QUANTIFICATION OF GENOTOXIC IMPURITIES IN FEXOFENADINE HYDROCHLORIDE BY SIMPLE AND SENSITIVE LIQUID CHROMATOGRAPHY TECHNIQUE

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ABSTRACT

Fexofenadine hydrochloride is synthesized using two key raw materials Methyl-2-(4-(4-chlorobutanoyl) phenyl)-2-methylpropanoate (KRM-I) and Diphenyl-4-piperidinemethanol (KRM-II). On basis of structure-activity relationship (SAR) KRM-I is potentially genotoxic impurity. One more mutagenic impurity that is bromobenzene is used for the synthesis of KRM-II. A sensitive High performance liquid chromatography (HPLC) method was developed and validated for the determination of both impurities in the active pharmaceutical ingredient fexofenadine hydrochloride. HPLC column Cosmosil MS-II C18, 250 mm X 4.6 mm, 5 µm particle size with ultraviolet detector (UV) was used. The proposed method is specific, linear, accurate, rugged and precise. The calibration curve of bromobenzene and KRM-I showed good linearity over the concentration range of 1.0 µg/g to 12.0 µg/g with respect to test solution concentration and the regression coefficient was 0.999 for both analytes. Method had very low limit of detection (LOD) and limit of quantification (LOQ) of both analytes which proves that the method is sensitive and suitable for quantification of both genotoxic impurities at trace level.

KEYWORDS: Fexofenadine; genotoxic impurity; method development and validation.

INTRODUCTION

Fexofenadine hydrochloride is histamine H1-receptorantagonist, chemically known as ± (4-[1-hydroxy-4-[(4-hydroxydiphenylmethyl)-1-piperidinyl]-butyl]-α, α-dimethyl, benzenacetic acid hydrochloride. Fexofenadine hydrochloride, having a chemical structure...
as shown in figure 1, has a molecular weight of 538.13 and its molecular formula is C$_{32}$H$_{39}$NO$_4$.HCl. The trade name, ALLEGRA (fexofenadine hydrochloride) is indicated for the relief of symptoms associated with seasonal and perennial allergic rhinitis. Symptoms treated effectively include sneezing, rhinorrhea, lacrimation, itchy, red eyes and itchy nose/palate/throat. ALLEGRA improves health-related quality of life and work/activity productivity.$^{[1, 2]}$ Fexofenadine hydrochloride drug substance is official in United State Pharmacopoeia (USP)$^{[3]}$ as well as European Pharmacopoeia (EP)$^{[4]}$.

Methyl-2-((4-(4-chlorobutanoyl) phenyl)-2-methylpropanoate (KRM-I) is an advanced intermediate used for synthesis of fexofenadine hydrochloride. The methyl ester group of KRM-I is structurally alert highly reactive potential genotoxic impurity (GTI). One more GTI that is bromobenzene which is used in the manufacturing of another advanced intermediate Diphenyl-4-piperidinemethanol (KRM-II). Bromobenzene is reasonably anticipated to be a human hepatotoxicant. It binds to DNA, RNA, and proteins of rat and mouse liver in vivo. Binding to a significant extent is also detected in mouse kidney. Bromobenzene is photoactivated by ultraviolet light at absorbance 254 nm to make it capable of interacting with DNA in vitro.$^{[5]}$ 8-hydroxy-2'-deoxyguanosine and double strand breakage was noticed in rat liver slices after its interaction with bromobenzene, ultimately leading to genotoxicity.

An in vitro model using a suspension of rabbit renal proximal tubules showed nephrotoxicity of bromobenzene due to its metabolite, 2-bromohydroquinone.$^{[6, 7]}$ Bromobenzene induced micronuclei in bone marrow of mice and was bound to DNA and RNA following intraperitoneal injection. Bromobenzene was second only to 1,2 dibromoethane in its relative in vivo reactivity with rat liver DNA, exhibiting higher reactivity than 1,2-dichloroethane, chlorobenzene, epichlorohydrin, and benzene.$^{[8]}$

Because of the known carcinogenicity and genotoxicity, the presence of residual bromobenzene and Methyl-2-((4-(4-chlorobutanoyl)phenyl)-2-methylpropanoate in fexofenadine hydrochloride drug substance must be checked or controlled as per European Medicines Agency (EMA), International Council for Harmonization$^{[9, 10]}$ and Food and Drug Administration (FDA) guidelines.$^{[11]}$ EMA and FDA guidelines proposed the use of the “threshold of toxicological concern” (TTC) concept for the limit of genotoxic / carcinogenic impurities. The concentration limit, in ppm, of genotoxic impurity in drug substance, is the ratio of TTC in μg per day to the expected dose of drug substance in gram per day. Considering the recommended daily maximum dose of 180mg fexofenadine hydrochloride
Bromobenzene and KRM-I must be limited to less than 8.3 μg/g in drug substance. To achieve quantification of such a trace level it is necessary to develop sensitive, accurate and robust analytical method. Fexofenadine hydrochloride is official drug substance in United State Pharmacopoeia (USP) and European Pharmacopoeia (EP). Both compounds bromobenzene and KRM-I are not controlled in the official monograph. During literature survey several analytical methods were found reported of estimation of bromobenzene but quantification of KRM-I at trace level is not reported. In most of the literature bromobenzene is tested by GC. Aromatic halogenated volatiles by gas chromatography using photoionization and electrolytic conductivity detectors. Determination of bromobenzene metabolites by HPLC is also reported. In present research, analytical method is developed to quantify the bromobenzene and KRM-I simultaneously at trace level using HPLC-UV detector. Bromobenzene which is used in the manufacturing of KRM-II is controlled in the KRM-II itself as well as it is checked in the active pharmaceutical ingredient (API) fexofenadine hydrochloride while the other GTI KRM-I is quantified and controlled in the API. The proposed analytical method is validated as per International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines ICH Q2-R1.

MATERIALS AND METHODS
Instrumentation: Shimadzu high performance liquid chromatography (HPLC) system LC-2010 CHT with UV detector with LC solutions software or its equivalent.

Chemical and reagents: HPLC column used of Nacalai Tesque Inc. Cosmosil MS II C_{18}, length 250 mm, internal diameter 4.6mm, particle size 5μm. Bromobenzene standard of purity 99.9% was procured from Spectrochem, Methyl-2-(4-(4-chlorobutanoyl)phenyl)-2-methylpropanoate (KRM-I) standard of purity 94% synthesized and characterized by R&D, Wanbury Ltd. Acetonitrile HPLC grade of Merck, sodium perchlorate monohydrate AR grade from Loba Chemie, sodium dihydrogen phosphate AR grade form Merck, triethylamine HPLC grade of Merck, orthophosphoric acid HPLC grade of Merck and purified HPLC grade water was used in the experimentation.

Chromatographic parameters
HPLC method was performed using Cosmosil MS II C_{18} HPLC column. Separation and peak symmetry was achieved with the mixture of mobile phase-A: buffer and mobile phase-B: Acetonitrile in gradient elution with timed programme T_{min}/A:B: T_0/40:60; T_{15}/40:60;
T_{17}/30:70; T_{22}/40:60 and T_{30}/40:60 with flow rate 1.0 mL/minute. The column temperature was maintained at 30°C and auto sampler temperature 10°C. Ultraviolet detection was performed at wavelength 220 nm and 246 nm. Injection volume was 50 μL, injector rinsing solution was methanol and run time 30.0 minutes.

**Preparation of buffer solution**
Buffer was prepared by dissolving 6.64g of sodium dihydrogen phosphate and 0.84g of sodium perchlorate monohydrate in 1000mL of water. Triethylamine 3.0 mL and orthophosphoric acid 2.0 mL was added and mixed. Filtered the solution and degassing was performed with the aid of sonicator to remove air bubbles.

**Solution preparation**

**Diluent preparation:** A mixture of equal quantity of buffer solution and acetonitrile was prepared.

**Standard stock solution A:** Accurately weighed and transferred 40 mg of Bromobenzene and KRM-I standard in to 100 mL volumetric flask. Sonicated to dissolve and diluted up to the mark with diluent.

**Standard stock solution B:** Transferred 5.0 mL of standard stock solution A in to a 50 mL volumetric flask and diluted up to the mark with diluent and mixed.

**Standard solution:** Transferred 1.0 mL of standard stock solution B in to a 100 mL volumetric flask and diluted up to the mark with diluent and mixed. (Bromobenzene and KRM-I are 8.0 ppm each with respect to test solution concentration).

**Test solution:** Accurately weighed 500 mg of fexofenadine test sample in to a 10 mL volumetric flask and diluted up to the mark with diluent. Solution was mixed and sonicated. Filtered the solution through 0.45μ syringe filter and injected the filtrate.

**Note:** All standard and test solutions were prepared freshly before analysis.

**Procedure**
Performed blank followed by standard solution six replicate and two test preparations. Run the blank and test solution chromatograph for 30.0 minutes and standard solution for 15.0 minutes. The peak of bromobenzene was integrated at wavelength 220 nm at retention time 10.9 minutes. The peak of KRM-I was integrated at wavelength 246 nm at retention time 11.9
minutes. Both desired peaks were integrated in standard and test solution at specified wavelength and calculated by external standard addition method.

**Acceptance criteria for system suitability**

% RSD of peak area of bromobenzene and KRM-I in six replicate standard injections was found less than 10.0

**RESULTS AND DISCUSSION**

Methyl-2-(4-(4-chlorobutanoyl)phenyl)-2-methylpropanoate (KRM-I) CAS No. 154477-54-0, molecular formula C\textsubscript{15}H\textsubscript{19}ClO\textsubscript{3}, molecular weight 282.76 and boiling point is 396.5\textdegree C. Bromobenzene CAS No. 108-86-1, molecular formula C\textsubscript{6}H\textsubscript{5}Br, molecular weight 157.01 and boiling point is 156\textdegree C. Refer figure 1 for the structure of bromobenzene and KRM-I. During literature survey it was found that most of the methods for determination of bromobenzene are by GC with FID or mass detector. Due to high boiling point gas chromatography (GC) is not a suitable technique for the testing of KRM-I. Both compounds are soluble in organic polar solvents. On basis of chemical properties of the compounds and to achieve simultaneous detection of both impurities reverse phase high performance liquid chromatography (RP-HPLC) was chosen as a starting point for method development. In RP-HPLC with UV detector on C8 and C18 column using mobile phase a mixture of sodium dihydrogen phosphate buffer of pH 2.0 and acetonitrile both analytes were easily separating with sharp and symmetrical peaks. Response of both peaks at low level was also sufficient with relative standard deviation (RSD) of precision was less than 2.0. Due to high concentration of sample matrix, interference was observed at the retention time (RT) of both analytes. For separation of matrix interference few trials were studied like by increasing concentration of buffer and preparing buffer by mixing two salts sodium dihydrogen phosphate and sodium perchlorate monohydrate. In mixture of two salts matrix peaks were found separated from both analytes but peak shape of KRM-I was became asymmetrical with tailing more than 4.0. In order to improve peak shape trials were taken with different pH of the mobile phase by adding triethylamine. The desired separation of both impurities without any blank or matrix interference and symmetrical peak shape was achieved with C18 column and the proposed mobile phase combination of buffer and acetonitrile.
ANALYTICAL METHOD VALIDATION

Selectivity

Selectivity is the ability of the method to determine accurately and specifically the analyte of interest in the presence of other components in a sample matrix that may be expected to be present in the sample matrix under the stated conditions. Selectivity of the method was evidenced by comparing chromatograms of blank, bromobenzene, KRM-I, diphenyl-4-piperidinomethanol (KRM-II), fexofenadine intermediate-I, fexofenadine intermediate-II, impurity-A, impurity-D and impurity-C separate injections. There is no interfering peak at the retention time of bromobenzene and KRM-I was observed. Bromobenzene and KRM-I was well resolved from all other specified impurity peaks which are used in the manufacturing process of fexofenadine. The interference with in the peak was checked using photodiode array detector (PDA). Both analytes were spiked at 100% level in the fexofenadine test solution and injected in the chromatograph. The peak purity index of bromobenzene and KRM-I was found 0.999322 and 0.999761 respectively in presence of all other specified and unspecified impurities of the test sample. It proves that no interference at the retention time of both analytes under given chromatographic parameters. The proposed method is selective and specific and therefore suitable for quantification of both analytes. Refer figure-2, 3 and 4 of blank, bromobenzene and KRM-I standard solution and spike solution chromatogram with peak purity.

Solution stability

Drug stability in active pharmaceutical ingredient is a function of storage conditions and chemical properties of the drug and its impurities. The solution stability till eight hours of bromobenzene and KRM-I had been checked by injecting standard solution. Bromobenzene and KRM-I standard solution was prepared fresh before injection and immediately injected and same solution was injected after every one hour interval. The sample cooler temperature was kept 10°C. The peak area of bromobenzene and KRM-I of freshly prepared standard solution was observed 46604 and 63239 and a upto six hours it was 45681 and 62611 respectively. No significant change in area was observed till six hours. After seven hours KRM-I area was found stable but bromobenzene area was found reduced to 44934. Same experiment was earlier carried out with sample cooler temperature 25°C and found that bromobenzene area was reduced about 5% after three hours. So that further testing was performed at sample cooler temperature 10°C where both analytes were found quite stable for six hours. On basis of solution stability data it is recommended that the sample cooler
temperature should be 10°C and standard and test solutions need to be prepared immediately before testing.

**LOD and LOQ**
The limit of detection (LOD) is the lowest concentration of the analyte in a sample that can be detected but not necessary quantified. The obtained LOD values of bromobenzene and KRM-I is discussed.

LOD = \(3.3 \times \frac{\sigma}{S}\)

The limit of quantitation (LOQ) is the lowest concentration or amount of the analyte that can be determined quantitatively within an acceptable level of repeatability precision and trueness.

LOQ = \(10.0 \times \frac{\sigma}{S}\)

Where, \(\sigma\) = the standard deviation of the response and \(S\) = slope of the calibration curve

LOD and LOQ concentration is reported in parts per million (ppm) with respect to Fexofenadine hydrochloride test concentration. LOD was found 0.40 ppm of both analytes bromobenzene and KRM-I with respect to test solution. LOQ was found 1.0 ppm of both analytes bromobenzene and KRM-I with respect to test solution. Precision of LOQ is checked by injecting six replicate injections of mixture of both analytes at their LOQ level. Relative standard deviation (RSD) of peak area of bromobenzene and KRM-I at LOQ level is observed 1.8% and 1.6% respectively which proves consistency and reproducibility of the method at trace level.

**Linearity**
Under the experimental conditions, the peak area vs. concentration plot for the proposed method was found to be linear over the range of LOQ level, 50%, 80%, 100%, 120% and 150% of the specified limit, with a regression coefficient of bromobenzene and KRM-I is 0.999 of both analytes. The linearity of bromobenzene and KRM-I is plotted. The horizontal x-axis or abscissa is typically chosen to represent the independent variable which is concentration of the analyte. The vertical y-axis or ordinate, is chosen to represent the dependent variable i.e. peak area, which changes as the independent variable is manipulated. The regression coefficient \((r^2)\) is more than 0.99 is generally considered as evidence of acceptable fit of the data to the regression line.
Accuracy
Accuracy can be defined as the closeness of agreement between a test result and the accepted reference value. Accuracy of the method was determined by recovery study. Analytical method may be considered validated in terms of accuracy if the mean value is within ± 20% of the actual value. During recovery study Fexofenadine hydrochloride batch was analyzed and then bromobenzene and KRM-I is spiked in the Fexofenadine hydrochloride at LOQ level, 50%, 100% and 150% with respect to the limit of bromobenzene and KRM-I. The recovery of bromobenzene is found 100.0%, 104.4%, 101.8% and 98.4% respectively and KRM-I is 107.2%, 103.5%, 107.9% and 105.8% respectively. It proves that the method is capable to quantify both the analyte accurately and results will be reliable even at trace level.

Ruggedness study
The (intra-laboratory tested) behavior of an analytical process when small changes in environment and/or operating condition are made. The ruggedness of the method was evaluated by estimating % RSD of bromobenzene and KRM-I standard solution tested by two different analysts on different days. % RSD of area of bromobenzene and KRM-I peak in standard solutions of both analysts should not be more than 10%. Six replicates of diluted standard solution were injected by each analyst. Relative standard deviation of area of bromobenzene was found 1.0% and 1.8% and KRM-I was found 0.6% and 1.0% on two different days. This shows that there is no variation in day to day analysis. Method validation summary is given in table-1. Ruggedness was checked using spiking solutions by two different analysts with six different preparations. Bromobenzene and KRM-I were spiked in the Fexofenadine hydrochloride test solution at 100% level. Finally RSD of total twelve preparations were calculated and found 3.7% of bromobenzene and 0.9% of KRM-I.

COMPLIANCE WITH ETHICAL STANDARDS
Conflicts of Interest: Author Ajit Anerala, Vishal Solse, Amol More, Nitin Pradhan declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Figure 1: - Bromobenzene and Methyl-2-(4-(4-chlorobutanyloxy)phenyl)-2-methylpropanoate (KRM-I).

Figure 2: - Blank chromatogram.
Figure 3: Bromobenzene and Methyl-2-(4-(4-chlorobutanoyl)phenyl)-2-methylpropanoate (KRM-I) standard solution chromatogram with peak purity.

Figure 4: Chromatogram of fexofenadine hydrochloride test solution is spiked with bromobenzene and methyl-2-(4-(4-chlorobutanoyl)phenyl)-2-methylpropanoate (KRM-I) standards at 100% level with peak purity.
Table 1:- Analytical method validation summary.

<table>
<thead>
<tr>
<th>S. NO.</th>
<th>PARAMETER</th>
<th>ACCEPTANCE CRITERIA</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Selectivity</td>
<td>Bromobenzene and KRM-I peaks should be well separated from all known and unknown peaks of fexofenadine hydrochloride.</td>
<td>Complies. Method is selective. Peak purity passing</td>
</tr>
<tr>
<td>2</td>
<td>Solution stability</td>
<td>Report Result</td>
<td>Solutions are stable up to 6 hour</td>
</tr>
<tr>
<td>3</td>
<td>Limit of detection (LOD)</td>
<td>Report Result</td>
<td>Bromobenzene = 0.40 ppm KRM-I = 0.40 ppm wrt test concentration</td>
</tr>
<tr>
<td>4</td>
<td>Limit of quantification (LOQ)</td>
<td>Report Result</td>
<td>Bromobenzene = 1.0 ppm KRM-I = 1.0 ppm wrt test concentration</td>
</tr>
<tr>
<td>5</td>
<td>LOQ precision (n=6)</td>
<td>%RSD for six replicates of LOQ level standard solutions is NMT:10.0%</td>
<td>RSD of bromobenzene = 1.8% KRM-I = 1.6%</td>
</tr>
<tr>
<td>6</td>
<td>Linearity (n=3)</td>
<td>Correlation: NLT 0.99</td>
<td>Bromobenzene = 0.999 KRM-I = 0.999</td>
</tr>
<tr>
<td>7</td>
<td>Accuracy (n=3)</td>
<td>Recovery should be between 80% to 120%</td>
<td>complies</td>
</tr>
<tr>
<td>8</td>
<td>Method precision (n=6)</td>
<td>%RSD for results of six spiked preparations is NMT:10%</td>
<td>RSD of bromobenzene = 1.0% KRM-I = 0.6%</td>
</tr>
<tr>
<td>9</td>
<td>Ruggedness Intermediate precision (n=12)</td>
<td>%RSD for results of twelve spiked preparations (Method precision and Intermediate precision) is NMT:10%</td>
<td>RSD of bromobenzene = 3.7% KRM-I = 0.9%</td>
</tr>
</tbody>
</table>

Ppm=parts per million, wrt=with respect to, RSD=relative standard deviation, n=number of replicate, NMT=Not more than and NLT=Not less than.

CONCLUSION

The proposed RP-HPLC analytical method with UV detector satisfies all validation parameters like system suitability, precision, specificity, accuracy, linearity of detector response and ruggedness. It indicates that the method is stable and suitable for the quantification of bromobenzene and KRM-I in fexofenadine hydrochloride. Hence, the
Validated method can be used for routine analysis of quantification of bromobenzene and KRM-I in quality control laboratories in the pharmaceutical industry.

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