

THE ROLE OF RELATIVE RESPONSE FACTOR IN RELATED SUBSTANCES METHOD DEVELOPMENT BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

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ABSTRACT

A study was conducted on Relative Response Factor by changing the High Performance Liquid Chromatography (HPLC) chromatographic method conditions like different HPLC columns, Flow rate, pH, Temperature, Buffer concentration, Detector wavelength, different Detectors (Ultraviolet & Photo Diode Array Detectors) and different Solvent grades and observed the variations in established RRF. The authors have studied the impact on RRF by changing the Robustness parameters and HPLC columns and all the results were compared. The comparison study has shown that any slight variations in method conditions, the impact is observed on established RRF values.

Key Words: RRF, Relative Response Factor, Correction factor, Response factor, Estimation of RRF.

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INTRODUCTION

Relative Response Factor (RRF) is an analytical parameter used in chromatographic procedures to control impurities/degradants in drug substance and drug product. RRF is used to correct the difference in detector response of impurities with analyte peak. RRF is established by slope method with linear range of solutions. Different Pharmacopoeias refer the term RRF differently.

As per United States Pharmacopoeia (USP) The Relative response factor, is the ratio of the responses of equal amounts of the Impurities and the drug substance¹. USP refers RRF as Correction factor or Response factor or Relative response factor.

As per European Pharmacopoeia (Ph.Eur) The Relative detector response factor, commonly referred as Response Factor, expresses the sensitivity of a detector for a given substance relative to a standard substance. The *correction factor* is reciprocal of the response factor². Ph.Eur refers RRF as Correction factor or Response factor.

As per British Pharmacopoeia (BP) The Response Factor is a relative term, being the response of equal weights of one substance relative to that of another in the conditions described in the test³. BP refers RRF as Response factor.

Establishment of RRF is required to avoid the stability issues with standards, to reduce the cost on preparation of Impurity Standards, to reduce Maintenance of Impurity Standards, due to the lack of donation of Impurity Standards, difficulty in synthesis and isolation of Impurity Standards, for convenience and time saving. Relative Response factor (RRF) is used in different stages: Phase 1 to Phase 4 studies, in drug purity tests, Mass balance tests, in limit tests, In stability indicating methods etc.

EXPERIMENTAL

An accurate and precised RS method was developed and the RRF values were established for Imatinib Tablets⁵. Imatinib Mesylate and Impurity – F (Desmethyl impurity) and Impurity – G (Isomeric impurity) as experimental drugs, and chromatographic conditions are followed as per Analytical Methodology. A study was carried to observe the variations in Relative Response Factor (RRF) values by changing

analytical parameters like change in HPLC columns with same stationary phase with different Column Manufacturers, different detector wavelengths, detectors, column temperature, flow rate, pH, buffer concentration and solvent grade.

Estimation of Relative Response factor (RRF)

RRF is estimated by Slope Method.

Slope Method:

- Before establishing RRF, inject Blank solution, Resolution solution and standard solution as per the test method and establish the System Suitability parameters.
- Prepare not less than seven preparations of Impurities individually and Drug Substance individually in the concentration range of 0.1 % to 1.0 % (eg... 0.1%, 0.2%, 0.3%, 0.5%, 0.7%, 0.8% and 1.0%) with respect to test concentration (or) Prepare not less than seven preparations in the range covering three points below the specification level (Above LOQ level) and three points above the specification level with respect to test concentration. Inject the above solutions individually into the HPLC system under test conditions as per the test method.
- Prepare not less than seven preparations of Drug substance individually and all Impurities mix solution (If mutually interfering peaks of Impurities are not there, then impurity mix solution can be injected) in the concentration range of 0.1 % to 1.0 % (eg... 0.1%, 0.2%, 0.3%, 0.5%, 0.7%, 0.8% and 1.0%) with respect to test concentration (or) Prepare not less than seven preparations in the range covering three points below the specification level (Above LOQ level) and three points above the specification level with respect to test concentration. Inject the above solutions (Standard and Impurity mix solution) into the HPLC system under test conditions as per the test method.
- Prepare not less than seven preparations containing mixture of Drug substance and Impurities (If mutually interfering peaks are not there, of standard and impurities then inject solution containing mixture of Drug substance and Impurities) in the concentration range of 0.1 % to 1.0 % (eg... 0.1%, 0.2%, 0.3%, 0.5%, 0.7%, 0.8% and 1.0%) with respect to test concentration (or) Prepare not less than seven preparations in the range covering three points below the specification level (Above LOQ level) and three points above the specification level with respect to test concentration. Inject the above solutions into the HPLC system under test conditions as per the test method.
- Plot a graph of concentration versus response for standard. Do not include 'Zero' in linearity plot. (Concentration on 'X' axis and response or area on 'Y' axis)
- Plot a graph of concentration versus response for impurity solutions. (Concentrations of impurity solutions on 'X' axis and response or area on 'Y' axis)
- A graph shall be constructed using all data points following the linear equation (i.e., $y = mx + c$) using least squares regression.
- Determine the slope of individual linearity plots.
- The Correlation coefficient is not less than 0.995 for both Standards and Impurities.

Useful Formulas:

Concentration of API/Impurity* = [Weight (mg)/ Dilution (mL)] X [Molecular weight of Base/ Molecular weight of salt] X Purity (On as is basis) (1)

* If molecule exists as salt base then calculate concentration as above.

Concentration of API/Impurity* = [Weight (mg)/ Dilution (mL)] X Purity (On as is basis) (2)

* If molecule does not exist as salt base then calculate concentration as above.

Relative response factor of impurity = [Slope of impurity solution in curve/ Slope of standard solution in curve] (3)

Note: If the impurity slope value is in numerator, Relative Response factor (RRF) value appears in the denominator (OR) The Relative Response Factor of the Impurity with respect to drug will appear as divide factor in the formula of impurity determination.

Relative response factor of impurity = [Slope of Standard solution in curve/ Slope of Impurity solution in curve] (4)

Note: If the impurity slope value is in denominator, Relative Response factor (RRF) value appears in the numerator (OR) The Relative Response Factor of the drug substance with respect to impurity will appear as multiplication factor in the formula of impurity determination.

Recovery Studies

- Calculate the potency of impurity as labeled or as mentioned in specification of drug substance and drug product. For free base as base, for salt as salt and for complex as complex.
- If the API exists as salt or as metal complex, calculate the potency by including salt or metal complex.
- If the impurity and Drug substance are in the same form (ex: both are in the form of bases or both are in same salt forms, etc...) calculate as per the regular practice.
- If the impurity is in salt base form and Drug substance in the form of base weight correction to be taken for impurity with respect to test concentration.
- If the impurity is in the form of free base and Drug substance in the form of salt base weight correction to be taken for drug substance with respect to test concentration.
- If the impurity and Drug substance both are in different forms (ex: both are in different base forms or different salt forms etc...) weight correction to be taken for drug substance and impurity with respect to test concentration.
- Recovery study is mandatory to confirm the established Relative Response Factor (RRF) are correct.
- Prepare a unspiked and spiked test preparations, inject in to the chromatographic system and record the chromatograms.
- Calculate % of each known impurity found in both unspiked and spiked test preparations by using Relative Response Factor (RRF) values and calculate % recovery.
- % of Recovery shall be between 95 – 105 %, if not investigate and repeat the experiment again after omitting the possible errors.
- If the recovery is within the range of 95% to 105 % then Relative Response Factor (RRF) shall be incorporated in calculation of % of known individual impurities.
- Compare the Relative Response Factor based on structural similarity of the particular compound relative to active pharmaceutical ingredient. If the RRF is significantly different which can not be explained based on structural difference then it needs to be investigated.
- When impurity is procured from outside laboratory (except Pharmacopoeial standards), testing like LC-MS, Chromatographic purity and TGA needs to be checked.
- If the impurity quantity is a constraint, perform the RRF establishment with specification level of Drug product or substance and below or above specification level.
- RRF values should be conformed by Second time.

Parameters that influence RRF⁴

- (i) Change in Column (same stationary phase from different Column Manufacturers)
- (ii) Change in Column particle Size
- (iii) Changes in Detector wavelengths
- (iv) Changes in Detector
- (v) Change in Solvent grade
- (vi) Change in Buffer concentration
- (vii) Change in Column temperature

- (viii) Change in Flow rate
- (ix) pH variation

Imatinib Mesylate is an Anti-neoplastic drug, with Molecular formula $C_{29}H_{31}N_7O.CH_3SO_3H$ and Molecular weight 589.7 g/mol. The finished product was formulated as Imatinib tablets. The finished product contains two known impurities, Imatinib Impurity – F (Desmethyl impurity) and Imatinib Impurity – G (Isomeric impurity). The structures and spectra of Imatinib Mesylate, Imatinib Impurity – G and Impurity – F are shown in the Fig. 1a to 1b, Fig. 2a to 2b and Fig. 3a to 3b respectively.

Imatinib Mesylate: 4-[(4-methylpiperazin-1-yl)methyl]-N-(4-methyl-3-[[4-(pyridin-3-yl)pyrimidin-2-yl]amino]phenyl) benzamide

Des methyl impurity (or) Imatinib Impurity – F: 4-[(piperizinyl)methyl]-N-[4-methyl-3-[(4-pyridinyl)-2-pyrimidinylamino]Phenyl]-benzamide

Isomeric impurity (or) Imatinib Impurity – G: 4-[4-(methyl-1-piperizinyl)methyl]-N-[5-methyl-4-[(4-pyridinyl)-2-pyrimidinylamino]Phenyl]-benzamide

Analytical methodology

Instrumentation

The analysis was carried out on a Waters Liquid Chromatography system equipped with 2695 pump and 2487 Ultraviolet detector connected with Empower2 software⁵. Reverse phase HPLC Column X-Bridge C18, 250 x 4.6 mm, I.D; particle size 5 μ m (Make: Waters (US), Microbalance (Make: Mettler Toledo, Model: XP6), Analytical Balance (Make: Mettler Toledo, Model: AB204S), pH Meter (Make: Lab India, Model: Pico+), Millipore vacuum pump (Make: Millipore, Lot number: X10422050), Mobile phase Filters-0.45 μ m (Make: Millipore, Model: HV 0.45 μ m), Hydrophilic PVDF(Millipore Millex-HV 0.45 μ m), Nylon (Millipore Millex-HN 0.45 μ m), Milli-Q Water System (Make: Millipore, Lot number: F0DA99893), Laboratory Centrifuge (Make: Remi, Model: R4C) were used for analysis.

Chemicals and solvents

HPLC Grade water (Millipore), Acetonitrile (Gradient Grade), Triethyl amine (HPLC Grade), Methanol (Gradient Grade) were obtained from Merck (India) Ltd., Mumbai and Sodium dihydrogen phosphate dihydrate of HPLC Grade (Qualigens) obtained from Fischer Scientific. Imatinib Mesylate, Impurity – G and Impurity – F working standards were supplied by Natco Pharma Limited, Hyderabad, India.

Buffer preparation

Accurately weigh and transfer about 3.0 g of Sodium dihydrogen phosphate dihydrate in 1000 mL of purified water and adjust the solution to pH 8.0 ± 0.05 with Triethylamine.

Solvent mixture: Prepare a mixture of Methanol and Acetonitrile in the ratio of 600:400 v/v and mix.

Mobile phase-A: Prepare a filtered and degassed mixture of Buffer and Solvent mixture in the ratio of 550:450 v/v and mix.

Mobile phase-B: Solvent mixture. Filter and degas.

Diluent: Mobile phase-A.

Impurity-G and Impurity-F Stock preparation

Accurately weigh and transfer each of 5.0 mg of Impurity G working standard and Impurity F working standard into a 25mL volumetric flask. Add about 15 mL of diluent and sonicate to dissolve. Cool the solution to room temperature and dilute to volume with diluent and mix well.

Standard preparation

Accurately weigh and transfer about 24.0 mg of Imatinib mesylate working standard into a 100mL volumetric flask. Add about 60 mL of diluent and sonicate to dissolve. Cool the solution to room temperature and dilute to volume with diluent and mix well.

Transfer 1.0 mL of the above solution into a 100 mL volumetric flask, and dilute to volume with diluent and mix well.

Sample preparation

Accurately weigh and transfer a quantity of powdered tablets equivalent to 100 mg of Imatinib into a 100 mL volumetric flask. Add about 60 mL of diluent, sonicate for 15 minutes with occasional shaking. Cool the solution to room temperature and dilute to volume with diluent and mix. Centrifuge the solution at 3500RPM for 10minutes. Use clear supernant liquid for analysis.

Chromatographic conditions

X-Bridge C18, 250 mm x 4.6 mm, 5 µm column was used for analysis at column temperature 30°C. The mobile phase was pumped through the column at a flow rate of 1.0mL/minute. The sample injection volume was 20 µL. The UV detector was set to a wavelength of 230nm for the detection and Chromatographic runtime 60minutes. Refer Gradient programming table (Table-1)

Procedure

Imatinib and its impurities shows significant absorbance at wavelength 230nm. The spiked chromatogram of Imatinib and its known impurities is shown in Fig. 10. The RRT and RRF values were shown in Table-2.

ESTABLISHMENT OF RELATIVE RESPONSE FACTOR (ANALYST-1)

Stock solution preparation (Mixture of Standard and Impurities)

Accurately weighed and transferred 5.002 mg of Imatinib Mesylate, 4.978 mg Imatinib impurity – F and 5.029 mg Imatinib impurity – G in to a 100 mL volumetric flask to it added 60 mL of diluent sonicated to dissolve and diluted volume with diluent. Purity and weights for Standard and Impurities are given in Table-3. For the dilutions from stock preparation to the respective concentrations (0.02% to 1.00% with respect to test concentration) for Imatinib Mesylate and its Impurities refer Table-4 to Table-6.

Linearity of detector response

A series of solutions containing mixture of Imatinib Mesylate and its Impurities were prepared in the concentration range of about 0.166 µg/mL to 8.290 µg/mL for Imatinib Mesylate, 0.189 µg/mL to 9.438 µg/mL for Imatinib Impurity – F and 0.186 µg/mL to 9.284 µg/mL for Imatinib Impurity – G. Linearity of detector response was established by plotting a graph concentration *versus* area and determining the correlation coefficient. The detector response was found to be linear with a correlation coefficient of 0.999, 1.000 and 0.999 for Imatinib, Impurity – F and Impurity – G respectively. Linearity graph is shown in Fig. 4 to Fig. 6. Linearity results of the method are presented in Table-4 to Table-6.

Preparation of Imatinib Mesylate stock solution

Stock solution preparation = $[5.002/100][99.0/100][493.6/589.7]1000 = 41.45\text{ppm}$

Diluted solutions = Stock solution (ppm) X [Dilution volume (mL)/ Diluted volume (mL)]

Note: Apply factor for calculation 493.6/589.7

Where, 493.6 = Molecular Weight of Imatinib; 589.7 = Molecular Weight of Imatinib Mesylate

Preparation of Imatinib Impurity - F stock solution

Stock solution preparation = $[4.978/100][94.8/100]1000 = 47.19\text{ppm}$

Diluted solutions = Stock solution (ppm) X [Dilution volume (mL)/ Diluted volume (mL)]

Preparation of Imatinib Impurity - G stock solution:

Stock solution preparation = $[(5.029/100)(92.3/100)]1000 = 46.42\text{ppm}$

Diluted solutions = Stock solution (ppm) X [Dilution volume (mL)/ Diluted volume (mL)]

RRF Calculation

Formula = [Slope of Impurity (known Impurity)/ Slope of Active]

RRF value for Imatinib Impurity-F = [(Slope of Impurity-F)/(Slope of Imatinib)]

$$= 61754.05/74167.65 = 0.83$$

RRF value for Imatinib Impurity-G = [(Slope of Impurity-G)/(Slope of Imatinib)]

$$= 68661.27/74167.65 = 0.93$$

* For slope values of the Imatinib and its Impurities refer Table-4 to Table-6. Recovery studies were established and the Results are reported in Table-7 (Analyst-1).

CONFORMATION OF RELATIVE RESPONSE FACTOR (ANALYST-2)

Stock solution preparation (Mixture of Standard and Impurities)

Accurately weighed and transferred 5.140 mg of Imatinib Mesylate, 5.110 mg Imatinib impurity – F and 5.050 mg Imatinib impurity – G in to a 100 mL volumetric flask to it added 60 mL of diluent sonicated to dissolve and diluted volume with diluent. Purity and weights for Standard and Impurities are given in Table-8. For the dilutions from stock preparation to the respective concentrations (0.02% to 1.00% with respect to test concentration) for Imatinib Mesylate and its Impurities refer Table-9 to Table-11.

Linearity of detector response

A series of solutions containing mixture of Imatinib Mesylate and its Impurities were prepared in the concentration range of about 0.170 µg/mL to 8.518 µg/mL for Imatinib Mesylate, 0.194 µg/mL to 9.689 µg/mL for Imatinib Impurity – F and 0.186 µg/mL to 9.322 µg/mL for Imatinib Impurity – G. Linearity of detector response was established by plotting a graph concentration *versus* area and determining the correlation coefficient. The detector response was found to be linear with a correlation coefficient of 0.999, 1.000 and 1.000 for Imatinib, Impurity – F and Impurity – G respectively. Linearity graph is shown in Fig. 7 to Fig. 9. Linearity results of the method are presented in Table-9 to Table-11.

Preparation of Imatinib Mesylate stock solution

Stock solution preparation = $[5.140 / 100][99.0 / 100][493.6 / 589.7]1000 = 42.59\text{ppm}$

Diluted solutions = Stock solution (ppm) X [Dilution volume (mL)/ Diluted volume (mL)]

Note: Apply factor for calculation 493.6/589.7

493.6 = Molecular Weight of Imatinib

589.7 = Molecular Weight of Imatinib Mesylate

Preparation of Imatinib Impurity - F stock solution

Stock solution preparation = $[5.110 / 100][94.8 / 100]1000 = 48.44\text{ppm}$

Diluted solutions = Stock solution (ppm) X [Dilution volume (mL)/ Diluted volume (mL)]

Preparation of Imatinib Impurity - G stock solution

Stock solution preparation = $[5.050 / 100][92.3 / 100]1000 = 46.61\text{ppm}$

Diluted solutions = Stock solution (ppm) X [Dilution volume (mL)/ Diluted volume (mL)]

RRF Calculation

Formula = [Slope of Impurity (known Impurity)/ Slope of Active]

RRF value for Imatinib Impurity-F = [(Slope of Impurity-F)/(Slope of Imatinib)]

$$= 61754.05/74167.65 = 0.83$$

RRF value for Imatinib Impurity-G = [(Slope of Impurity-G)/(Slope of Imatinib)]

$$= 68661.27/74167.65 = 0.93$$

* For slope values of the Imatinib and its Impurities refer Table-9 to Table-11. Recovery studies were established and the Results are reported in Table-12 (Analyst-2).

The parameters which are emphasized in the establishment of Relative Response Factor (RRF), changes the Relative Response Factor (RRF) values on changing the parameters. The study visualizes the RRF values deviating on changing the method parameters.

The following experimental parameters were changed and effect on established Relative Response Factor (RRF) was studied.

(1) Change in Column (same stationary phase with different Column Manufacturers).

- X-Bridge C18, 250 x 4.6mm, 5 μ (Make: Waters, USA)
- X-Terra RP-18, 250 x 4.6mm, 5 μ (Make: Waters, USA)
- Inert Sustain C18, 250 x 4.6mm 5 μ (Make: LCGC, Japan)
- Cosmicsil BDS C18, 250 x 4.6mm, 5 μ (Make: Cosmic Age Analytica, USA)
- Purosphere star C18, 250 x 4.6mm, 5 μ (Make: Merck, Germany)
- Cosmicsil Aster C18, 250 x 4.6mm, 5 μ (Make: Cosmic Age Analytica, USA)
- Cosmicsil Agate C18, 250 x 4.6mm, 5 μ (Make: Cosmic Age Analytica, USA)
- Hypersil BDS C18, 250 x 4.6mm, 5 μ (Make: Thermo scientific, UK)
- Kromasil 100, 5-C18, 250 x 4.6mm, 5 μ (Make: Kromasil, Sweden)
- X-Bridge Shield RP-18, 250 x 4.6 mm, 5 μ (Make: Waters, USA)
- YMC Pack ODS AQ, 250 x 4.6mm, 5 μ (Make: YMC, Japan)
- Atlantis T 3 C18, 250 x 4.6mm, 5 μ (Make: Waters, USA)
- X-Bridge C18, 250 x 4.6mm, 3.5 μ (Make: Waters, USA)
- Develosil ODS MG-5, 250 x 4.6mm, 5 μ (Make: Nomura Chemicals, Japan)
- Sunfire C18, 250 x 4.6mm, 5 μ (Make: Waters, USA)

All chromatograms are shown in Fig. 10 to Fig. 25 and the % variation in Relative Response Factor (RRF) values along with the comparison are shown in the Table-13.

(2) Robustness study:

- Change in Particle Size (30 % Variation)
- Change in Flow rate (1.0 \pm 0.2 mL)
- Change in Temperature (30 $^{\circ}$ C \pm 5 $^{\circ}$ C)
- Change in Wavelength (230 \pm 3nm)
- Change in pH (\pm 0.2)
- Change in Different Detectors (UV – 2487 and PDA – 2998 (Make: Waters))
- Change in Buffer concentration (\pm 10% Variation) and
- Change in Solvent grade (HPLC grade & Gradient grade).

The respective chromatograms are shown in Fig. 26 to Fig. 39 and the % variation in Relative Response Factor (RRF) values along with the comparison are shown in the Table-14 to Table-21.

RESULTS AND DISCUSSIONS

The authors studied the impact of RRF by using various HPLC columns and Robustness parameters. Column to column variation study was conducted with same stationary phase with different Column Manufacturers. Significant variations were observed in RRF values because retention times of Imatinib and its impurities changed drastically.

Silica used in the HPLC columns is different for each Column Manufacturer. No two columns exhibit identical retention behavior (PQRI Approach)⁶. Each column can differ in Relative Retention, hydrophobicity, steric interaction, hydrogen bond – acidity and basicity and relative silanol ionization or cation exchange capacity. In USP Approach⁶, the NIST Standard Reference Material (SRM) 870 used to carry out the evaluation of C18 columns were used. This procedure uses a mixture of five organic compounds (Uracil, toluene, ethylbenzene, quinizarin and amitriptyline) in methanol to characterize column performance. This test mixture is intended primarily for the characterization of C18 columns used in reversed-phase liquid chromatography.

Significant variations were observed in RRF values while changing column temperature and flow rates. Slight variations were observed in changing different detectors, solvent grades, wavelength, particle size, pH of the Mobile phase and buffer concentrations. Detector lamp energies also influence RRF values. The authors established RRF's for above robustness parameters and different HPLC Columns with linear range from 0.02 to 1.0% with respect to test concentration of RRF solutions.

United States Pharmacopoeia Forum⁷ does not indicate when Relative Response Factor (RRF) can be rounded off to 1.0. Some sponsors round off the Relative Response Factor (RRF) values to 1.0 when they are in the range of 0.80-1.2, some do so when they are in the range 0.90-1.1 and others do so when they are in the range of 0.95-1.05. State the Relative Response Factor (RRF) values in monographs to one decimal place if it is equal to or greater than 1.0 and to two decimal places if it is less than 1.0. That is, the Relative Response Factor (RRF) value will be expressed to two significant digits. The ICH Q3A (R) guideline indicates that the impurity results should be reported to one decimal place if they are at or above 1.0 and to two decimal places if they are below 1.0. In USP, the symbol 'F' is used to designate Relative Response Factor.

European Pharmacopoeia (Ph. Eur)² requires that, if the value of the Relative Response Factor (RRF) is different from 0.8-1.2, it should be included in the monographs.

British Pharmacopoeia (BP)³ states that Response Factors of less than 0.2 or more than 5 are not used. If the difference between the response of an impurity and that of the substance being examined is outside these limits, a different method of determination, such as a different detection wavelength (λ) or a different method of visualization is used.

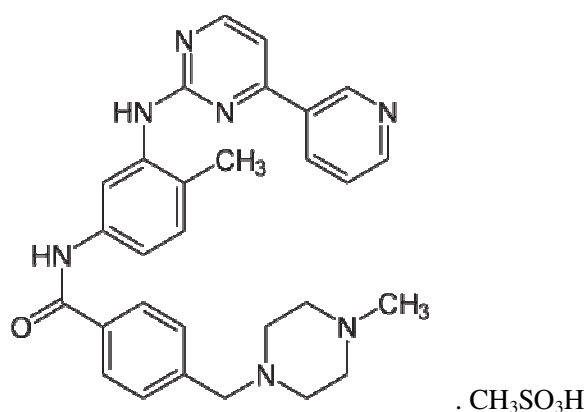


Fig.-1a: Imatinib Mesylate

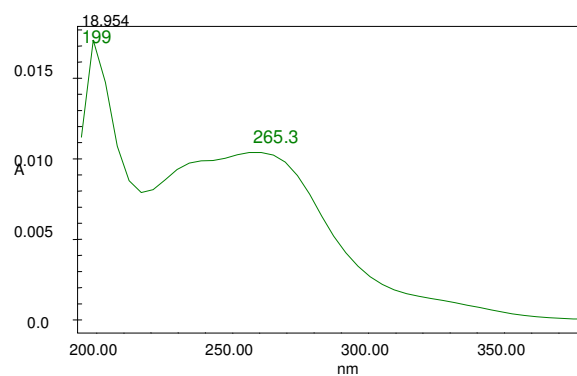


Fig.-1b: Imatinib Mesylate Spectra

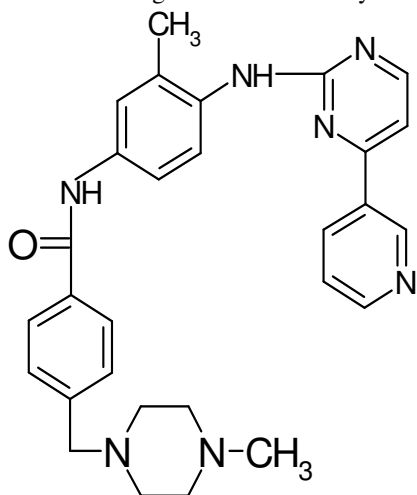


Fig.-2a: Imatinib Impurity – G
(Isomeric impurity)

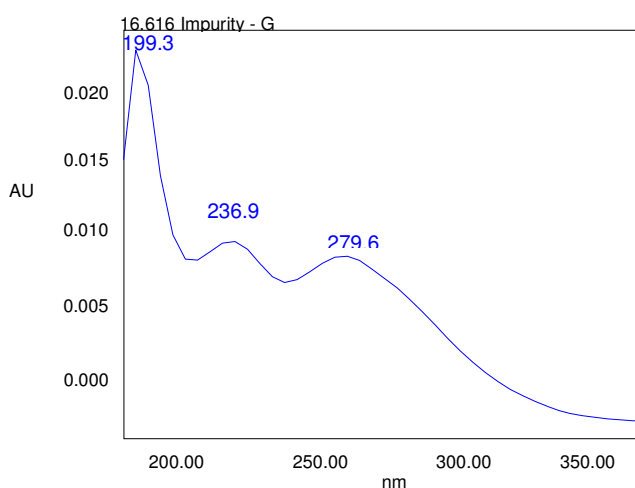


Fig.-2b: Imatinib Impurity – G Spectra

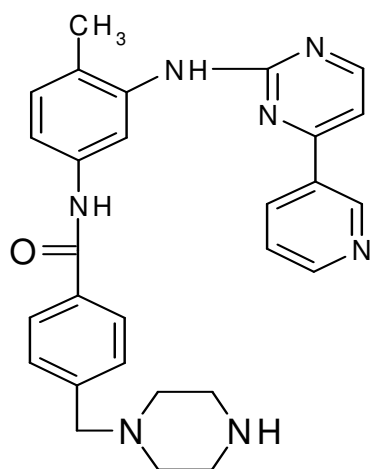


Fig.-3a: Imatinib Impurity – F
(Desmethyl impurity)

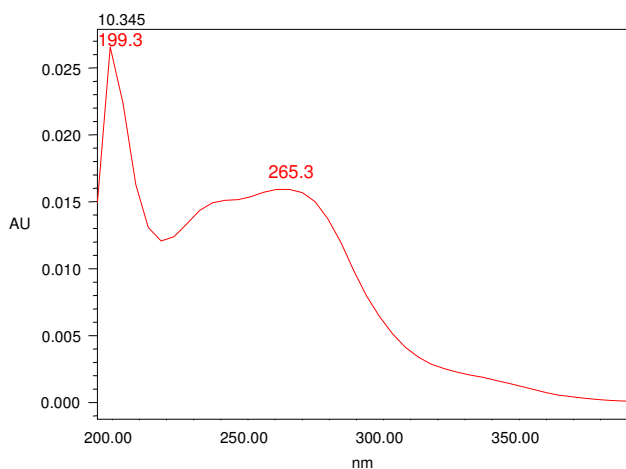


Fig.-3b: Imatinib Impurity – F Spectra

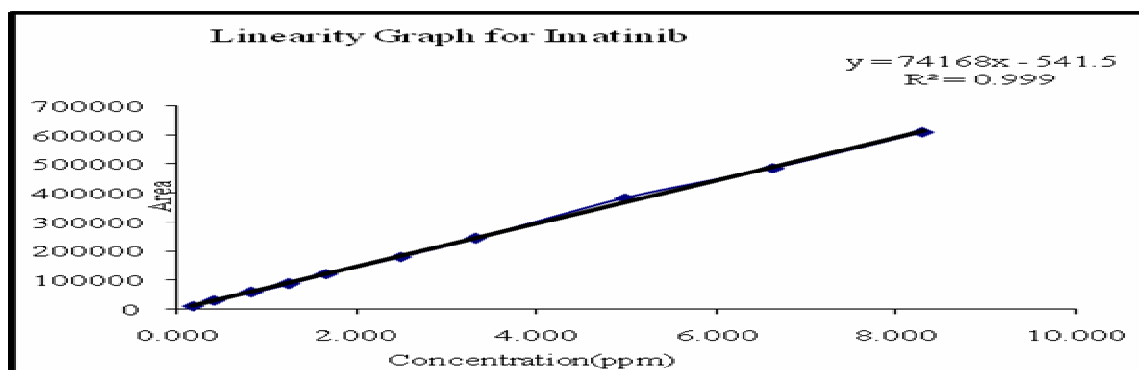


Fig.-4: Linearity graph for Imatinib (Analyst-1)

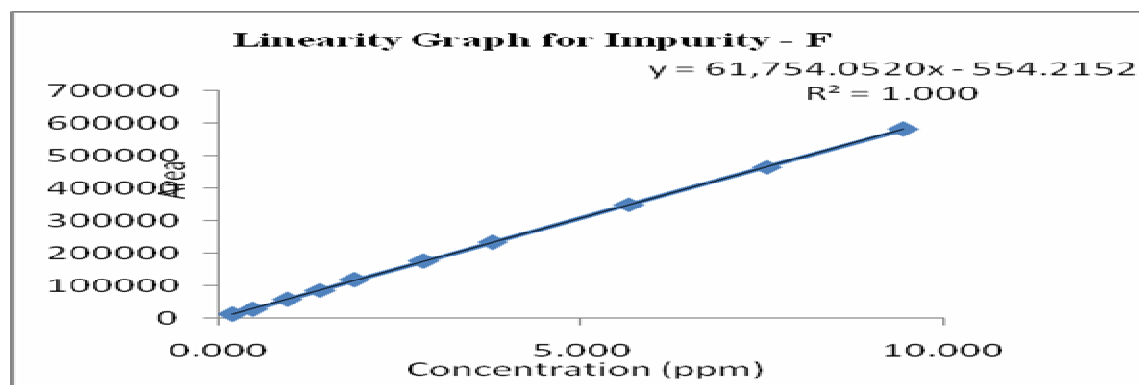


Fig.-5: Linearity graph for Imatinib Impurity – F (Analyst-1)

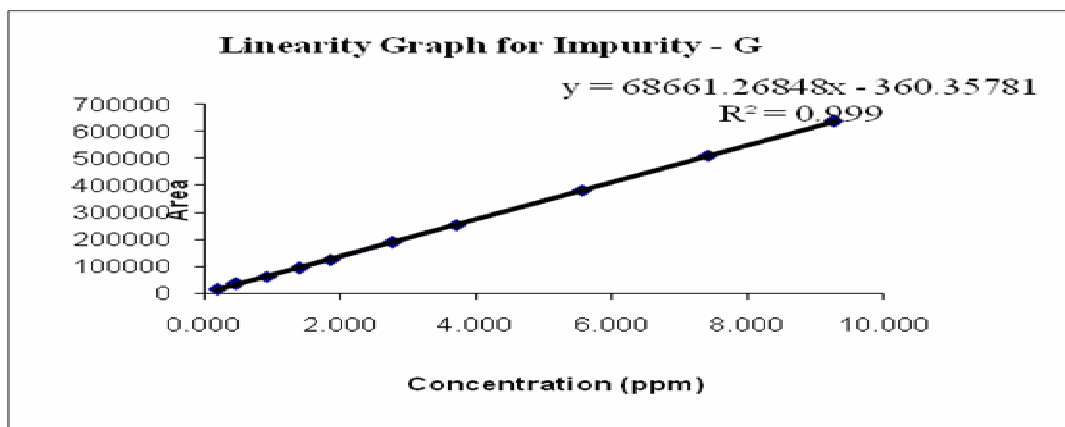


Fig.-6: Linearity graph for Imatinib Impurity – G (Analyst-1)

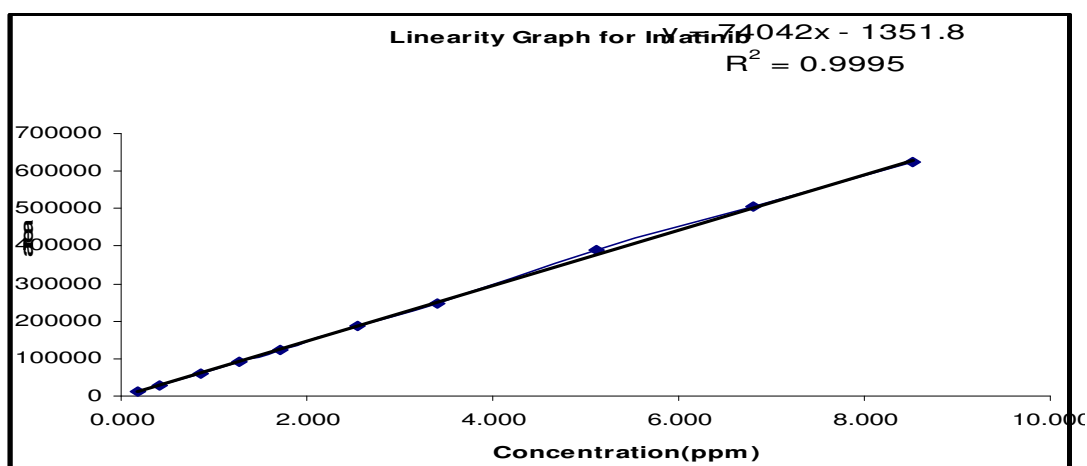


Fig.-7: Linearity graph for Imatinib (Analyst-2)

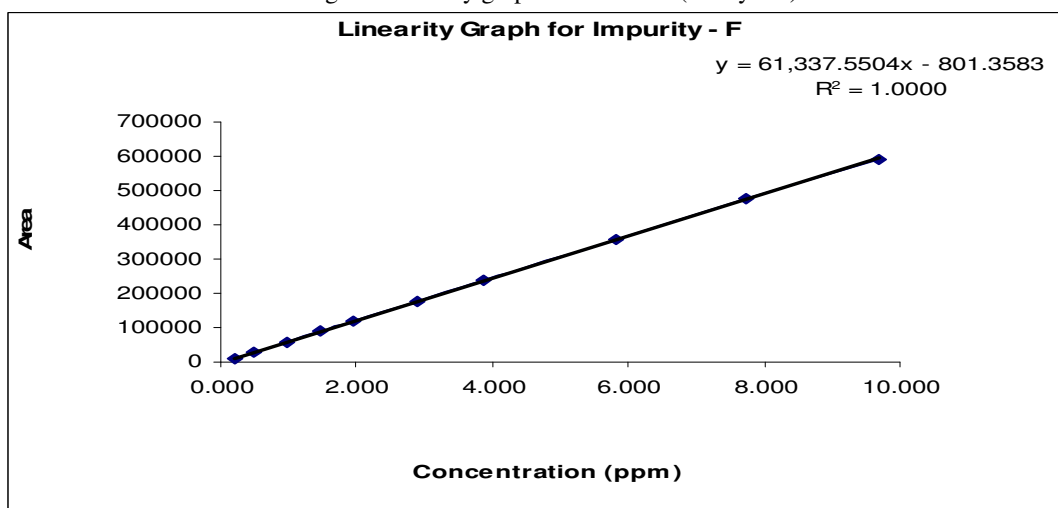


Fig. -8: Linearity graph for Imatinib Impurity – F (Analyst-2)

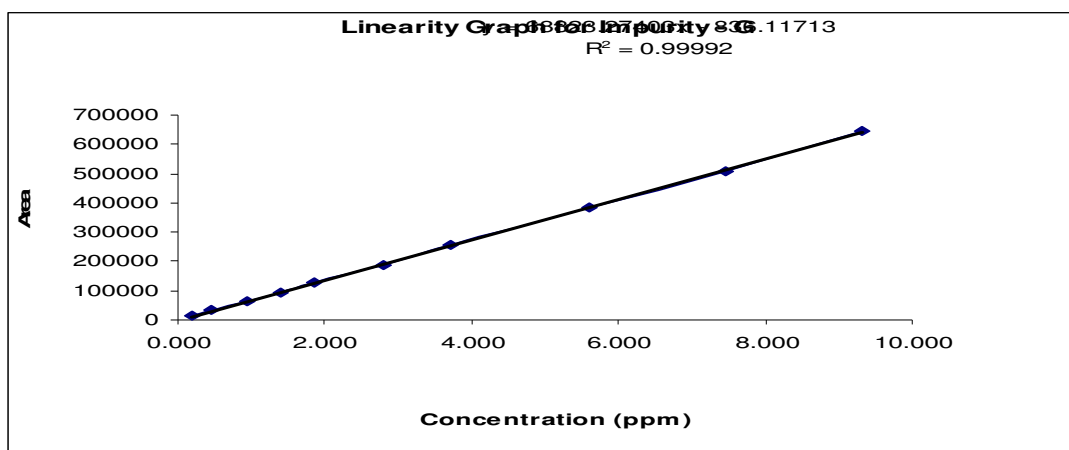


Fig. -9: Linearity graph for Imatinib Impurity – G (Analyst-2)

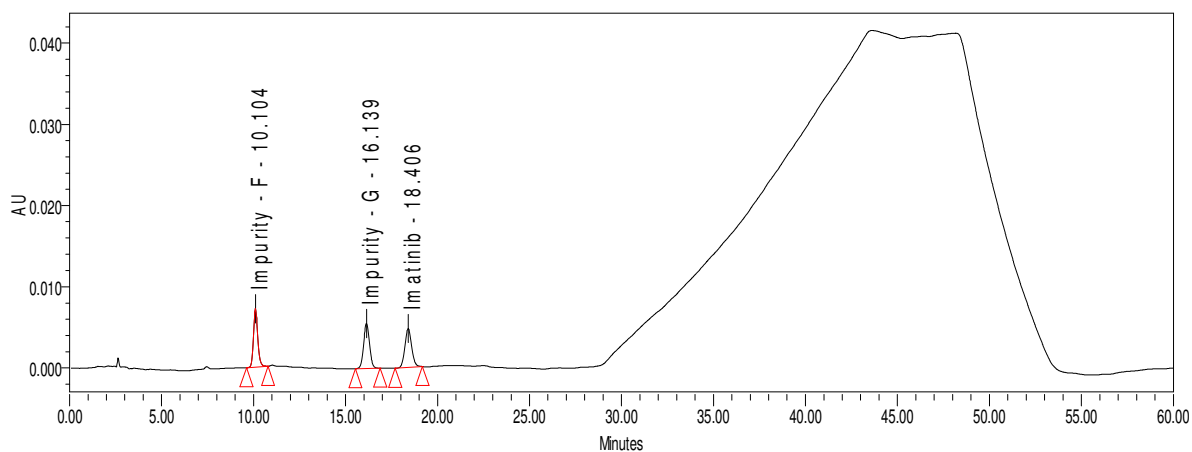


Fig.-10: X-Bridge C18, 250 x 4.6mm, 5 μ (Make: Waters, USA) (Analyst-1)

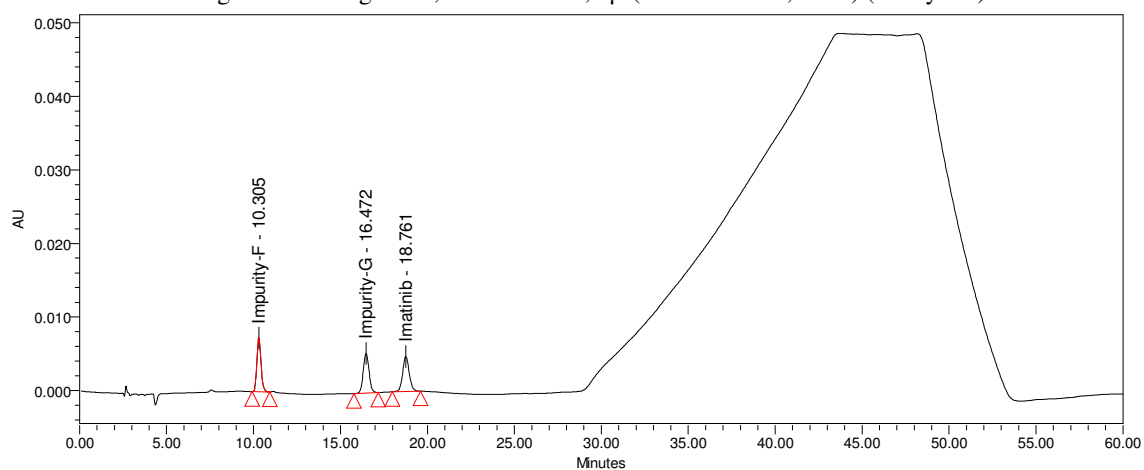


Fig.-11: X-Bridge C18, 250 x 4.6mm, 5 μ (Make: Waters, USA) (Analyst-2)

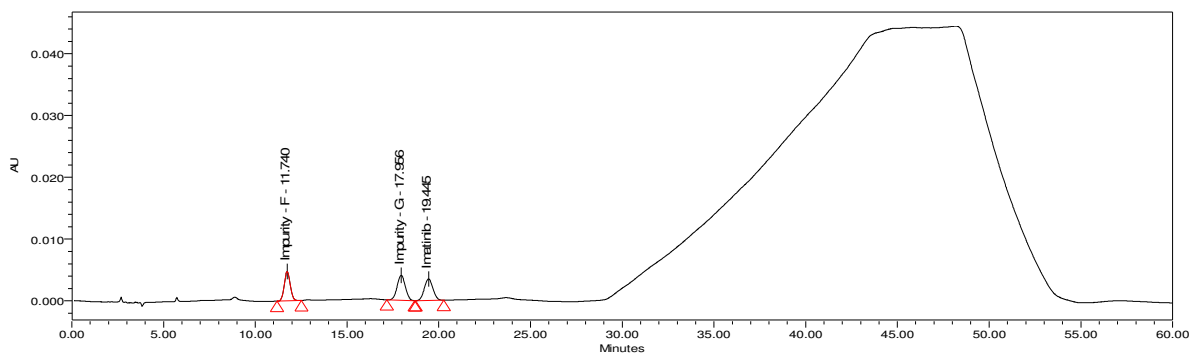


Fig.-12: X-Terra RP 18, 250 x 4.6mm, 5 μ (Make: Waters, USA)

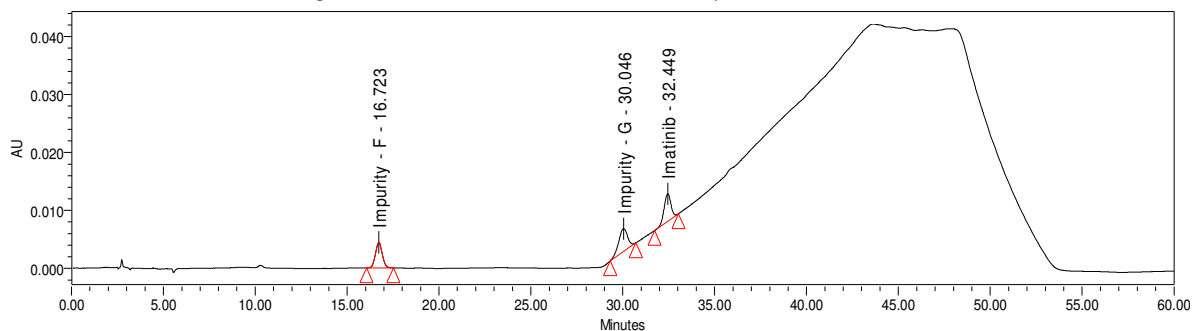


Fig.-13: Inert Sustain C18, 250 x 4.6mm, 5 μ (Make: LCGC, Japan)

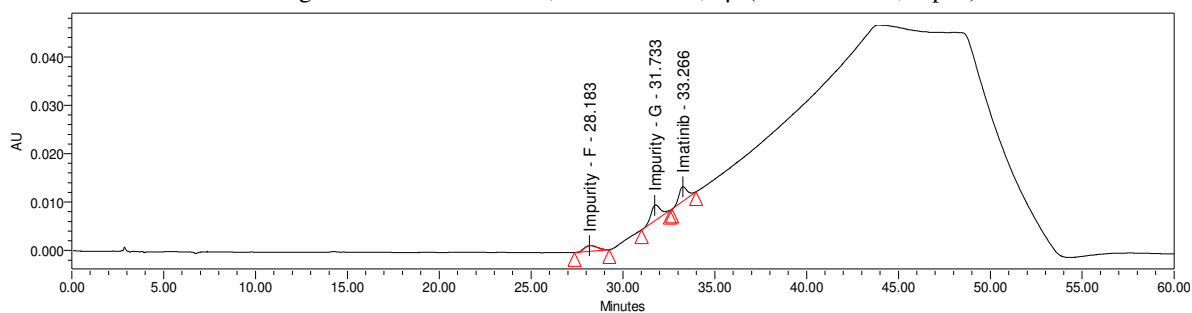


Fig.-14: Cosmicil BDS, C18, 250 x 4.6mm, 5 μ (Make: Cosmic Age Analytica, USA)

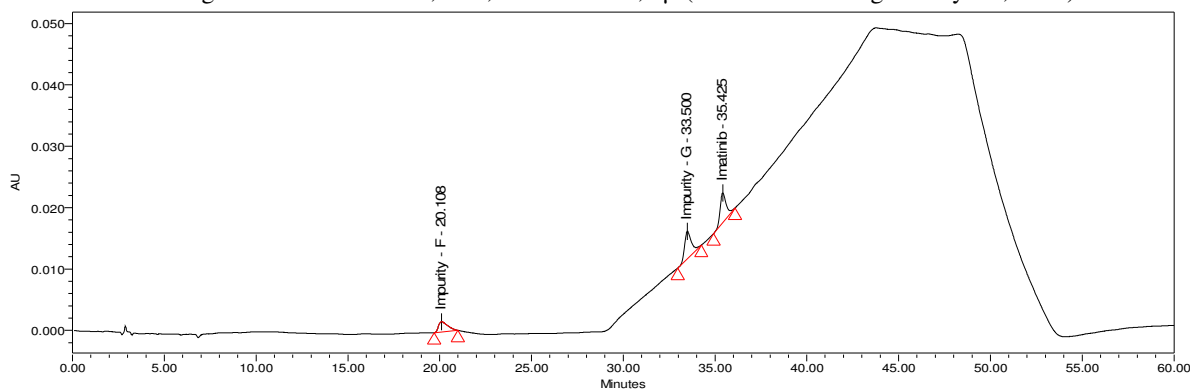


Fig.-15: Purosphere star C18, 250 x 4.6mm, 5 μ (Make: Merck, Germany)

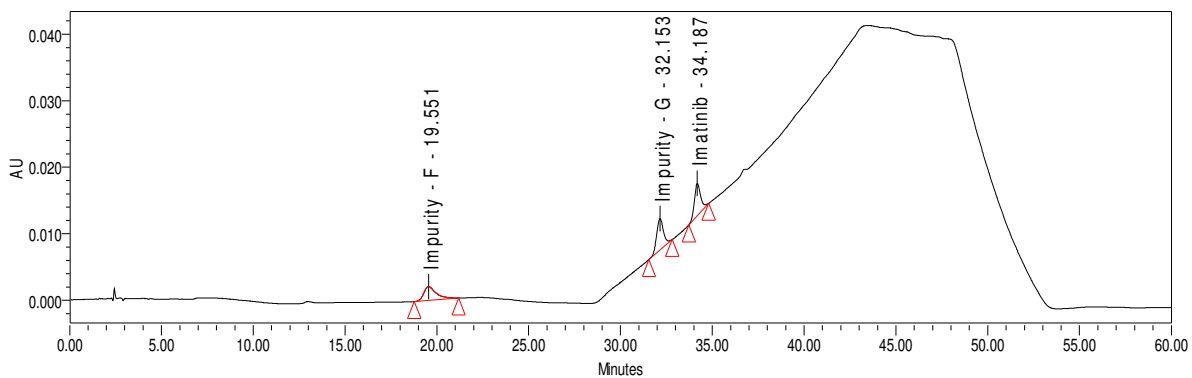


Fig.-16: Cosmicil Aster C18, 250 x 4.6mm, 5 μ (Make: Cosmic Age Analytica, USA)

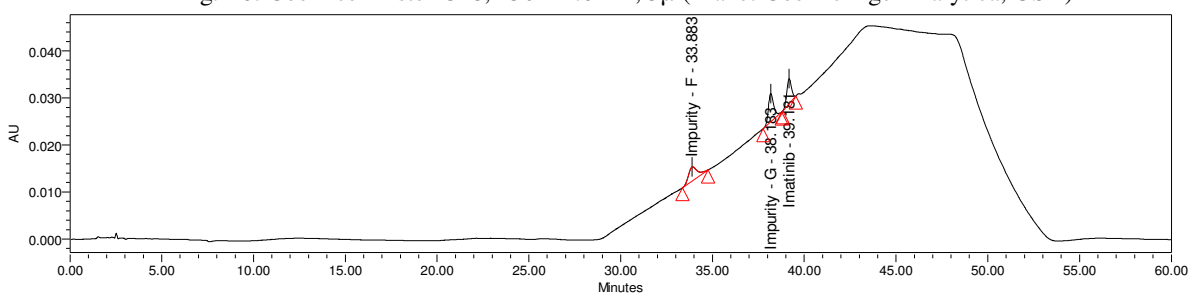


Fig.-17: Cosmicil Agate C18, 250 x 4.6mm, 5 μ (Make: Cosmic Age Analytica, USA)

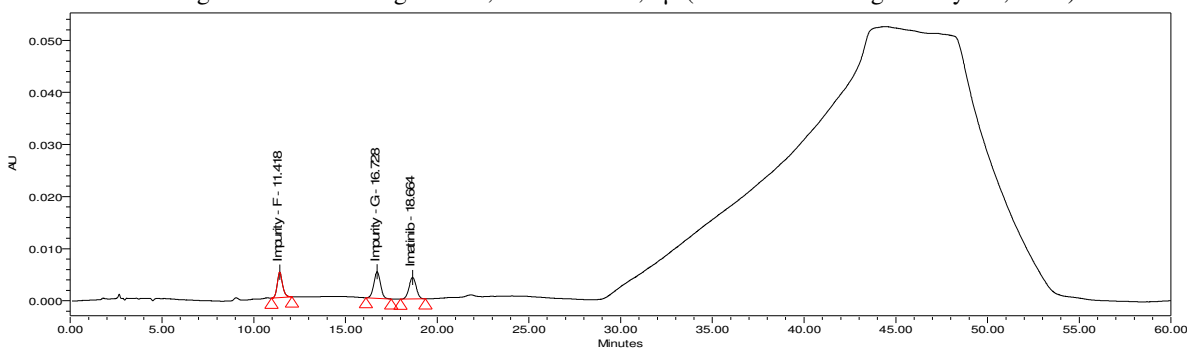


Fig.-18: Hypersil BDS C18, 250 x 4.6mm, 5 μ (Make: Thermo scientific, UK)

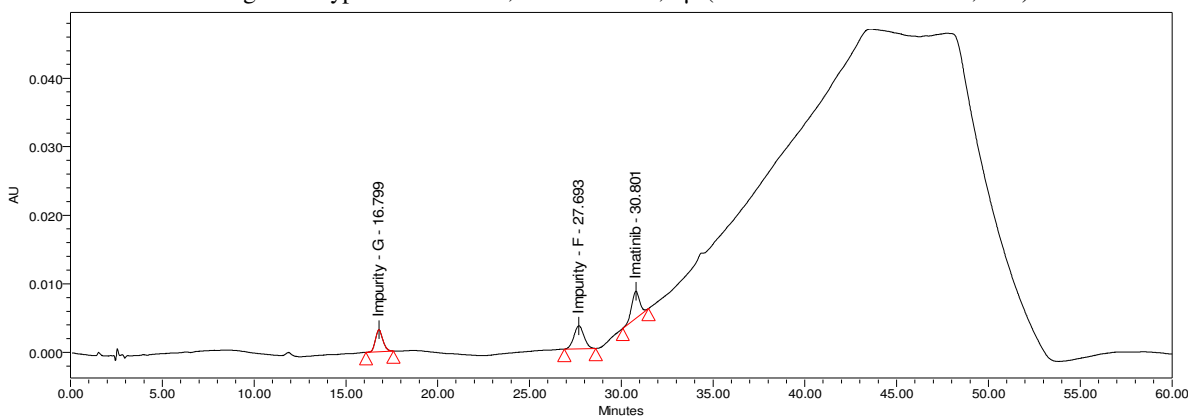


Fig.-19: Kromasil 100, 5-C18, 250 x 4.6mm, 5 μ (Make: Kromasil, Sweden)

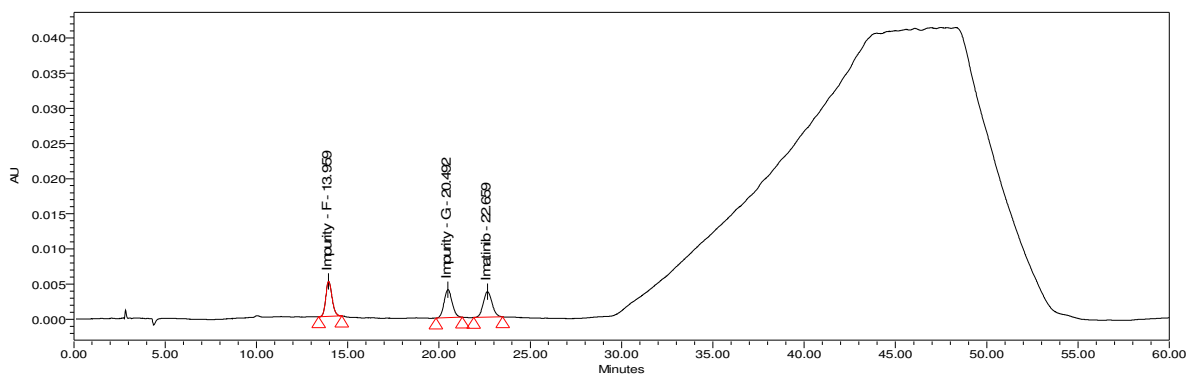


Fig.-20: X-Bridge Shield RP-18, 250 x 4.6mm, 5 μ (Make: Waters, USA)

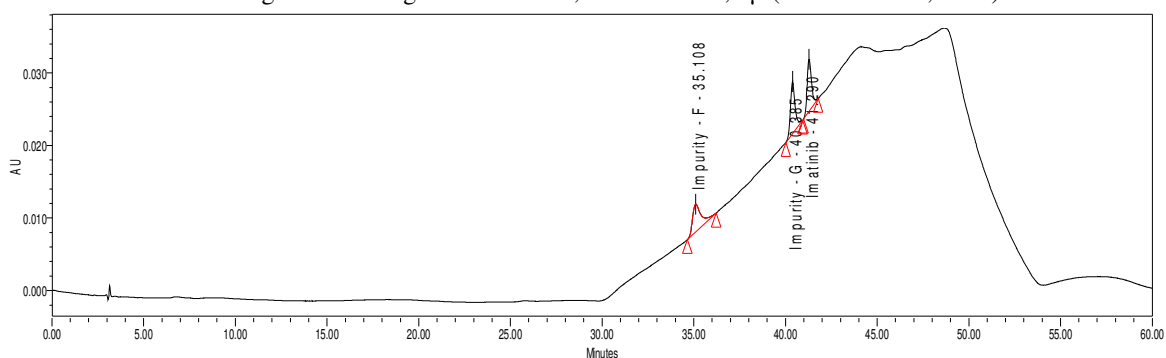


Fig.-21: YMC Pack ODS AQ, 250 x 4.6mm, 5 μ (Make: YMC, Japan)

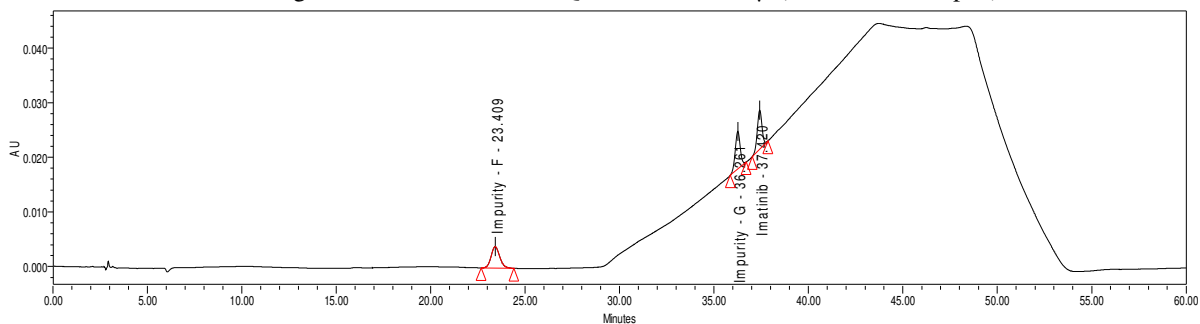


Fig.-22: Atlantis T3 C18, 250 x 4.6mm, 5 μ (Make: Waters, USA)

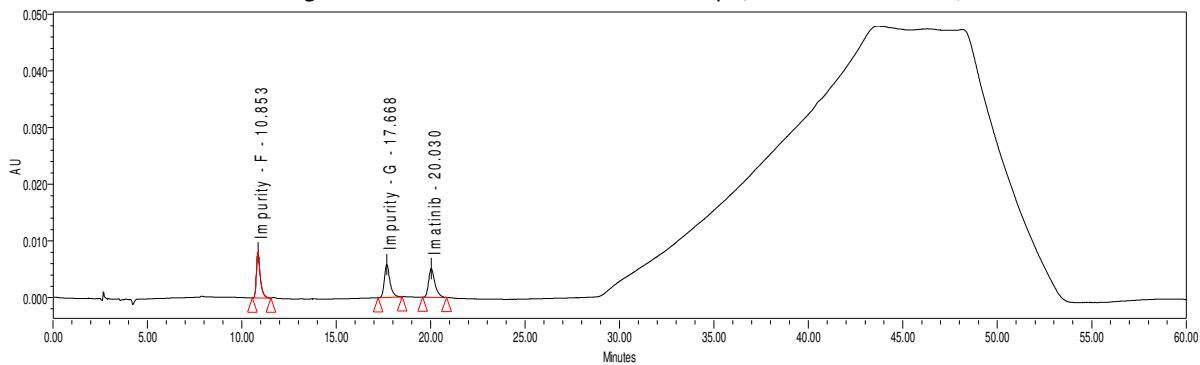


Fig.-23: X-Bridge C18, 250 x 4.6mm, 3.5 μ (Make: Waters, USA)

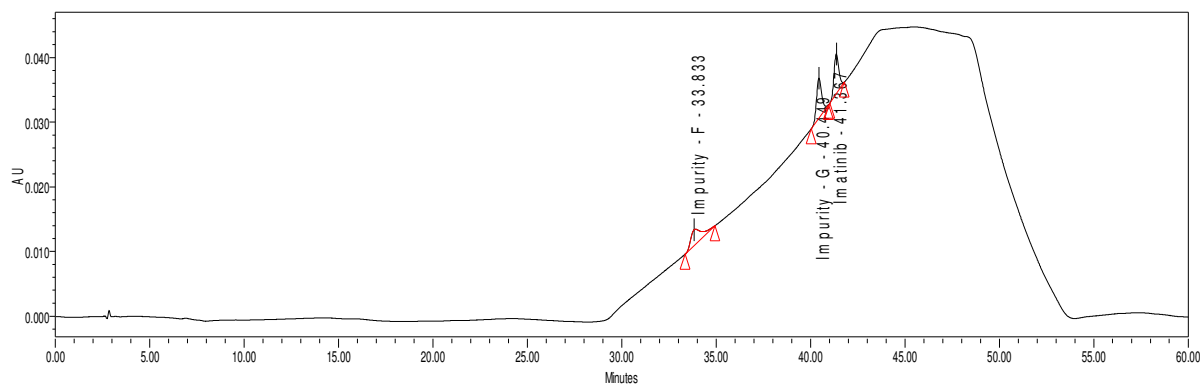


Fig.-24: Develosil ODS MG-5 250 x 4.6mm, 5 μ (Make: Nomura Chemicals, Japan)

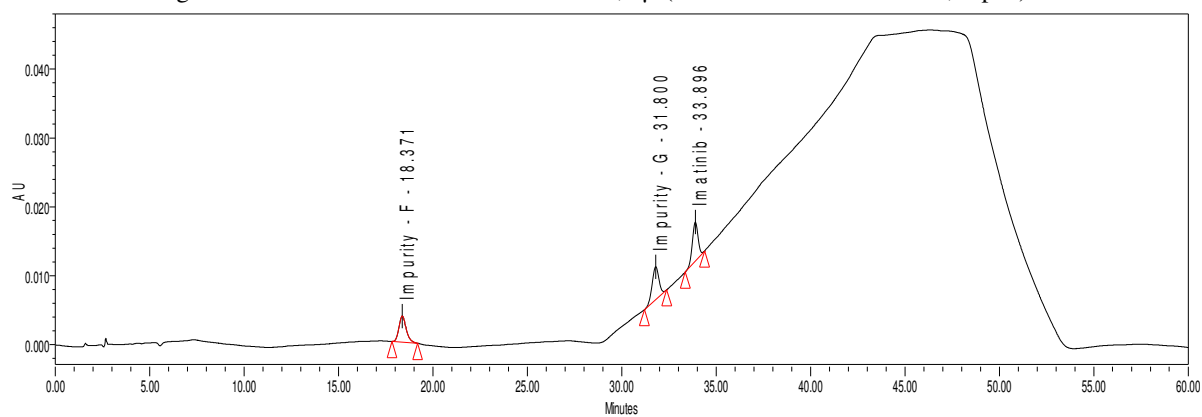


Fig.-25: Sunfire C18, 250 x 4.6mm, 5 μ (Make: Waters, USA)

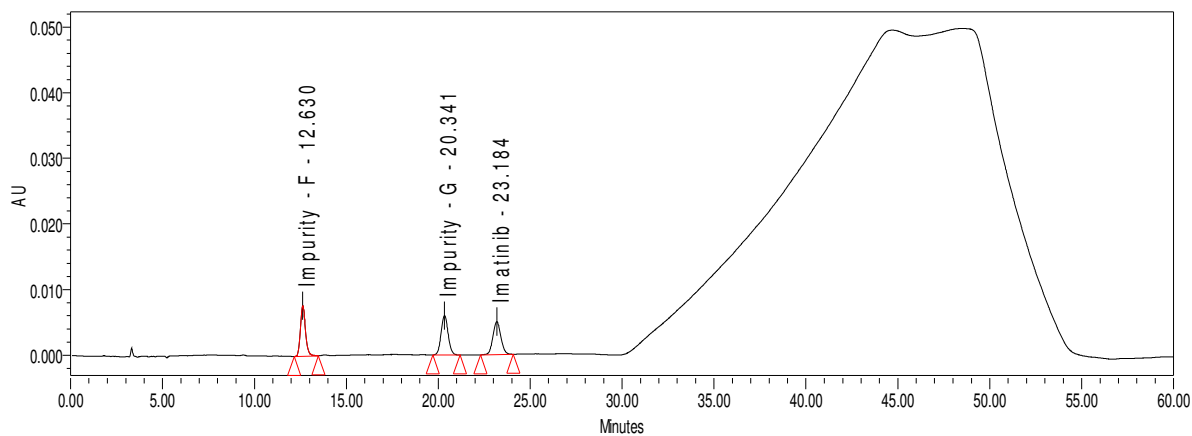


Fig.-26: Low Flow-0.8mL (Column: X-Bridge C18, 250 x 4.6mm, 5 μ (Make: Waters, USA))

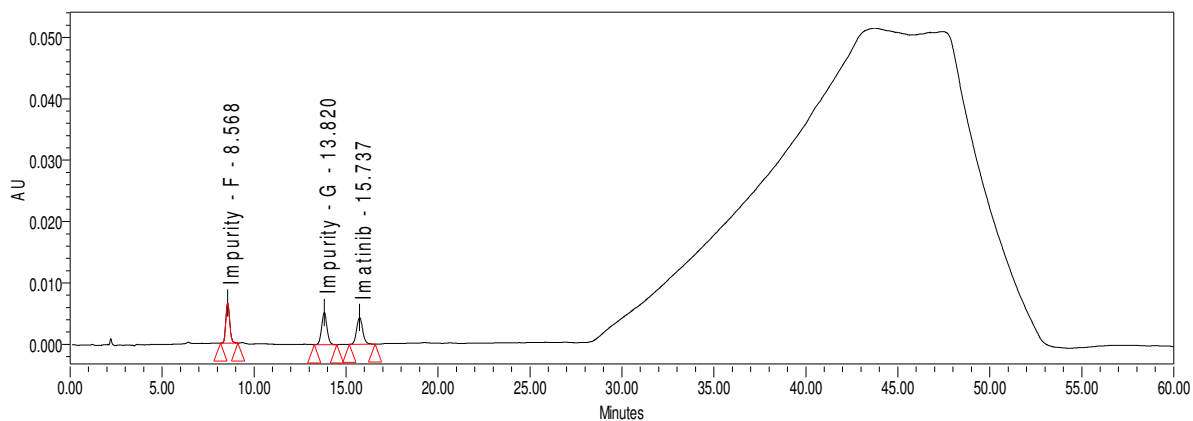


Fig.-27: High Flow-1.2mL (Column: X-Bridge C18, 250 x 4.6mm, 5 μ (Make: Waters, USA))

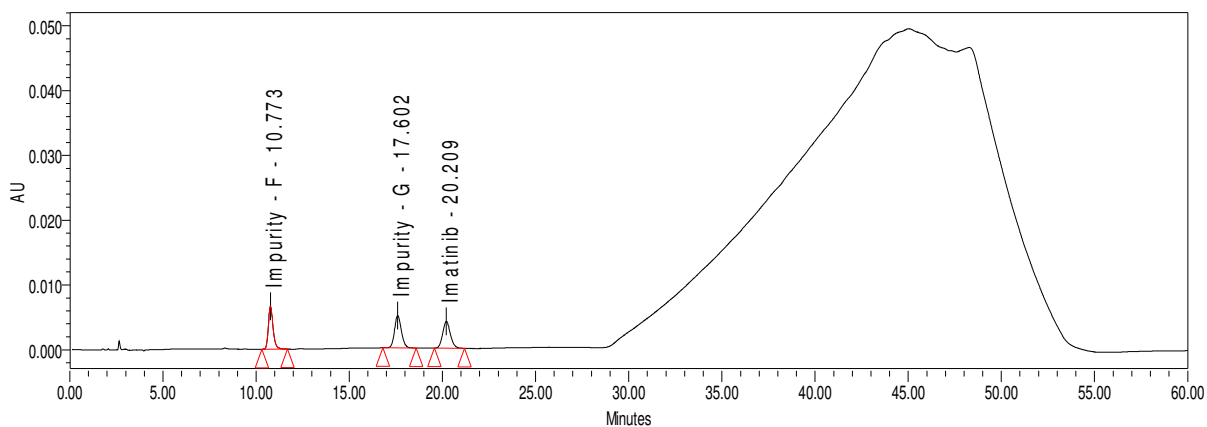


Fig.-28: Low Temperature-25°C (Column: X-Bridge C18, 250 x 4.6mm, 5 μ (Make: Waters, USA))

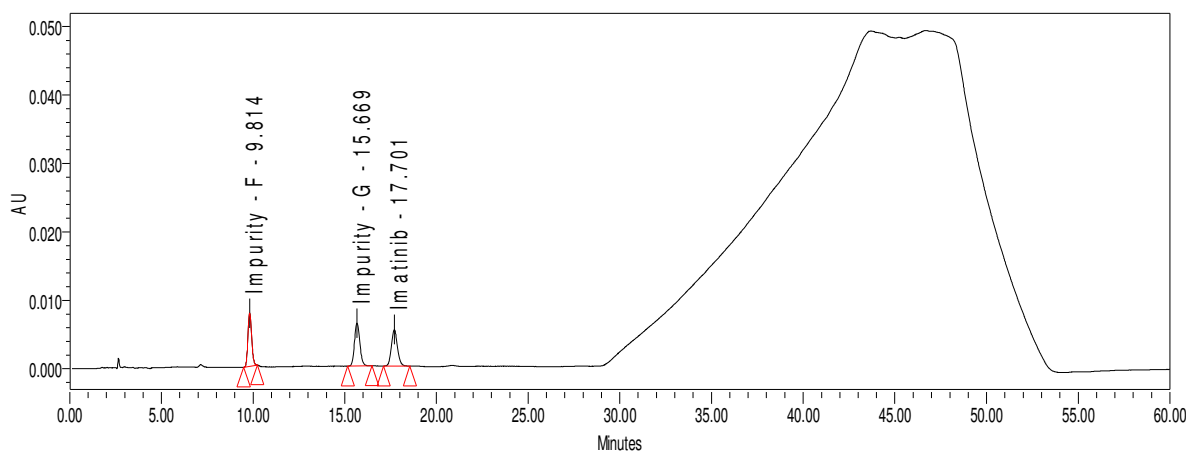


Fig.-29: High Temperature-35°C (Column: X-Bridge C18, 250 x 4.6mm, 5 μ (Make: Waters, USA))

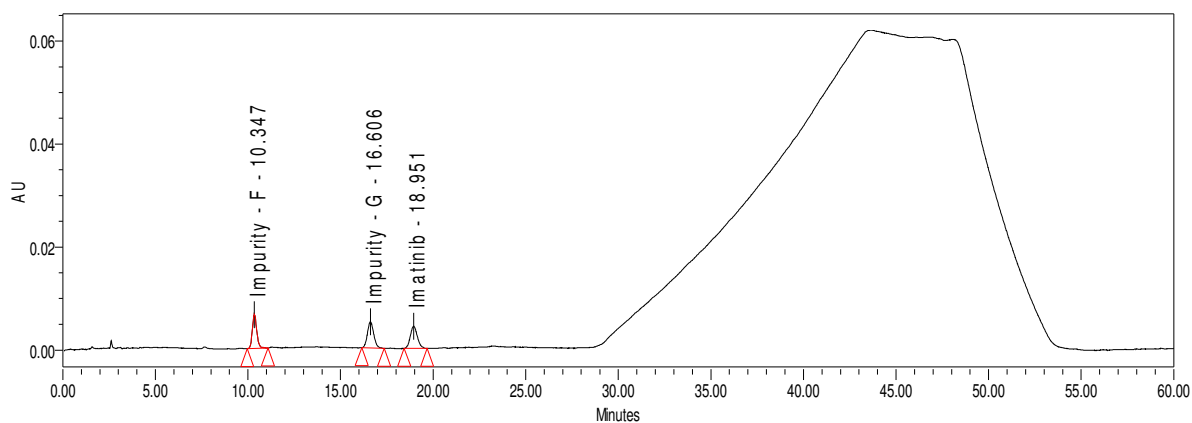


Fig.-30: Low Wave length-227nm (Column: X-Bridge C18, 250 x 4.6mm, 5 μ (Make: Waters, USA))

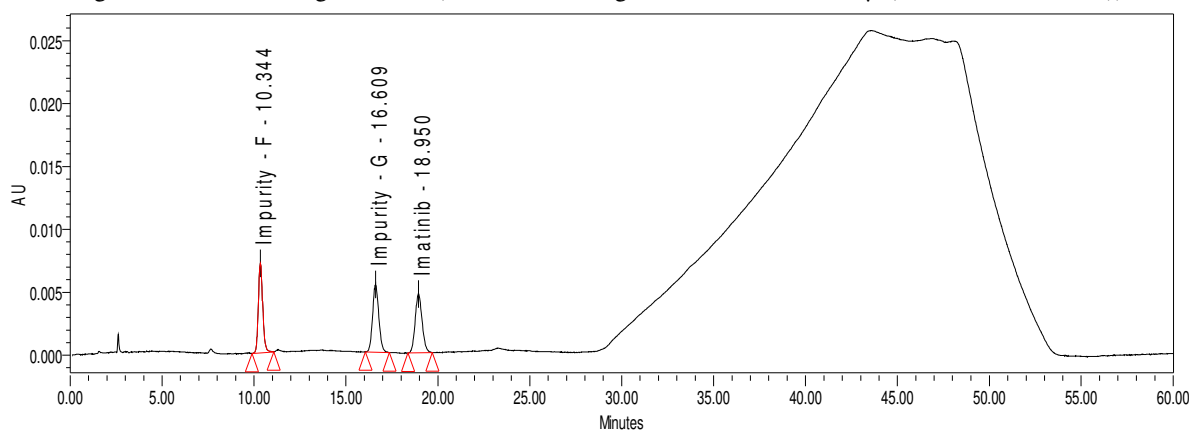


Fig.-31: High Wave length-233nm (Column: X-Bridge C18, 250 x 4.6mm, 5 μ (Make: Waters, USA))

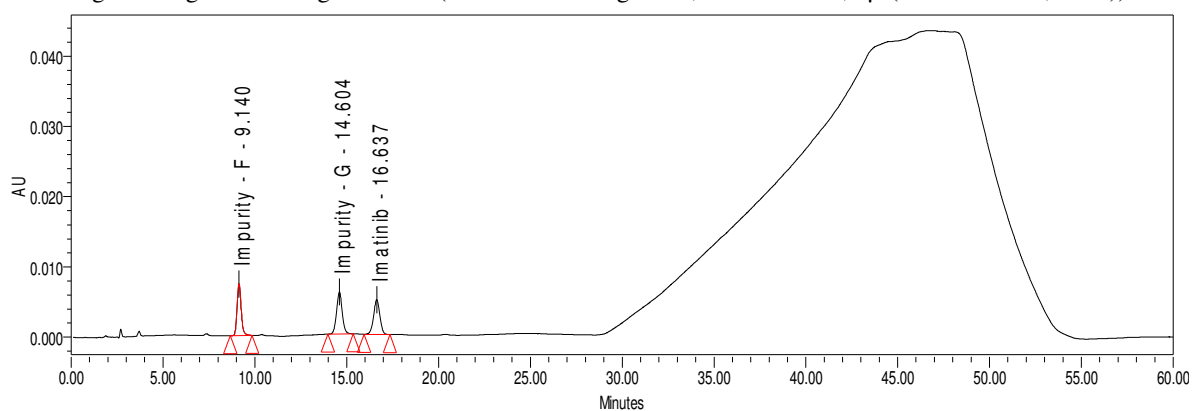


Fig.-32: Low pH-7.8 (Column: X-Bridge C18, 250 x 4.6mm, 5 μ (Make: Waters, USA))

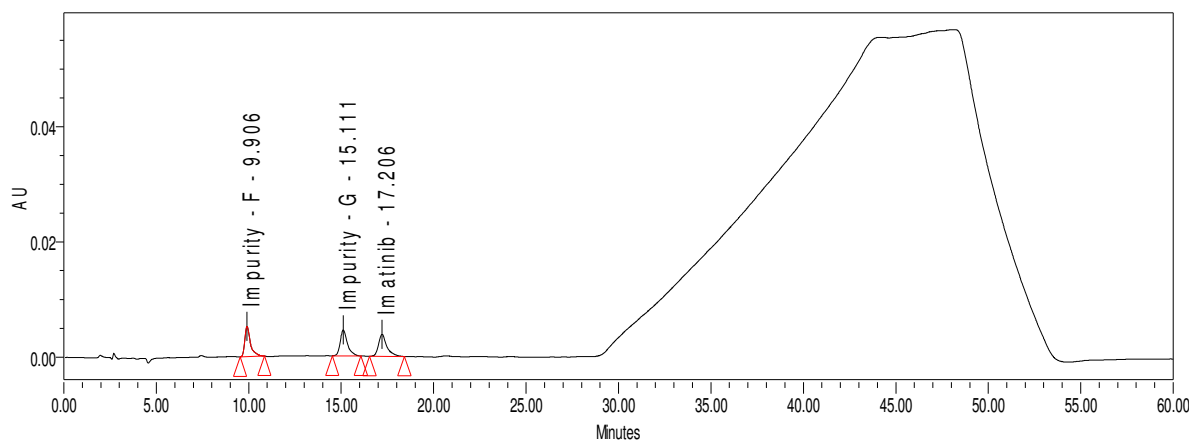


Fig.-33: High pH-8.2 (Column: X-Bridge C18, 250 x 4.6mm, 5 μ (Make: Waters, USA))

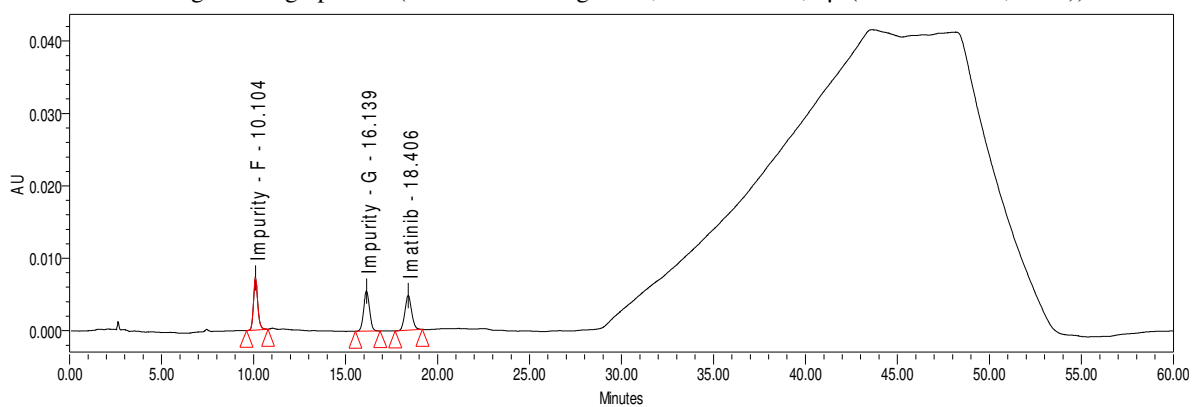


Fig.-34: Alliance 2487 -UV Detector (Make: Waters, Column: X-Bridge C18, 250 x 4.6mm, 5 μ (Make: Waters, USA))

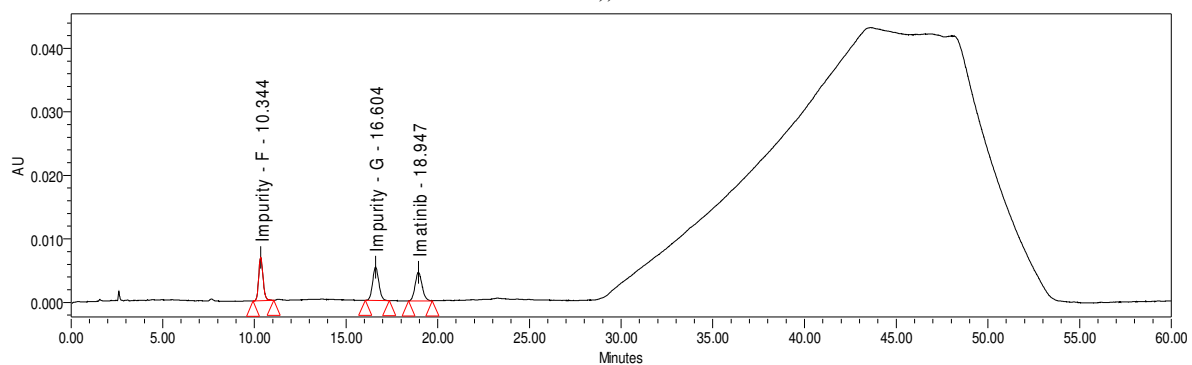


Fig.-35: Alliance 2998 -PDA Detector (Make: Waters, Column: X-Bridge C18, 250 x 4.6mm, 5 μ (Make: Waters, USA))

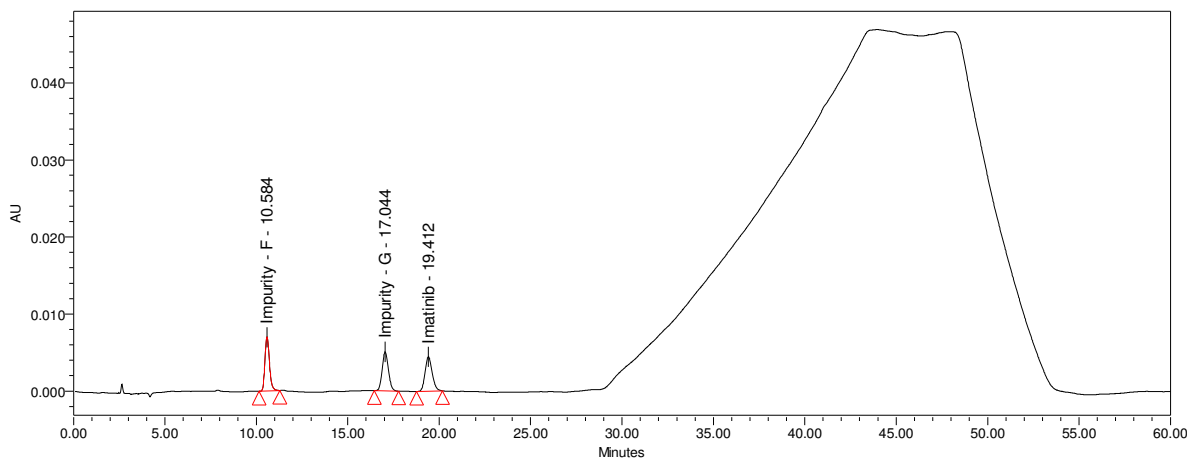


Fig.-36: Low Buffer concentration (Column: X-Bridge C18, 250 x 4.6mm, 5 μ (Make: Waters, USA))

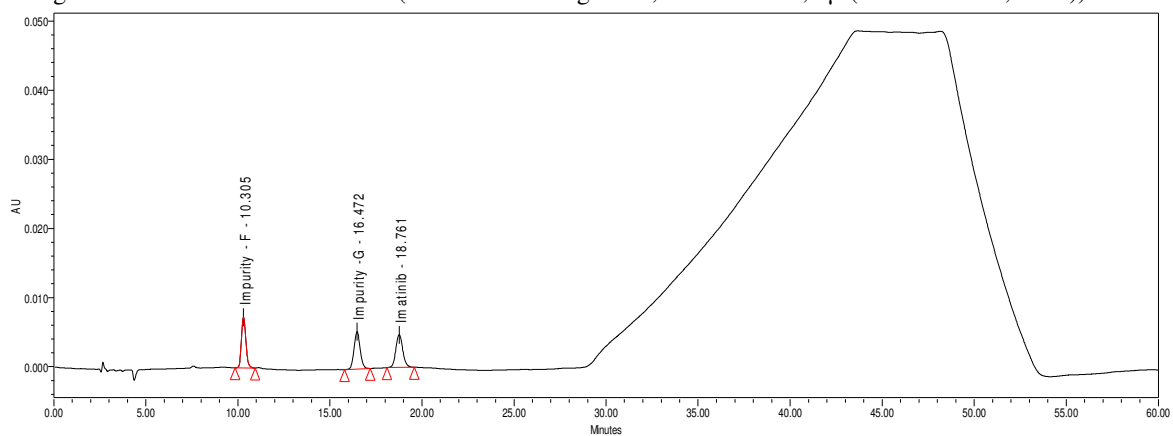


Fig.-37: High Buffer concentration (Column: X-Bridge C18, 250 x 4.6mm, 5 μ (Make: Waters, USA))

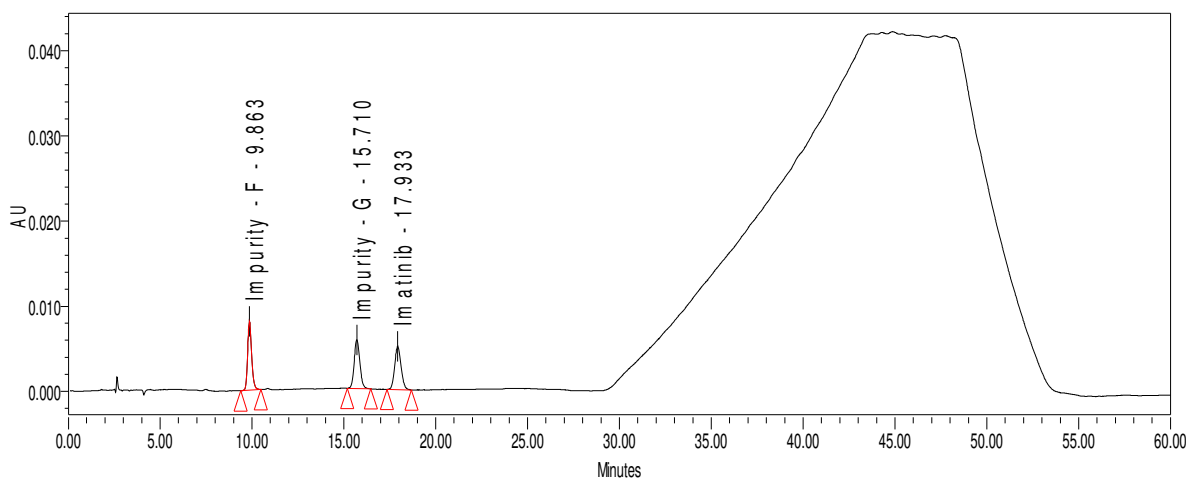


Fig.-38: Solvent Grade (HPLC grade) (Column: X-Bridge C18,250x 4.6mm, 5 μ (Make: Waters,

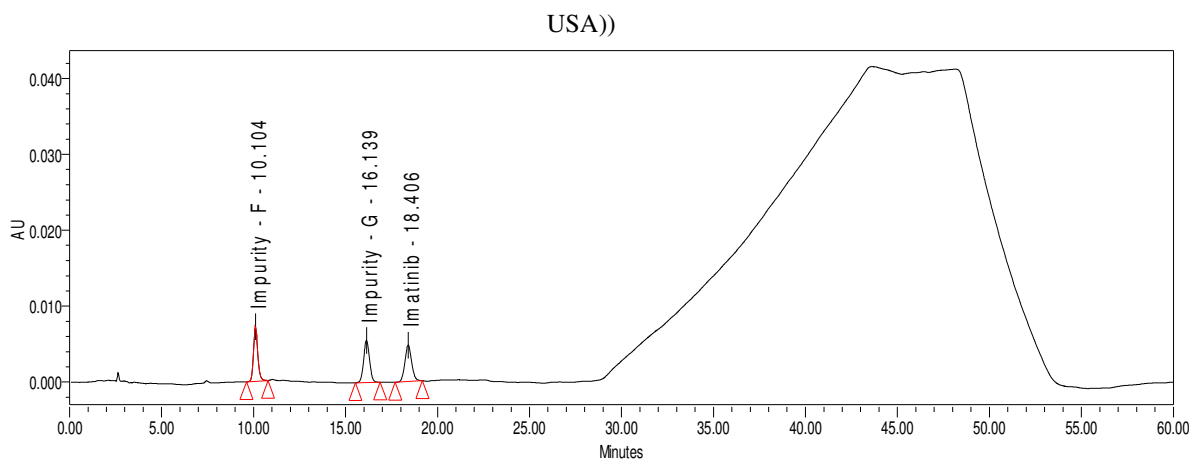


Fig.-39: Solvent Grade (Gradient grade) (Column: X-Bridge C18, 250 x 4.6mm, 5 μ (Make: Waters, USA))

Table-1 : Gradient programming table

Time(minute)	Flow (mL/minute)	Mobile phase -A(%)	Mobile phase -B(%)
0.0	1.0	100	0
25.0	1.0	100	0
40.0	1.0	65	35
45.0	1.0	65	35
50.0	1.0	100	0
60.0	1.0	100	0

Table-2: Identify the each known impurity in the chromatogram with the following relative retention times (RRT) (As per existing Method)

S.No	Name of the Component	RRT (min)	RRF
1.	Imatinib	1.00	1.00
2.	Impurity-F	0.54	0.83
3.	Impurity-G	0.87	0.93
4.	Imatinib Retention time is about 18.5 minutes		

Table-3: Purity and sample weight of Imatinib Mesylate and its Impurities (Analyst-1)

Name of the sample	Wt. of Sample (mg)	Purity (%)
Imatinib Mesylate	5.002	99.0
Imatinib impurity - F	4.978	94.8
Imatinib impurity - G	5.029	92.3

Table-4: RRF calculation for Imatinib (Analyst-1)

Imatinib				
Concentration(%)	Dilution (mL)	Diluted To(mL)	Concentration(ppm)	Area
0.02	1.0	250	0.166	12416
0.05	1.0	100	0.414	31708
0.10	1.0	50	0.829	59047
0.15	1.5	50	1.243	87704
0.20	2.0	50	1.658	122994
0.30	1.5	25	2.487	181123
0.40	2.0	25	3.316	244259
0.60	3.0	25	4.974	383231
0.80	4.0	25	6.632	487411
1.00	4.0	20	8.290	610344
SLOPE				74167.65
Correlation Coefficient				0.999

Table-5: RRF calculation for Imatinib Impurity – F (Analyst-1)

Impurity - F				
Concentration (%)	Dilution(mL)	Diluted To(mL)	Concentration(ppm)	Area
0.02	1.0	250	0.189	10913
0.05	1.0	100	0.472	26525
0.10	1.0	50	0.944	57650
0.15	1.5	50	1.416	86226
0.20	2.0	50	1.888	117962
0.30	1.5	25	2.831	175732
0.40	2.0	25	3.775	232984
0.60	3.0	25	5.663	349311
0.80	4.0	25	7.551	465288
1.00	4.0	20	9.438	581793
SLOPE				61754.05
Correlation Coefficient				1.000

Table-6: RRF calculation for Imatinib Impurity – G (Analyst-1)

Impurity - G				
Concentration (%)	Dilution (mL)	Diluted To (mL)	Concentration(ppm)	Area
0.02	1.0	250	0.186	14473
0.05	1.0	100	0.464	36056
0.10	1.0	50	0.928	62950
0.15	1.5	50	1.393	92852
0.20	2.0	50	1.857	123825
0.30	1.5	25	2.785	189187
0.40	2.0	25	3.713	254276
0.60	3.0	25	5.570	381190
0.80	4.0	25	7.427	511777
1.00	4.0	20	9.284	637268
SLOPE				68661.27
Correlation Coefficient				0.999

Table-7: Recovery (Analyst-1)

%Recovery Found		
	Impurity -F	Impurity - G
Centrifuge	99.7	99.8
Hydrophilic PVDF(Millipore Millex-HV 0.45µm)	99.9	102.1
Nylon(Millipore Millex-HN 0.45µm)	99.0	104.1

Table-8: Purity and sample weight of Imatinib Mesylate and its Impurities (Analyst-2)

Name of the sample	Wt. of Sample (mg)	Purity (%)
Imatinib Mesylate	5.140	99.0
Imatinib impurity - F	5.110	94.8
Imatinib impurity - G	5.050	92.3

Table-9: RRF calculation for Imatinib (Analyst-2)

Imatinib				
Concentration (%)	Dilution (mL)	Diluted To (mL)	Concentration (ppm)	Area

0.02	1.0	250	0.170	12572
0.05	1.0	100	0.426	29540
0.10	1.0	50	0.852	60534
0.15	1.5	50	1.278	92774
0.20	2.0	50	1.704	123583
0.30	1.5	25	2.555	185746
0.40	2.0	25	3.407	247904
0.60	3.0	25	5.111	388229
0.80	4.0	25	6.815	506254
1.00	4.0	20	8.518	622524
SLOPE				74041.88
Correlation Coefficient				0.9998

Table-10: RRF calculation for Imatinib Impurity – F (Analyst-2)

Imatinib Impurity – F				
Concentration (%)	Dilution (mL)	Diluted To (mL)	Concentration (ppm)	Area
0.02	1.0	250	0.194	10562
0.05	1.0	100	0.484	28160
0.10	1.0	50	0.969	57877
0.15	1.5	50	1.453	88600
0.20	2.0	50	1.938	118490
0.30	1.5	25	2.907	177479
0.40	2.0	25	3.875	238141
0.60	3.0	25	5.813	357229
0.80	4.0	25	7.751	474635
1.00	4.0	20	9.689	592080
SLOPE				61337.55
Correlation Coefficient				1.0000

Table-11: RRF calculation for Imatinib Impurity – G (Analyst-2)

Imatinib Impurity – G				
Concentration (%)	Dilution (mL)	Diluted To (mL)	Concentration (ppm)	Area
0.02	1.0	250	0.186	12845
0.05	1.0	100	0.466	34238
0.10	1.0	50	0.932	62341
0.15	1.5	50	1.398	95139
0.20	2.0	50	1.864	127364
0.30	1.5	25	2.797	189673
0.40	2.0	25	3.729	255081
0.60	3.0	25	5.593	383854

0.80	4.0	25	7.458	509639
1.00	4.0	20	9.322	644025
SLOPE				68823.27
Correlation Coefficient				1.0000

Table-12: Recovery (Analyst-2)

%Recovery Found		
	Impurity -F	Impurity - G
Centrifuge	99.6	99.7
Hydrophilic PVDF(Millipore Millex-HV 0.45 μm)	100.1	102.0
Nylon(Millipore Millex-HN 0.45μm)	99.1	103.9

Table-13: The following table shows the variation in RRF values on changing the column

Columns	RRF Values		% Observed		% Variation (±)	
	Impurity F	Impurity G	Impurity F	Impurity G	Impurity F	Impurity G
Column to column variation						
X- Bridge C18, 250 x 4.6 mm, 5μ	0.83	0.93				
X- Terra RP 18, 250 x 4.6 mm, 5μ	0.84	0.98	101.2	105.4	-1.2	-5.4
Inert Sustain C18, 250 x 4.6 mm, 5μ	0.82	0.97	98.8	104.3	1.2	-4.3
Cosmicsil BDS C18, 250 x 4.6 mm, 5μ	0.59	0.99	71.1	106.5	28.9	-6.5
Purosphere Star C18, 250 x 4.6 mm, 5μ	0.69	0.94	83.1	101.1	16.9	-1.1
Cosmicsil Aster C18, 250 x 4.6 mm, 5μ	0.75	0.97	90.4	104.3	9.6	-4.3
Cosmicsil Agate C18, 250 x 4.6 mm, 5μ	0.94	1.13	113.3	121.5	-13.3	-21.5
Hypersil BDS C18, 250 x 4.6 mm, 5μ	0.76	0.99	91.6	106.5	8.4	-6.5
Kromasil 100-5 C18, 250 x 4.6 mm, 5μ	0.74	0.94	89.2	101.1	10.8	-1.1
X-Bridge Shield RP-18, 250 x 4.6 mm, 5μ	0.8	0.94	96.4	101.1	3.6	-1.1
YMC Pack ODS AQ, 250 x 4.6mm, 5μ	0.88	1.04	106	111.8	-6.0	-11.8
Atlantis T 3 C18, 250 x 4.6mm, 5μ	0.86	0.94	103.6	101.1	-3.6	-1.1
Develosil ODS MG-5, 250 x 4.6mm, 5μ	0.81	0.98	97.6	105.4	2.4	-5.4
Sunfire C18, 250 x 4.6mm, 5μ	0.75	0.94	90.4	101.1	9.6	-1.1

Table-14: Change in Particle Size

Parameter	RRF Values		% Observed		% Variation (±)	
	Impurity F	Impurity G	Impurity F	Impurity G	Impurity F	Impurity G
Change in Particle Size						
X- Bridge C18, 250 x 4.6 mm, 5μ	0.83	0.93				
X- Bridge C18, 250 x 4.6 mm, 3.5μ	0.85	0.95	102.4	102.2	-2.4	-2.2

Table-15: Change in Flow rate

Parameters	RRF Values		% Observed		% Variation (±)	
	Impurity F	Impurity G	Impurity F	Impurity G	Impurity F	Impurity G
Change in Flow rate						
As per Method (1.0mL/min)	0.83	0.93				
Low Flow (0.8mL/min)	0.86	0.96	103.6	103.2	-3.6	-3.2
High Flow (1.2mL/min)	0.84	0.96	101.2	103.2	-1.2	-3.2

Table-16: Change in Column Temperature

Parameters	RRF Values		% Observed		% Variation (±)	
	Impurity F	Impurity G	Impurity F	Impurity G	Impurity F	Impurity G
Change in Column Temperature						

As per Method (30°C)	0.83	0.93				
Low Temperature (25°C)	0.90	0.99	108.4	106.5	-8.4	-6.5
High Temperature (35°C)	0.79	0.97	95.2	104.3	4.8	-4.3

Table-17: Change in Wave Length

Parameters	RRF Values		% Observed		% Variation (±)	
	Impurity F	Impurity G	Impurity F	Impurity G	Impurity F	Impurity G
Change in Wave Length						
As per Method (230nm)	0.83	0.93				
Low Wave Length (227nm)	0.82	0.94	98.8	101.1	1.2	-1.1
High Wave Length (233nm)	0.83	0.92	100.0	98.9	0.0	1.1

Table-18: Change in pH of Mobile phase

Parameters	RRF Values		% Observed		% Variation (±)	
	Impurity F	Impurity G	Impurity F	Impurity G	Impurity F	Impurity G
Change in pH of Mobile phase						
As per Method (8.0)	0.83	0.93				
Low pH (7.8)	0.84	0.95	101.2	102.2	-1.2	-2.2
High pH (8.2)	0.85	0.96	102.4	103.2	-2.4	-3.2

Table-19: Changes in Detector

Parameters	RRF Values		% Observed		% Variation (±)	
	Impurity F	Impurity G	Impurity F	Impurity G	Impurity F	Impurity G
Changes in Detector						
As per Method (UV 2487)	0.83	0.93				
PDA 2998	0.83	0.92	100	98.9	0.0	1.1

Table-20: Changes in Buffer concentration

Parameters	RRF Values		% Observed		% Variation (±)	
	Impurity F	Impurity G	Impurity F	Impurity G	Impurity F	Impurity G
Changes in Buffer concentration						
As per Method (20mM)	0.83	0.93				
Low Buffer concentration (18mM)	0.84	0.94	101.2	101.1	-1.2	-1.1
High Buffer concentration (22mM)	0.82	0.93	98.8	100.0	1.2	0.0

Table-21: Changes in Solvent Grade

Parameters	RRF Values		% Observed		% Variation (±)	
	Impurity F	Impurity G	Impurity F	Impurity G	Impurity F	Impurity G
Changes in Solvent Grade						
As per Method (Gradient Grade)	0.83	0.93				
HPLC Grade	0.86	0.93	103.6	100.0	-3.6	0.0

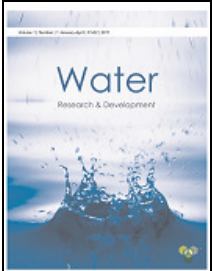
CONCLUSION

Relative Response Factor (RRF) is a common analytical parameter frequently used in many chromatographic procedures. The parameter is critical for quantitative or limit tests for impurities because in many cases the corresponding reference standards are not available. The RRF parameter is sensitive to the experimental parameters and hence the value changes on deviating the chromatographic conditions. While establishing the RRF, one should follow the exact method conditions without deviating from the original.

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