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Method Development and Validation for the Identification of Selected Genotoxic Impurities in Bulk and Pharmaceutical Formulations

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Abstract:

Background: Safety and efficacy are the two most important aspects to evaluate in any drug substances and drug product. Hence it is crucial to maintain a good quality of drugs that are utilized to treat different diseases.

Introduction: This paper describes a single RP-HPLC method for the identification of selected genotoxic impurities (GIs), *p*-Anisidine (ANI) and benzidine (BEN) in each of the drugs i.e. aripiprazole (ARP) and phenylbutazone (PBZ).

Method: All the analysis parameters were carried out using Phenomenex Kinetics (250 mm × 4.6 mm, particle size 5 µm) C_{18} column. Satisfactory peak symmetry was produced, the mobile phase, buffer (potassium dihydrogen phosphate), acetonitrile and methanol (30:50:20 v/v/v) ratio at pH 3.5 at a wavelength of 254 nm.

Results: The method was validated and the parameters like accuracy, precision, linearity, LOD, and LOQ was followed as per ICH guidelines. The retention time of ARP and ANI was found to be 2.455 and 1.925 min and for PBZ and BEN, it was found to be 5.776 and 2.046 min. The method was found to be linear at the concentration range of (2 - 10 µg mL^{-1}) for both drugs (ARP and PBZ) as well as their impurities (1 - 5 µg mL^{-1}).

Conclusion: The method developed for determining the selected impurities in ARP and PBZ is simple, reliable, sensitive, and precise. Satisfactory recovery % and RSD values confirmed the suitability of the method developed to identify related GIs in pharmaceutical products.

Keywords: RP-HPLC, Genotoxic impurities, Aripiprazole, Phenylbutazone, *p*-Anisidine, Benzidine

1.0 INTRODUCTION

The identification of GIs for the safety and also the purity of drug substances and products are emphasized by various regulatory authorities. The purity of a pharmaceutical product and substances is intended to limit the number of impurities, as their presence can affect the drug even in small quantities [1-3]. GIs should be controlled because they pose a cancer risk for human beings, and even low levels of such impurities can be of major toxicological concern in the final active drug ingredient (API) [4-6]. It is therefore very important that GIs are identified in drugs and monitored, to guarantee safety for our community. Based on this approach we have developed a single HPLC method for the identification of various GIs in various drugs and this is carried out to avoid multiplication of the method. If we follow different methods the cost of solvents, manpower for adopting the methods will increase. Therefore, developing one single method for analyzing more than one impurities and drugs can be beneficial for the society and the drug industries. Most of the current approaches used expensive reagents for the study and they also suffer from many drawbacks such as longer run time, usage of an inorganic buffer which can affect the column lifetime drastically after prolong use. Day by day the drug industry is expanding to develop new drugs extracted from natural products or chemical drugs, but the product must be as pure as possible and each year there is a growing number of drugs on the market [7, 8]. Purity was therefore always regarded as an important factor in ensuring drug quality. The available drugs in the market may be new or these drugs can be a structural change of existing entities. With the use of many chemicals (organic or inorganic) during the manufacturing process, some of the unwanted substances are transported into the final bulk product or API as impurities [9, 10].

1.1 Sources of GI's [11-13]

There are many sources from which the impurities can form in the API they mainly fall in the following categories.

- Unreacted chemicals or intermediates used during manufacturing the API.
- Degradants formed during or after the manufacturing process.
- Reactions of certain chemicals and solvents can also lead to the formation of unrelated products.
- Degradants formed due to environmental conditions or during storage.

1.2 ARP [14-17]

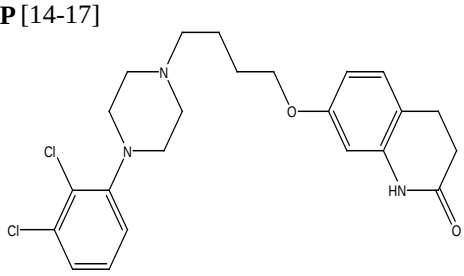

Fig. 1 Structure of ARP

ARP is a drug used to treat schizophrenia and bipolar disorder. The drugs when degraded can yield impurities that are genotoxic. These impurities when present in large amounts can potentially affect the health of the individual during long term exposure. The main compound which can form when ARP gets degraded is ANI. This impurity shared a similar structure relationship with the parent compound. Therefore needs to be identified.

1.3 ANI [18]

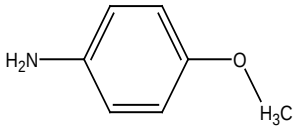

Fig. 2 Structure of ANI

ANI is a known PGI which belongs to class 3 type of genotoxic impurities because it is having a similar structural activity relationship with the drug ARP. Due to which the cyclic ring structure contains an amine group they are highly reactive and poses a health risk by forming an adduct with the DNA bases resulting in mutagenicity and carcinogenicity.

1.4 PBZ [19-21]

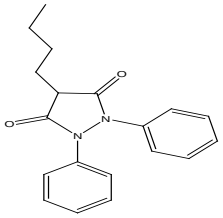

Fig. 3 Structure of PBZ

This drug is having an anti-inflammatory, antipyretic, and analgesic activities. It is known to be effective especially in the treatment of ankylosing spondylitis. Degraded PBZ results in several types of impurities where benzidine is one of the impurities which is known to be a GTI and needs to be identified.

1.5 BEN [22]

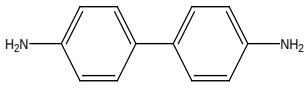

Fig. 4 Structure of BEN

BEN exist as an impurity E in PBZ. BEN belongs to class 3 GTI as it bears a similar structural relationship with the drug PBZ. This impurity is very potent even in small concentration, therefore, needs to be identified if it is present in the selected drug compound.

2.0 EXPERIMENTAL

2.1 Chemicals and Reagents

HPLC grade Acetonitrile was purchased from Merck Specialties Private Limited, Mumbai, HPLC grade Methanol from Finar Limited, Ahmedabad, HPLC grade Water from Loba Chemi Private Limited, Mumbai, Orthophosphoric acid was procured from Yarrow Chem Products, Mumbai, Potassium dihydrogen phosphate buffer from Yarrow Chem Products, Mumbai, Sodium hydroxide (NaOH) from Yarrow Chem Products, Mumbai. The standard API i.e. ARP and PBZ were procured from Sigma Aldrich. The marketed formulation dose of ARP 5 mg and PBZ 100 mg was purchased from a pharmacy store. And the GIs was procured from Sigma Aldrich. All the chemicals were of analytical grade and were used as received.

2.2 Instrumentation

All the analytical studies were performed on the HPLC instrument (Shimadzu, Japan) equipped with an SPD 20A UV-visible detector and LC-20AT pump, manual Rheodyne injector with 20 µL loop, Phenomenex Kinetics C_{18} column (250 mm × 4.6 mm id, 5 µm particle size) and LC solution software.

2.3 Preparation of solutions

2.3.1 Preparation of buffer solution (pH 3.5, 10 mM)

About 0.136 g of potassium dihydrogen phosphate was accurately weighed, transferred to a 100 mL volumetric flask containing HPLC grade water, and was sonicated to dissolve the buffer. It was then adjusted to a pH of 3.5 ± 0.05 units with the help of orthophosphoric acid. The solution was decanted through a 0.45 µ membrane filter and degassed with the help of the sonicator.

2.3.2 Preparation of mobile phase:

A variety of rigorous tests were conducted to optimize the mobile phase. Various solvents such as methanol, water, and acetonitrile in different ratios and various pH levels of the mobile phase ratios using varying buffer solutions to obtain sharp peak and baseline separation of the components and without the intervention of the excipients. Satisfactory peak symmetry was produced, well resolved, and free from tailing in the mobile phase, Buffer (potassium dihydrogen phosphate): Acetonitrile: methanol (30:50:20 v/v/v) at pH 3.5 in isocratic mode.

2.3.3 Standard stock solutions of impurities:

10 mg of each impurities ANI and BEN were weighed and transferred into individual 10 mL volumetric flask. Then 5 mL of diluent was added into each flask. After sonication, volume was made up to the mark with diluents to get a concentration of 1000 µg mL^{-1}. A portion of 1 mL of the standard stock solution of the individual impurities was transferred to a separate volumetric flask of 10 mL and it was suitably diluted to get a concentration of 100 µg mL^{-1}. From the second stock solution, 1 mL solution from each flask was transferred into an individual volumetric flask of 10 mL and it was suitably diluted to get a concentration of 10 µg mL^{-1} for both the impurities. From here further dilution is made as required.

2.3.4 Standard solutions of drugs:

Powder equivalent to about 10 mg of drugs ARP and PBZ was weighed and suitably diluted with the diluent to get a concentration of 1000 µg mL^{-1}. A portion of 1 mL of standard stock solution of ARP and PBZ was transferred into a separate volumetric flask of 10 mL and it was suitably diluted to get a concentration of 100 µg mL^{-1}. From the second stock solution, 1 mL solution was transferred into a volumetric flask of 10 mL and it was suitably diluted to get a concentration of 10 µg mL^{-1} for each drug.

2.3.5 Sample drugs solutions:

10 tablets of each of the drugs ARP and PBZ were weighed separately and the average weight was find out and weight equivalent to about 10 mg of each drug was transferred into two 10 mL volumetric flask, mixed with diluent, sonicated for 10 min and volume was made up to 10 mL with the same solvent. An aliquot of the supernatant solution was diluted to get a concentration of each of 10 µg mL^{-1}.

3.0 METHOD VALIDATION

This procedure is to determine whether the employed method for the test is suitable for the proposed work. Guidelines for the analytical method validation have been addressed by the regulatory bodies such as ICH, FDA, USP and these guidelines are needed to be fulfilled before the method can be applied for the analysis.

3.1 Validation parameters for RP-HPLC [23]

After the development of the method, validation of the method for the identification of GIs in ARP and PBZ was performed following ICH parameters. The developed HPLC method has to be validated by various parameters to ensure that the performance characteristic of the method meets the requirements for the intended analytical application.

3.1.1 System suitability:

For system suitability, a known volume of the sample containing a known amount of ARP, PBZ along with their impurities were injected separately into the column and the factors such as resolution, number of theoretical plates, % RSD was calculated for three replicates each and the observed data is used for checking system suitability of the developed method.

3.1.2 Specificity:

Specificity was conducted to evaluate and to ensure that the samples analyzed are not affected by the impurities and diluents. 20 µL of diluent and sample solution of ARP, PBZ (10 µg mL^{-1}), and their related impurities ANI and BEN (5 µg mL^{-1}) were injected into HPLC system and the chromatograms are recorded. The interference of retention time was checked.

3.1.3 Linearity:

The linearity of the analytical procedure is its capacity to produce test outcomes that are directly proportional to the analyte concentration in the sample. Linearity study was performed for both drugs ARP and PBZ (2-10 µg mL^{-1}) along with their related impurities (1-5 µg mL^{-1}). Linearity was checked at 5 different concentrations of standard solutions namely 20%, 40%, 60%, 80%, and 100%. Each level was determined 3 times and the average peak area and % RSD was calculated for each level. A graph concentration vs peak area was then plotted and the regression coefficient (r^2) was determined.

3.1.4 Accuracy:

The accuracy was carried out by spiking the drugs solution with the impurities at different levels. The accuracy of the method was determined by calculating the % recovery at each level. Further, the average % recovery was calculated for each sample.

3.1.5 Precision:

This was carried out to determine repeatability by injecting 3 concentration range (2, 6, 10 µg mL^{-1}) of ARP, PHY, and their related impurities (1, 3, 5 µg mL^{-1}) for 3 times into the HPLC system. % RSD and peak area for the 3 injections were then calculated.

3.1.6 Limit of detection (LOD):

LOD is the minimum amount of the analyte that can be possibly detected, but not necessarily quantified as an exact value, under the optimized experimental conditions.

The LOD is calculated by applying the formula,

$LOD = 3.3\times \sigma/S$

Where, σ = standard deviation, S = slope

3.1.7 Limit of quantification:

The quantification limit of an analytical technique is the lowest quantity of analyte in a sample that can be detected with sufficient accuracy and precision.

LOD is determined by applying the formula,

$LOQ = 10\times \sigma/S$

Where, σ = standard deviation, S = slope

3.1.8 Robustness:

The robustness of the analytical method is carried out by small alteration of method parameters such as flow rate changes, mobile phase ratio, and wavelength to a level of ±2%. The robustness studies were performed for the solution containing ARP (10 µg mL^{-1}) with ANI (5 µg mL^{-1}), PHY (10 µg mL^{-1}) with BEN (5 µg mL^{-1}).

4.0 RESULTS AND DISCUSSIONS

4.1 Method development

The aim was to develop an RP-HPLC method for the identification of the selected GIs both in ARP and PBZ. As per the research, the impurities in the drugs either have similar structural alerts or exist as starting material and process impurities. The HPLC method was developed by using reversed-phase Phenomenex kinetics (ODS) C_{18} column (250 × 4.6 mm, 5µm) with a mobile phase consisting of Buffer (potassium dihydrogen phosphate): acetonitrile: methanol (30:50:20 v/v/v) in isocratic mode. The flow rate was kept at 0.8 mL/min and UV detection at 254 nm. The HPLC method was validated by using the following parameters as per the ICH guidelines.

4.2 Final optimized method

The method was carried out in isocratic mode and the parameters for the optimized method was shown in table 1.

Table 1. Final Optimized method

Parameters	Specifications
Column	Phenomenex kinetics (ODS) C18 (250 × 4.6 mm, 5µm)
Mobile phase	Buffer: Acetnitrile: Methanol (30:50:20) pH 3.5
Inject volume	20 µL
Flow rate	0.8 mL min^{-1}
Wavelength	254 nm
Detector	UV - Detector

4.3 Method Validation

4.3.1 System suitability

Where the theoretical plates were determined by statistical analysis to check the method suitability concerning the applied system. The system suitability test was found within the acceptance criteria for both drugs and their impurities. System suitability parameters for HPLC**,** where the theoretical plates were determined by statistical analysis to check the method suitability for the applied system. The system suitability test was found within the acceptance criteria for both drugs and their impurities. The result is summarized in Table No. 2 & 3.

Table 2. System suitability test for ARP, ANI and Internal standard

Parameters	ARP	ANI	Internal standard
Retention time (min)	2.455	1.925	4.628
Tailing factor	1.393	1.387	1.386
Theoretical plates	16878.437	13802.623	26674.896
Resolution	2.911	-	8.909

Table 3. System suitability test for PBZ, BEN and internal standard

Parameters	PBZ	BEN	Internal standard
Retention time (min)	5.776	2.046	4.616
Tailing factor	1.487	1.487	1.414
Theoretical plates	32454.198	14569.897	27195.397
Resolution	3.740	-	11.072

4.3.2 Specificity

This parameter was performed to assess and ensure that the drugs, impurities, internal standard, and diluents do not have any interference between them**.** There was no visible peak in chromatogram with diluent and excipients used in capsule formulation, indicating a high degree of specificity for the proposed method. The specificity of the method was determined and there was no visible peak in chromatogram with diluent and excipients used in tablet formulation, indicating a high degree of specificity for the proposed method. The result is summarized in fig. 6 and 7.

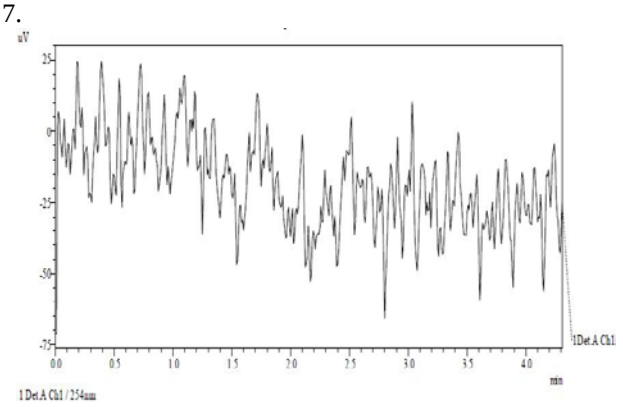

Fig. 5 Chromatogram of diluents

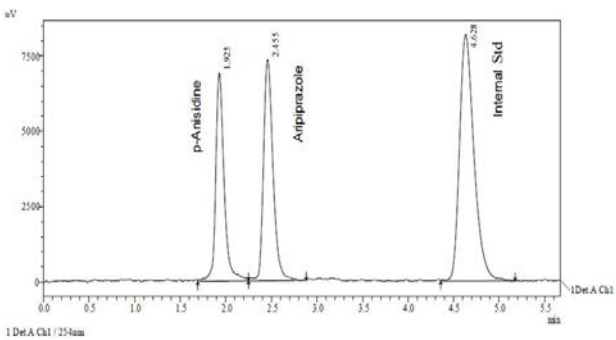

Fig. 6 Chromatogram for specificity test of ARP and ANI

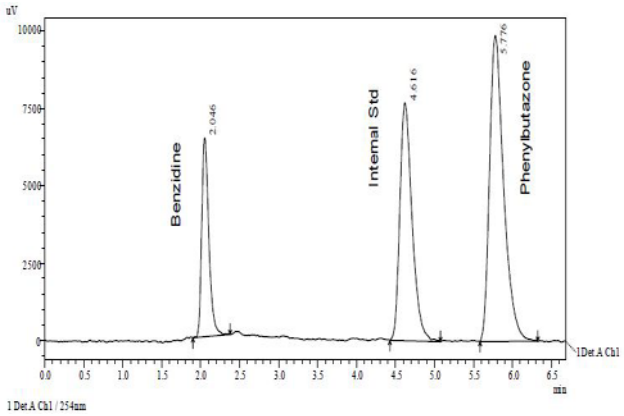

Fig. 7 Chromatogram for specificity test of PBZ and BEN

4.3.3 Linearity

The linearity studies were performed to ensure that the test results are directly proportional to the concentration of the analyte. 20 µL of each standard solution of ARP (2 to 10 µg mL^{-1}) and PBZ (2 - 10 µg mL^{-1}) along with their impurities was injected into the HPLC system. The peak area v/s concentration was plotted to get a standard calibration curve. Linearity for ARP and PBZ as well as their impurities were analyzed at 2 - 10 µg mL^{-1} concentration. The r^2 value for ARP and ANI was found to be 0.9987 and 0.9996 and for PBZ and BEN, it was found to be 0.9987 and 0.9991 respectively, which is well within the acceptable limits. The result obtained was shown in Table.4, 5, 6, 7, and Fig no 8, 9, 10, and 11.

Table 4. Data for the linearity of ARP

Conc (µg mL^{-1})	Mean ± SD
2	160654 ± 113598.1
4	326154 ± 230622.9
6	472041 ± 333779.1
8	627920 ± 444000.8
10	758320 ± 536206.1

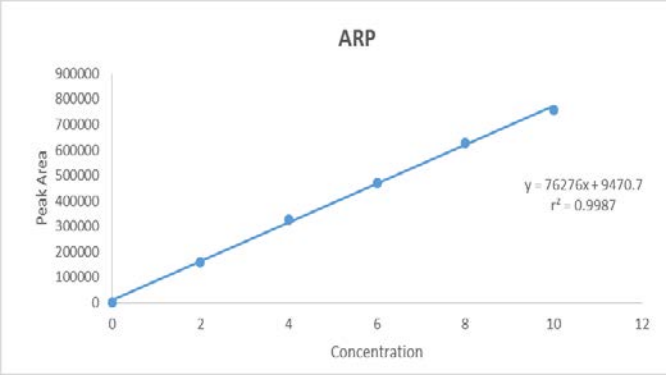

Fig. 8 Calibration graph of ARP

Table 5. Data for the linearity of ANI (GIs)

Conc (µg mL^{-1})	Mean ± SD
1	153307 ± 108403
2	309571 ± 218896.9
3	456471 ± 322769.5
4	600591 ± 424676.3
5	742337 ± 524904.5

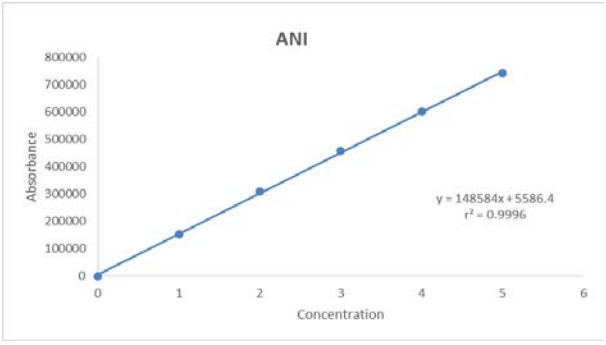

Fig. 9 Calibration graph of *p*-Anisidine

Table 6. Data for the linearity of PBZ

Conc (µg mL^{-1})	Mean ± SD
2	164339 ± 116203.8
4	347391 ± 245639.7
6	502261 ± 3555147.9
8	678102 ± 479484.9
10	814257 ± 575759.6

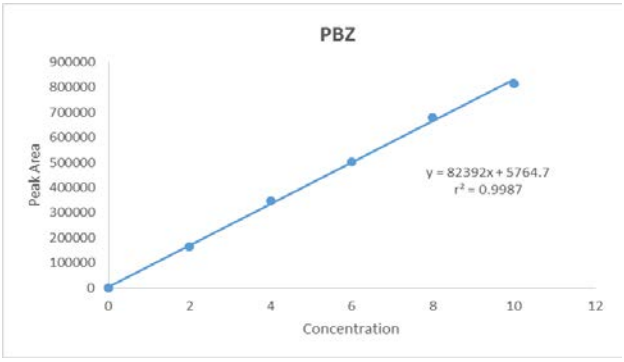

Fig. 10 Calibration graph of PBZ

Table 7. Data for the linearity of BEN (GIs)

Conc (µg mL^{-1})	Mean ± SD
1	152109 ± 107555.9
2	324177 ± 229224.9
3	487723 ± 344868
4	621252 ± 439285.8
5	789102 ± 557972.3

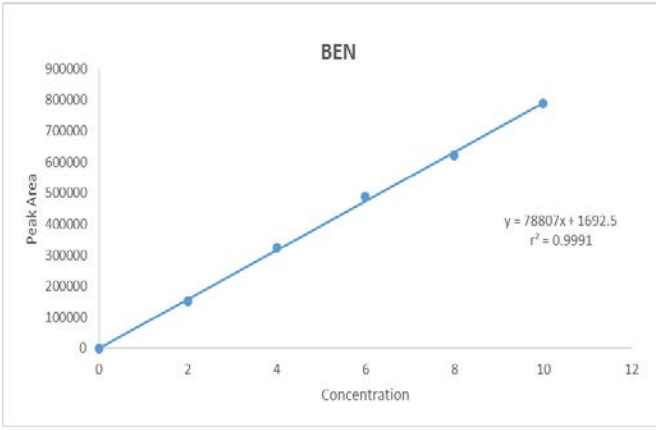

Fig. 11 Calibration graph of BEN

4.3.4 Accuracy

Accuracy studies were performed to determine the degree of closeness of test results with that of the true value which is expressed as % recovery. The percentage recovery at each level should be between 98 - 102 %. The accuracy was determined through the percentage recovery and is between 98 – 102 % which were found to be well within the acceptance limit, indicating practically no product interaction with one another or the excipients found in the formulation. Results for the accuracy studies were presented in Table. 8, 9, 10, and 11.

Table 8. Data of accuracy study for ARP

Spik	Amoun	Amount	Mean ±	Total	%
80 %	10	8	133441	18.3	101.8
100	10	10	150850.	19.8	99.1
120	10	12	212258.	22.3	101.5

Table 9. Data of accuracy study for ANI

Spik	Amount	Spiked	Mean ±	Total	%
80 %	5	3	103166	18.2	101.1
100	5	5	178827.	19.6	98
120	5	7	300950	22.2	100

Table 10. Data accuracy study for PBZ

Spike level	Amount of drug sample taken (µg)	Amount of standard drug taken (µg)	Mean ± SD	Total conc. found (µg)	% Recovery
80 %	10	8	169408.3 ± 1772.024	17.7	98.4
100 %	10	10	191592.1 ± 2993.473	20.5	102.7
120%	10	12	196358.7 ± 2949.524	21.7	98.7

Table 11. Data of accuracy study for BEN

Spike level	Amount of impurity taken (µg)	Spiked Amount (µg)	Mean ± SD	Total conc. found (µg)	% Recovery
80 %	5	3	151909.2 ± 2682.274	17.9	99.4
100 %	5	5	163929.2 ± 3795.263	20.1	100.9
120 %	5	7	172952.3 ± 3097.658	21.9	99.5

4.3.5 Precision

Precision was performed both by intraday precision and interday precision. The % RSD value of peak areas for repeated injections of ARP and PBZ along with their related impurities was found to be < 2 %, which specify that the suggested method was precise.

4.3.6 Limit of detection (LOD) and limit of quantification (LOQ)

The LOD values for ARP, ANI, PBZ, and BEN was recorded at 0.4, 0.2, 0.5 and 0.4 µg mL^{-1} and similarly limit of quantification values for ARP, ANI, PBZ, and BEN were found to be 1.4, 0.8, 1.5 and 1.2 µg mL^{-1} respectively, which indicates that the concentration in small level is precise and shows acceptable accuracy.

4.3.7 Robustness

The next validation parameters for the HPLC method is Robustness which was determined by altering the ratio of the mobile phase, the flow rate, and the wavelength slightly in the HPLC method. There was a small change in retention time found for both the drug and their related impurities and falls within the acceptance criteria.

4.3.8 Application of RP-HPLC method for assay of ARP and PBZ in marketed formulation.

Regarding the percentage purity for ARP and PBZ, it was found to be within acceptance criteria 98 – 102 %. Hence, the method can be employed for the identification of the selected GIs in ARP and PBZ formulations

4.4 Discussion

Safety and efficacy are the two most important aspects to evaluate in any drug substances and drug product. Hence it is crucial to maintain a good quality of drugs that are utilized to treat different diseases. At the same time, it requires the need for the development of newer, efficient, and robust methods for the estimation of impurities present in various drugs to improve the product quality and manufacturing efficiency of the drug product. Therefore, an attempt has been carried out to identify and quantify the impurities, degradation, and analysis of the drug by the HPLC method. The Development of an analytical method for the impurity identification and Quantification by chromatography has become mandatory for various country regulatory bodies because of accuracy in reproducing the results and also plays an important role to control the qualities of drugs. The aim was to develop an RP-HPLC method for the identification of the selected genotoxic impurities both in ARP and PBZ. As per the research, the impurities in the drugs either have similar structural alerts or exist as starting material and process impurities. The proposed method was validated according to the ICH Guidelines.

The HPLC method was developed by using reversed-phase Phenomenex kinetics (ODS) C_{18} (250 × 4.6 mm, 5µm) analytical column with a mobile phase consisting of Buffer (potassium dihydrogen phosphate): acetonitrile: methanol (30:50:20 v/v/v) in isocratic mode. The flow rate was kept at 0.8 mL min^{-1} and UV detection at 254 nm. The HPLC method was validated by using the following parameters as per the ICH guidelines.

System suitability parameters for HPLC**,** where the theoretical plates were determined by statistical analysis to check the method suitability concerning the applied system. The system suitability test was found within the acceptance criteria for both drugs and their impurities. The specificity of the method was determined and there was no visible peak in chromatogram with diluent and excipients used in tablet formulation, indicating a high degree of specificity for the proposed method. Linearity for ARP and PBZ as well as their impurities were analyzed at 2 - 10 µg mL-1 concentration. The r^2 value for ARP and ANI was found to be 0.9987 and 0.9996 and for PBZ and BEN, it was found to be 0.9987 and 0.9991 respectively, which is well within the acceptable limits. The accuracy was determined through the percentage recovery and is between 98 – 102 % which were found to be well within the acceptance limit, indicating practically no product interaction with one another or the excipients found in the formulation. Precision was performed both by intraday precision and interday precision. The % RSD value of peak areas for repeated injections of ARP and PBZ along with their related impurities was found to be < 2 %, which specify that the suggested method was precise.

The LOD values for ARP, ANI, PBZ, and BEN was recorded at 0.4, 0.2, 0.5 and 0.4 µg mL^{-1} and similarly limit of quantification values for ARP, ANI, PBZ, and BEN were found to be 1.4, 0.8, 1.5 and 1.2 µg mL^{-1} respectively, which indicates that the concentration in small level is precise and shows acceptable accuracy. The next validation parameters for the HPLC method is Robustness which was determined by altering the ratio of the mobile phase, the flow rate, and the wavelength slightly in the HPLC method. There was a small change in retention time found for both the drug and their related impurities and falls within the acceptance criteria.

Regarding the percentage purity for ARP and PBZ, it was found to be within acceptance criteria 98 – 102 %. Hence, the method can be employed for the identification of the selected GIs in ARP and PBZ formulations.

5.0 CONCLUSION

The selected genotoxic impurities are not found in the formulations after carrying out the assay. However, the single method developed for both the drugs can be used for routine analysis since the linearity found in both the APIs and its related impurities was less than 1 which shows the good linearity regression. This established method achieves maximum recovery and the average recovery percentage for each compound was close to 100 %. This technique can also be used for repetitive analysis. Most of the current approaches used expensive reagents for the study and they also suffer from many drawbacks such as longer run time, usage of an inorganic buffer which can affect the column lifetime drastically after prolong use. The method developed for determining the selected impurities in ARP and PBZ is simple, reliable, sensitive, and precise. Satisfactory recovery % and RSD values confirmed the suitability of the method developed to identify related genotoxic impurities in pharmaceutical products.

The validation for its acceptable performance was carried out using the developed analytical method to confirm the suitability for the indented purpose. Validation parameters such as system suitability, specificity, linearity, accuracy, precision, limit of detection, quantification limit, and robustness have been conducted, and established method requirements have been assessed to fulfill the criteria for both ARP and PBZ analysis and their impurities. The findings of solution stability tests showed that sample solutions for both assays and associated material analysis were stable up to 24 h. This developed method achieves full recovery and the mean percentage recovery for each portion was nearly 100 percent. The experiment data for assay shows < 2 per % RSD (relative standard deviation) for drugs and impurities. In all the deliberately varied experimental parameters such as flow rate (±0.2 mL min^{-1}), organic solvent composition (±2 percent of organic solvent method), and mobile phase buffer pH (±0.2), all analytes and impurities were properly resolved and elution orders remained constant. These findings correspond well to the method's precision. This approach should also be used for repetitive analysis and one of the key reasons is that the method developed does not require the use of costly reagents. Even, as compared with other approaches, our suggested approach takes less time to assess both the drugs and their impurities at once. The process developed for evaluating the associated genotoxic impurities in ARP and PBZ is uncomplicated, reliable, sensitive, and precise.

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