	32,210	0.30	0.3002	100.08
0	32,300	32,170	0.30	0.3041	101.37
0	32,300	35,250	0.30	0.3026	100.86
				Average	100.77
				Standard deviation	0.651
				%RSD	0.65

RSD=Relative standard deviation

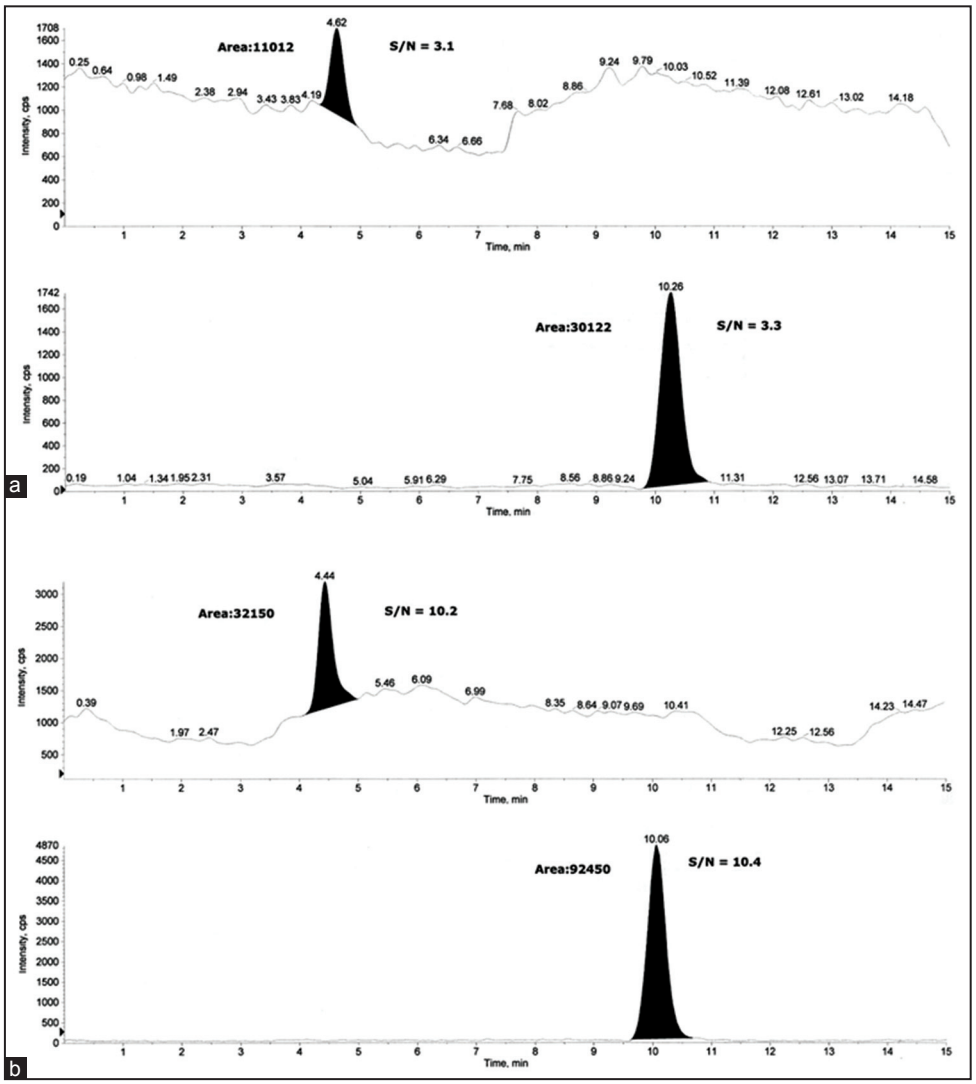

Figure 3: (a) Limits of detection chromatogram of para chloroaniline (b) limits of quantitation chromatogram of para chloroaniline.

Table 2: Solution stability data of para chloroaniline in diluent.

Sample area, injection time (h)	Standard area	Spiked area	Theoretical concentration	Measured concentration	% recovery
Level-I 0	32527	32010	0.3	0.2952	98.41
Level-II 0	32527	31990	0.3	0.2950	98.35
12 h					
Level-I 0	33450	33110	0.3	0.2970	98.98
Level-II 0	33450	32980	0.3	0.2958	98.59
24 h					
Level-I 0	31450	31020	0.3	0.2959	98.63
Level-II 0	31450	30980	0.3	0.2955	98.51
48 h					
Level-I 0	32180	32240	0.3	0.3006	100.19
Level-II 0	32180	31980	0.3	0.2981	99.38

Table 3: Linearity of para chloroaniline.

Concentration (ppm)	Peak area (au)
0.3	32140
3	315400
4	421500
5	540100
6	651200
7.5	810500
Correlation	0.9998
Slope	108715.9236
Intercept	−5671.8047

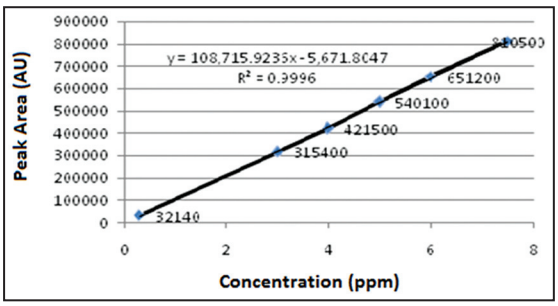

Figure 4: Linearity graph of para chloroaniline.

approximately 3 times less than LOQ. In addition, the relative efficiency of SIM versus multiple reaction monitoring (MRM) (selected reaction monitoring [SRM]) modes in sensitivity improvement was also evaluated. We found that, in SIM mode the LOD was 0.1 ppm, whereas with SRM/MRM was 0.3 ppm, the corresponding chromatograms are not shown.

3.2.4. Recovery studies

The recovery studies by the standard addition method were performed to evaluate accuracy and specificity, accordingly the accuracy of the method was determined in triplicate at LOQ level in bulk drug sample. The recoveries were calculated. Excellent recovery values of para chloroaniline (100-102%) was obtained. At such a low levels these recoveries and relative standard deviation (%RSD) is <1.0 was satisfactory. Sample and accuracy at LOQ chromatograms are shown in Figure 3 and the %RSD were calculated from the average of triplicate analysis, which were shown in Table 1. Further, the stability of para chloroanilinee was found as 48 h and the stability of this impurity at different time intervals is presented in Table 2.

3.2.5. Linearity and range

The linearity test for the method was performed according to the guidelines laid by ICH. This method was evaluated at six different concentrations of analytes with in the range of 0.3-7.5 ng/mL. These standard solutions were prepared by suitable dilution of stock solution with mobile phase. The linearity of the plot was evaluated using least squares linear regression analysis by SIM. The linearity of para chloroaniline was satisfactorily established with a six point calibration curve between LOQ and 150% of analyte concentrations (60, 80, 100, 120 and 150%). The calibration curve was produced by plotting the average of triplicate para chloroaniline injections against the concentrations expressed in percentage. The slope, intercept and correlation coefficient values were derived from linear least-square regression analysis and the data and graph were presented in Table 3 and Figure 4. It reveals that good correlation existed between the peak areas concentration of para chloroaniline. Repeatability was checked by calculating the %RSD of six determinations by injecting six freshly prepared solutions containing 0.3 ppm of para chloroaniline on the same day. The low %RSD values confirm the good precision of the developed method.

4. CONCLUSIONS

The present development study is based on validation of a highly sensitive, specific, reproducible and high-throughput LC-MS/MS method to quantification of para chloroaniline in APIs. It has been established that it is highly sensitive with a LOD of 0.1 ppm. Trace level ammonium acetate is added to the mobile phase to enhance ionization and detection. Selected sample solvents were assessed for the effect on standard stability with and without presence of API. As a systematic approach, it is very important to utilize the comprehensive chromatographic knowledge gained throughout the lifecycle of the development of a drug candidate based on continuous understanding of the API manufacturing process. The method which is able to quantify them at ppm level is developed and validated. We can conclude that the developed method could be very useful for monitoring of para chloroaniline in EFV in its pure and tablet form.

5. ACKNOWLEDGMENTS

The authors extend their grateful acknowledgments to Wellquest Clinical Research Laboratories, Hyderabad for providing necessary facilities for carrying out this study.

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****Bibliographical Sketch***

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