Development of validation of a rapid and simple analytical separation method for anticancer alkylating agents using application of total error concept

Islam SOFIQU1*, Vedigounder MURUGAN1*

1 Department of Pharmaceutical Chemistry, College of Pharmaceutical Sciences, Karnataka, Bengaluru-560078, India.
* Corresponding Author. E-mail: Sofi59964@gmail.com; Tel. +00-805-098-47-39.

ABSTRACT: A simple and sensitive high-performance liquid chromatographic (HPLC) method is established to separate and quantify the related substance present in two alkylating agents. The use of the traditional approach of analytical validation, in practice or in the literature, is communal. However, statistical verification, that looks separately the two errors (such as absolute bias and repeatability) to make a decision, presents a risk to declare that an analytical method is valid while it is not, or equally. To minimize this peril, a new approach based on the concept of total error was proposed. In this paper, we reveal the applicability and simplicity of the both methods based on the total error approach: accuracy profile and uncertainty profile. Proposed study demonstrated by validation case of a liquid chromatographic LC method for the quantification of related compounds present in two alkylating agents. Both methods showed good linearity response (> 0.995) with repeatability (%relative standard deviation less than 2%) and accuracy (94 to 106%). Accuracy profile was found within the range of ±10% and risk profile ±5% between the two series respectively. The excellence of the total error approach was presented since it enables successfully to validate the analytical procedure as well the calculation of the measurement uncertainty at each concentration level.

KEYWORDS: Related substances; Quantification; Total error; HPLC; Validation

1. INTRODUCTION

Cancer accounts for millions of mortality globally and is the second most leading cause of fatality according to world regulatory agencies. Antineoplastic alkylating agents offer a great therapeutic interest for treatment of cancer patients. Ifosfamide and cyclophosphamide are the two molecules which show promising medical importance, hence both are known to be commonly used antineoplastic agents [1,2].

Related substances and related compounds are structurally similar substance, which can be acquired through manufacturing, storage condition form or during handling. Presence of these substances will always have an impact on quality, safety and efficacy of the product. Therefore, it is necessary to have an accurate chromatographic method to integrated these known substances in such kind of material. This helps to identify the risk associated with the toxicity of any drug substances [3].

Review of scientific literature suggested the presence of three potential related substances named Ifosfamide Imp A, ifosfamide Imp B and ifosfamide Imp D in ifosfamide and related compounds named related compound A, related compound B and related compound D in cyclophosphamide. These are few listed substances that are detected through thin layer chromatography method and currently there is no simple procedure involving HPLC, to detect and quantify the presence of this compounds. Recent studies showed the usages of complicated analytical methods like quadrupole time of flight mass spectroscopy [4,5], HPLC with electrospray ionization technique, tandem mass spectroscopy to identify those analytes [6,7]. However, these methods were found to be difficult for use in terms of reproducibility, separation and robustness. To counter all these challenges faced by conventional detector an attempt has been made to develop a method using ultraviolet or evaporative light scattering detector to quantify those compounds along with the main analyte. HPLC with UV detector is a general all purpose modern application for measuring the absorption of light at different wavelength to identify the analyte.
HPLC method are capable of reporting precise and accurate results as compared to thin layer
chromatography (TLC), since TLC technique based on the visual comparison of spots intensity matching which can be less quantitative in practice [8]. HPLC with UV detector provide good sensitivity for light absorbing compounds and they are easy to operate as compared to other detectors like refractive index detector, fluorescence detector or electron capture detector. Most of the alkylating agents are hydrophilic compounds because they contain amine group. Due to their highly polar nature, the use of simple chromatographic technique is not quite easy. The lack of chromophore group in their structure made it difficult to detect it in the UV-visible region [9,10]. LC technique with ELSD detector provides good sensitivity with stable baseline for non volatile analytes. HPLC/UV or HPLC/ELSD technique appears to be feasible for attaining the maximum sensitivity [11]. A review of the physical characteristics of each antineoplastic agents along with the related compounds of interest was conducted to review the existing analytical methods [12]. The aim of this work was to develop an single run LC method capable of quantifying these substances.

Analytical method validation is considered as a critical measurement especially for the quantitativa
tive methods in vision of monitoring aspect. Therefore, usages of statistical tool like total error concept provides an additional tool for assessing the performance of developed methods. In this study, e-noval web-based software was used to perform the statistical calculations. Validation solutions were prepared in triplicate and performed in two different series to show the applicability of total error approach [13]. Accuracy and uncertainty profiles are used as powerful tools to assess the performance of the proposed method [14]. The utilization of these statistical tools helps to assess the results of the alternative methods successfully to determine the reliability and feasibility of these methods.

The objective of this paper is to adapt the described HPLC/UV method for cyclophosphamide and HPLC/ELSD method for ifosfamide in order to separate and quantify the related compounds from the main analyte. Then validate the same method to provide assurance about the accuracy and reliability of the results. Prominent advantage of the proposed method is simplicity, rapid and easy solution preparation procedure, with high level of sensitivity and applicability for wider use with easily accessible instruments.

2. RESULTS

2.1. Chromatographic method optimization of ifosfamide

In the present work, the optimized chromatographic conditions were achieved after various trials. The primary objective of this study was to develop a simple LC method using UV detector. Different mobile phase compositions have been evaluated in various trials to achieve the optimized chromatographic condition, but no peaks were eluted in any of the chromatographic condition due to the lack of chromophore group in the structure of related substances of ifosfamide. Hence ELSD provides an auxiliary detector option for detection, in characterizing the molecules that lacks an active chromophore. Combination of trifluoroacetic acid and methanol is found to be suitable mobile phase during this analysis since it is volatile in nature and greatly increase in the detector response.

Presence of high purity C18 stationary phase in agilent ZORBAX columns are useful for the separation of basic and highly polar compounds by reverse phase chromatography. Mobile phase combination of trifluoroacetic acid and appropriate concentration of methanol helps to achieve symmetrical peaks. The flow rate of nebulizer gas and the temperature of the drift tube plays major role during method development. The droplets are transported from the atomization chamber to the drift tube by a carrier gas for evaporation. The flow rate of the carrier gas affect the formation of droplets in the atomizer while the temperature in the drift tube determine whether the evaporation of the mobile phase was complete. Flow rate of the nebulizer gas (0.5, 0.7, 0.9, 1.0 ml/min) and temperature of the drift tube (50, 55, 60°C) were studied. The temperature of the nebulization was set at 55°C and flow rate of inert gas (nitrogen) was at 25 psi in optimized condition. LC technique involving isocratic elution and ELSD detection is found to be an appropriate technique for the quantification of related substances in ifosfamide. All peaks are well separated in impurity mixture solution as shown in Figure 1. Also, no interference peaks observed at the retention time of ifosfamide and its related substances.
2.2. Chromatographic method optimization of cyclophosphamide

The primary target is to develop a suitable single run LC method for separation of each related compounds of cyclophosphamide. Related compound mixture solutions were run in acidic buffer (0.02M sodium acetate buffer, pH 4.5) and in neutral buffer (0.02 M potassium dihydrogen phosphate buffer, pH 7.0) to check the chromatographic elution of the analytes. It was observed that none of the analytes were eluted in acidic buffer. Thus, solvent mixture comprising of basic media was used to achieve the separation of each compound. Mobile phase-A comprises of 0.02M potassium dihydrogen phosphate buffer, pH 7.0 along with mobile phase-B, acetonitrile: water proportions of 60:40% was used as optimized combination to separate each analyte. It was observed that the latter composition gave well resolved peaks due to its increase in polarity.

A longer gradient elution run time is used in Atlantis T3 column interaction due to the complex nature of the molecule. Better separation of each analytes from the complex mixtures was achieved with the combination of buffer and organic constituents.

Mobile phase flow rate 0.8 ml/min provided satisfactory resolution between the related compound and the compound of interest. Column temperature was maintained at 40°C, which was inferred to be the optimum temperature for intended analytical purpose. Injection volume was set as 50 µL to run the analysis. The wavelength range for the cyclophosphamide molecule exist in between 195 to 200 nm. Because of the limited absorption of UV radiation provided by cyclophosphamide and its related compounds, it was decided to use 195 nm as the suitable wavelength for quantification.

Atlantis HILIC and Atlantis T3 column were identified as the suitable analytical column for the analysis. Atlantis HILIC column are useful for the polar compound separation and works on the principle of liquid-liquid partition (where stationary phase has water adsorbed capacity on its surface and interacts with the mobile phase which in this case is buffer(water)), thus not letting the polar analytes to interact with the column for a long time. Atlantis T3 showed better separation than Atlantis HILIC as the peaks were well resolved, since Atlantis T3 column is coated with silica-based C18 and found superior as they retain and separate water-soluble polar organic compounds, but also provide higher performance across a wide range of pH.

Figure 2 and 3 reveals the chromatogram obtained using Atlantis HILIC column and Atlantis T3 column for related compound mixture solution.

An original approach using accuracy profile based on tolerance interval for the error measurement, including bias and standard deviation for intermediate precision was applied to demonstrate the method trueness, precision, accuracy and linearity. Proposed method is stated to be suitable for use within the range for which the accuracy profile computed by means of β-expectation tolerance interval including accuracy acceptance limit set at ±10%.

![Figure 2](http://dx.doi.org/10.29228/jrp.140)

J Res Pharm 2022; 26(2): 431-443
2.3. System suitability test

According to ICH guideline, system suitability is an integral part of analytical procedure in the course of optimizing the condition of the proposed method. It is injected to check the correct performance of the system. System suitability was tested by injecting six replicates of system suitability solution. The %relative standard deviation of peak area, tailing factor and theoretical plate count were determined for each peak. Capacity factor is calculated to the peak of interest located with respect to the void volume. Individual standard solution injections of related substance were injected to check the retention time (Rt). System suitability results provides assurance that the system performance is appropriate for use and results are presented in Table 1.

| Parameter                        | Limit of acceptance | Ifosfamide | Measured results |
|----------------------------------|---------------------|------------|------------------|
|                                  |                     |            | Ifosfamide   | Imp A | Imp B | Imp D |
| Theoretical plates (N)           | ≥ 2000              | 17456      | 10785          | 9874  | 10032 |
| Tailing Factor (T)               | ≤ 1.5               | 1.1        | 1.1            | 1.1   | 1.2   |
| %RSD                             | ≤ 5.0%              | 1.9        | 2.1            | 1.7   | 1.8   |
| Resolution between               |                     |            |                |       |       |
| Ifosfamide and Imp D peak        | NLT 2.0             | 3.5        | Not Applicable |
| Capacity factor (k)              | NLT 2.0             |            | 4.7            |
|                                  |                     |            |                |       |       |
|                                  |                     |            |                |       |       |
|                                  |                     |            |                |       |       |
|                                  |                     |            |                |       |       |
|                                  |                     |            |                |       |       |
|                                  |                     |            |                |       |       |

Table 1. HPLC system suitability test results.

Note: n=6, RSD: relative standard deviation
2.4. Validation method

LC technique is considered as the most reliable method for detecting and quantifying the presence of related substances compared to TLC method. Under the optimized chromatographic condition, a legalization study was carried out to evaluate the performance characteristics of the proposed method.

The method was successfully assessed under optimized conditions using the e-Noval statistical tool with acceptance criteria of 95% confidence interval (5% risk) with an accuracy profile of ±10% for validation study [15,16]. This provided a greater confidence to the performance of proposed methods [17].

2.4.1. Specificity

Specificity is the ability of a method to measure the degree of interference from the other substances. To prove the specificity or selectivity of the method, an injection of diluent along with system suitability solution and each individual standard solutions were injected. No significant interference peak was observed at the retention time of components due to diluent for each analyte and all peaks were well separated from each other.

2.4.2. Linearity

Linearity of the analytical procedure was evaluated in the concentration range of 1.25 to 3.75 ppm for related substances of Ifosfamide and 12.5 to 150 ppm for related compounds of cyclophosphamide. In order to demonstrate linearity results for each analyte, regression line was fitted using theoretical and experimental concentration of each analyte. The correlation coefficient of each analyte is plotted in Table 2. Correlation coefficient value more than 0.995 is considered as the evidence of an acceptable value for the data obtained from regression line. The data of the linearity test involved is attached to Appendix A of supplementary data.

Table 2. Results of linearity.

| Related substance | Intercept | Slope | Standard error | Correlation coefficient | Residual sum of square | Linear range       |
|-------------------|-----------|-------|----------------|-------------------------|------------------------|--------------------|
| A                 | -0.01825  | 0.9759| 0.0218         | 0.9995                  | 0.02175                | 1.265 to 3.785 ppm |
| B                 | 0.005788  | 0.9711| 0.0223         | 0.9996                  | 0.02007                | 1.266 to 3.814 ppm |
| D                 | -0.006081 | 0.9713| 0.0249         | 0.9994                  | 0.02500                | 1.262 to 3.643 ppm |

| Related substance | Intercept | Slope | Standard error | Correlation coefficient | Residual sum of square | Linear range       |
|-------------------|-----------|-------|----------------|-------------------------|------------------------|--------------------|
| A                 | -0.3159   | 1.050 | 0.4077         | 0.9999                  | 17.30                  | 12.51 to 150.1 ppm |
| B                 | 0.3005    | 0.9774| 0.1815         | 1.000                   | 1.788                  | 12.53 to 150.2 ppm |
| D                 | -0.3577   | 0.9780| 0.0955         | 1.000                   | 0.8467                 | 12.51 to 150.1 ppm |

2.4.3. Precision test

Method repeatability was determined using the six replicates injection at 100% of the test concentration level. The total precision of the method was expressed as the relative standard deviation. Precision and Intermediate precision results are presented in Table 3 and 4. Percentage relative standard deviation value of two different day analysis was less than 5% which is considered as acceptable value.
Table 3. Results of Precision and QL (Ifosfamide).

| Concentration level | Precision (%RSD) | Intermediate precision (%RSD) |
|---------------------|------------------|-------------------------------|
|                     | A                | B                             | D                | A                | B                  | D                  |
| 1.0                 | 0.1754           | 0.2484                        | 0.1881           | 0.1764           | 0.2484             | 0.1881             |
| 2.0                 | 0.1204           | 0.1047                        | 0.07819          | 0.1200           | 0.1297             | 0.2094             |
| 3.0                 | 0.06203          | 0.09657                       | 0.1267           | 0.06508          | 0.1122             | 0.2422             |
| 4.0                 | 0.1483           | 0.05015                       | 0.09439          | 0.1671           | 0.05015            | 0.1744             |
| 5.0                 | 0.1552           | 0.04239                       | 0.08568          | 0.1552           | 0.04239            | 0.1080             |

| Related substances (Ifosfamide) | Concentration (ppm) | Signal to Noise (S/N) |
|--------------------------------|---------------------|----------------------|
| A                             | 1.265               | 142                  |
| B                             | 1.266               | 97                   |
| D                             | 1.262               | 165                  |

Table 4. Results of Precision and QL (Cyclophosphamide).

| Concentration level | Precision (%RSD) | Intermediate precision (%RSD) |
|---------------------|------------------|-------------------------------|
|                     | A                | B                             | D                | A                | B                  | D                  |
| 1.0                 | 0.06662          | 0.1209                        | 0.01813          | 0.07789          | 0.1209             | 0.01813             |
| 2.0                 | 0.1407           | 0.01823                       | 0.03873          | 0.1407           | 0.06768            | 0.03873             |
| 3.0                 | 0.09738          | 0.02828                       | 0.02416          | 0.09738          | 0.2551             | 0.09876             |
| 4.0                 | 0.04061          | 0.01177                       | 0.06976          | 0.04061          | 0.2001             | 0.06976             |
| 5.0                 | 0.1454           | 0.09716                       | 0.05967          | 0.1454           | 0.09716            | 0.05967             |

| Related compound (Cyclophosphamide) | Concentration (ppm) | Signal to Noise (S/N) |
|-------------------------------------|---------------------|----------------------|
| A                                   | 12.51               | 80                   |
| B                                   | 12.51               | 16                   |
| D                                   | 12.51               | 39                   |

2.4.4. The limit of detection and quantification

Direct LC analysis of cyclophosphamide and ifosfamide was carried out successfully with the proposed method. LC method appears to be most feasible for attaining the lower limit of detection and quantification. The LOQ for each related substances were estimated at a signal-to-noise ratio of 10:1 by injecting serial dilutions with known concentration solution. LOD was determined based on the standard deviation (SD) of the response and slope (S) of the regression line as per the ICH guideline according to the formula LOD=3.3 X SD/S.

The final concentration of each analyte along with the signal to noise ratio are presented in Table 3 and Table 4. All the peaks have still existed in this lowest concentration, however, the noises could influence in the measurement.

2.4.5. Accuracy

Accuracy refer to the closeness of agreement between the test results and the accepted reference value. Accuracy of the proposed method was evaluated by calculating the percentage recovery of each analyte. To calculate the accuracy, each analyte was spiked to the test solution at various concentration level. Stock test solution was prepared by using one gram powder for injection reconstituted with 50 mL water for injection. Then transferred 1.25 mL of stock solution into 5 mL and made up the volume up to the mark with Milli Q water. Related compounds were spiked to test solution at 0.25%, 0.50%, 1.0%, 1.25% and 1.5% impurity level. Each solutions were tested three times and calculated the average recovery at each level. The recovery of each analyte was found within 90-110% respectively. This proves that the method is capable to quantify each analyte accurately. Summary of accuracy results are presented in Table 5. Risk was measured at each series for average of three replicate analysis performed at level of two series data. Results are presented in Table 5.
for both the compounds. Predictive interval of risk level at each level was found to be within tolerance limit of ±5%. Trueness results gives information about the systematic error. Table 5 result reveals the trueness level in absolute bias and relative bias form in different concentration level. The maximum bias was less than ±10% for each compound.

The chromatographic data of the accuracy test involved is attached to Appendix A of Supplementary 1.

**Table 5.** Accuracy results data.

| Mean Concentration (ppm) | Absolute bias | Relative bias | Recovery (%) | Relative Beta-expectation tolerance limits (%) | Risk (%) |
|--------------------------|---------------|---------------|--------------|-----------------------------------------------|----------|
| Ifosfamide RS A          |               |               |              |                                               |          |
| 1.265                    | -0.07423      | -5.868        | 94.13        | [-6.374, -5.361]                              | 0.02970  |
| 1.898                    | -0.05751      | -3.031        | 96.97        | [-3.375, -2.686]                              | 0.00006417 |
| 2.530                    | -0.05180      | -2.047        | 97.95        | [-2.244, -1.850]                              | 0.0000831 |
| 3.163                    | -0.06647      | -2.102        | 97.90        | [-2.656, -1.547]                              | 0.001966 |
| 3.785                    | -0.1460       | -3.858        | 96.14        | [-4.304, -3.412]                              | 0.0004999 |
| Ifosfamide RS B          |               |               |              |                                               |          |
| 1.266                    | -0.02444      | -1.930        | 98.07        | [-2.643, -1.216]                              | 0.0006652 |
| 1.899                    | -0.04075      | -2.146        | 97.85        | [-2.640, -1.651]                              | 0.005689 |
| 2.537                    | -0.06971      | -2.747        | 97.25        | [-3.136, -2.359]                              | 0.001443 |
| 3.176                    | -0.1317       | -4.146        | 95.85        | [-4.290, -4.002]                              | 0.00000455 |
| 3.814                    | -0.07096      | -1.861        | 98.14        | [-1.982, -1.739]                              | 0.0000023 |
| Ifosfamide RS D          |               |               |              |                                               |          |
| 1.262                    | -0.05396      | -4.274        | 95.73        | [-4.814, -3.734]                              | 0.002009 |
| 1.894                    | -0.05955      | -3.145        | 96.86        | [-5.287, -1.002]                              | 1.424    |
| 2.530                    | -0.04422      | -1.748        | 98.25        | [-3.515, 0.01944]                             | 0.6664   |
| 3.161                    | -0.05662      | -1.791        | 98.21        | [-3.009, -0.5735]                             | 0.3594   |
| 3.643                    | -0.1469       | -4.032        | 95.97        | [-4.456, -3.608]                              | 0.01287  |
| Cyclophosphamide RC A    |               |               |              |                                               |          |
| 12.51                    | 0.7195        | 5.752         | 105.8        | [5.479, 6.024]                                | 0.0007097 |
| 50.03                    | 2.333         | 4.662         | 104.7        | [4.258, 5.066]                                | 0.00005840 |
| 100.1                    | 3.351         | 3.348         | 103.3        | [3.068, 3.628]                                | 0.00000430 |
| 125.1                    | 5.873         | 4.695         | 104.7        | [4.578, 4.811]                                | 0.0000023 |
| 150.1                    | 8.111         | 5.403         | 105.4        | [4.985, 5.821]                                | 0.0001315 |
| Cyclophosphamide RC B    |               |               |              |                                               |          |
| 12.53                    | -0.1753       | -1.402        | 98.60        | [-1.749, -1.054]                              | 0.00000440 |
| 50.01                    | -0.5971       | -1.193        | 98.81        | [-2.033, -0.354]                              | 0.3315   |
| 100.1                    | -1.758        | -1.757        | 98.24        | [-5.571, 2.058]                               | 1.942    |
| 125.1                    | -2.811        | -2.247        | 97.75        | [-5.326, 0.8312]                              | 1.616    |
| 150.2                    | -3.056        | -2.036        | 97.96        | [-2.315, -1.757]                              | 0.00000210 |
### Table 6. Summary of system suitability result (Ifosfamide and Cyclophosphamide) at low column temperature (28°C).

| Parameter                  | Limit of acceptance | Ifosfamide | Measured results | Cyclophosphamide | Measured results |
|----------------------------|---------------------|------------|------------------|------------------|------------------|
| Theoretical plates (N)     | ≥ 2000              | 19785      | 9748             | 21326            | 16587            |
| Tailing Factor (T)         | ≤ 1.5               | 1.1        | 1.0              | 1.2              | 1.2              |
| %RSD                       | ≤ 5.0%              | 2.1        | 2.4              | 2.3              | 2.6              |
| Resolution between Ifosfamide and Imp D peak | NLT 2.0 | 3.6 | Not Applicable |
| Capacity factor (k)        | NLT 2.0             | 4.5        |                  |                  |                  |

2.4.6. Robustness test

According to ICH, the robustness of an analytical method is its ability to withstand small but deliberate changes in the experimental variables. In this study, the robustness was evaluated by an experimental design examining the system suitability solution by simultaneous influence on varied column temperature condition (30±2)°C and results are summarized in Table 6 and 7. Results were found within the acceptable limit.
Table 7. Summary of system suitability result (Ifosfamide and Cyclophosphamide) at high column temperature (32°C).

| Parameter                        | Limit of acceptance | Ifosfamide | Measured results | Imp A | Imp B | Imp D |
|----------------------------------|---------------------|------------|------------------|-------|-------|-------|
| Theoretical plates (N)           | ≥ 2000              | 21547      | 13542            | 8954  | 10126 |
| Tailing Factor (T)               | ≤ 1.5               | 1.1        | 1.1              | 1.1   | 1.2   |
| %RSD                             | ≤ 5.0%              | 1.1        | 1.8              | 1.7   | 1.8   |
| Resolution between Ifosfamide and Imp D peak | NLT 2.0          | 3.3        | Not Applicable   |
| Capacity factor (k)              | NLT 2.0             |            | 4.3              |

| Parameter                        | Limit of acceptance | Cyclophosphamide | Measured results | Imp A | Imp B | Imp D |
|----------------------------------|---------------------|-----------------|------------------|-------|-------|-------|
| Theoretical plates               | ≥ 2000              | 19785            | 12546            | 7426  | 8564  |
| Tailing Factor                   | ≤ 1.5               | 1.2              | 1.3              | 1.3   | 1.6   |
| %RSD                             | ≤ 5.0%              | 1.2              | 0.7              | 0.8   | 1.9   |
| Resolution between Cyclophosphamide and Imp A peak | NLT 5.0          | 10.2            | Not Applicable   |
| Capacity factor (k)              | NLT 2.0             |                 | 4.4              |

3. DISCUSSION

3.1. Validation method

Based on the proposed optimum condition of the LC system, validation study was carried out to investigate the method performance. Validation parameters namely specificity, precision, linearity, accuracy, range, limit of quantification and robustness test was evaluated and results are presented. All the results mentioned are evaluated against the standard requirement and results showed that method is suitable for use.

Related compounds of cyclophosphamide and related substances of ifosfamide molecules are highly polar in nature, non-volatile and lack the presence of chromophore in their structure which makes the analysis quite problematic. Structure of its related substance are closely related to each other and do not possess UV absorbing chromophores which leads to the challenging quantification. Proposed chromatographic method can separate the analogue of each component. The quantification of ifosfamide was carried out using analytical column (Make: Agilent ZORBAX column (250 X 4.6 mm; 5 µm particle size)) at a flow rate 0.7 ml/min and column temperature at 25°C. The quantification of cyclophosphamide was carried out using analytical column (Make: Atlantis T3 column (250 X 4.6 mm; 5 µm particle size)) at a flow rate 0.8 ml/min and maintained column temperature 40°C at 195 nm for analysis of cyclophosphamide and its related compounds. Separation of each analyte from the parent analyte was easily accomplished using the proposed chromatographic condition. The method was successfully validated under optimized conditions using the e-noval statistical tool with acceptance criteria of 95% confidence interval (5% risk) with an accuracy profile of ±10%. This provided a greater confidence in the performance of methods. To prove the selectivity of the method, an injection of diluent along with system suitability solution and individual standard solution was injected. No interference peak was observed at the retention time of components of each analyte. Method repeatability was determined by using the six replicate injection at the test concentration solution. The results for precision parameter was expressed as the percentage relative standard deviation. %RSD value of both different day analysis was less than 5% which is considered as acceptable value. Linearity covered the concentration range from 1.25 ppm to 3.75 ppm for Ifosfamide and 12.5 ppm to 150 ppm for cyclophosphamide. Then linearity was evaluated to calculate the correlation coefficient, slope, intercept, standard error and range. Correlation coefficient of each analyte was found to be more than 0.995 which is considered as an evidence of acceptable value obtained from regression line. To calculate the accuracy, each related compounds were spiked to the test solution at QL, 75%, 100%, 125% and 150% level. Each solutions were tested for three times and average recovery was calculated.
at each level. The recovery of each analyte was found within 90-110%. Results were found within the acceptable limits. Robustness results demonstrates that the developed method is robust.

4. CONCLUSION

An HPLC procedure for quantification of the related compounds in cyclophosphamide and ifosfamide was developed and validated using total error concept. Proposed method was effectively employed to get the suitable resolution between each analyte peak. The newly developed method has shown several advantages like, short run time, usages of UV and ELSD detector compared to the complicated procedure. Method presentation and trustworthiness of the results on outmoded validation are confronted due to difficulty in the assessment of results and unexpected bias in regular day-to-day use. Therefore, it has been shown that the usage of the accuracy profile built on β-expected tolerance intervals shows a predicted region where a predefined quantity of results observed over the use in routine analysis. The measurement of uncertainty profile provides complete information about an analytical result. Total error concept is decisively engrained to identify the difference between the measured value and the true value. The method has been proven to fulfill all validation parameter requirement and revealed a robust chromatography system and has been shown as an adequate method for quantification of related substances in ifosfamide and related compounds in cyclophosphamide.

5. MATERIAL AND METHODS

5.1. Chemicals for HPLC analysis

Ifosfamide (99% purity) obtained from Sigma Aldrich, ifosfamide Imp A (95% purity), ifosfamide Imp B (95% purity) procured from LGC, ifosfamide Imp D procured from SimSon pharma (97% purity). HPLC grade of methanol (99% purity) and trifluoroacetic acid (95% purity) were purchased from Merck, India. Cyclophosphamide and its related compounds A, B and D were procured from Sigma Aldrich with a purity of 100.0%. HPLC grade of acetonitrile (99% purity) was supplied by Bio Solve. Analytical grade of potassium dihydrogen phosphate (99% purity) and potassium hydroxide (99% purity) were procured from Merck.

5.2. Instruments

The instrument used in this study includes HPLC Water e2695 consist of ELSD and PDA detector, Agilent ZORBAX SB C18 column particle size 5 μm, 150 X 4.6 mm, Atlantis T3 C18 column particle size 5 μm, 250 x 4.6 mm, ultrasonic bath, analytical balance (make: Mettler Toledo), pH meter (Metrohm).

5.3. Procedures

5.3.1. Solution preparation for analysis of ifosfamide

a. Preparation of mobile phase

The mobile phase was prepared by using the combination of 0.1 M trifluoroacetic acid and methanol in the ratio of 92:8 v/v. Filtered the mobile phase through 0.45-micron filter paper and degassed by the instrument. Mobile phase was used as diluent during this analysis.

b. Preparation of standard stock solutions

Stock solution of ifosfamide, ifosfamide Imp A, ifosfamide Imp B, ifosfamide Imp D were prepared by using the diluent at each of concentration 0.1 mg/mL.

System suitability standard solution was prepared by maintaining the final concentration of 10 ppm for each analyte.

Precision, linearity and accuracy solutions were prepared by spiking each related substance into the sample solution having concentration of 1.25 ppm, 1.875 ppm, 2.5 ppm, 3.125 ppm and 3.75 ppm respectively.

5.3.2. Solution preparation for analysis of cyclophosphamide

c. Preparation of mobile phase

The mobile phase composition being 0.02 M potassium dihydrogen phosphate (pH 7.0) as mobile phase A and a ratio of 60:40% v/v of acetonitrile: water mixture as mobile phase B. Filtered the mobile phase through 0.45-micron filter paper and degassed by the instrument. Milli-Q water was used as diluent.
d. **Preparation of standard stock solutions**

Stock solution of cyclophosphamide, related compound A, related compound B, related compound D were prepared by using the diluent at each of concentration 1000 ppm.

System suitability solution was prepared by maintaining the final concentration of 50 ppm for each analyte.

Precision, linearity and accuracy solutions were prepared by spiking each related substance into the sample solution having concentration of 12.5, 50, 75, 100 and 150 ppm respectively.

**5.4. System optimization**

HPLC method optimization was performed by determining the wavelength, mobile phase, flow rate, column temperature in Waters e2695 instrument. The selection of a reverse phase stationary material (C18) was based on the preliminary investigation and literature survey. Reverse phase C18 column was able to separate the related compounds from the main analyte. Usages of suitable concentration of organic phase and buffer results better separation between the peaks. Based on the desirability function, injection volume, column temperature and buffer strength was assessed to check the method performance characteristics.

Signal intensity in ELSD is influenced by pressure and temperature of the nebulizer gas. Nebulizer temperature at 55°C and nitrogen gas pressure at 25 psi yield the improvement of signal to noise (S/N) ratio also baseline noise. Thus the method allows quantification of related substances in ifosfamide.

The optimized HPLC method was successfully validated and found specific, sensitive, linear, accurate, precise and robust.

**5.5. Validation of the method**

5.5.1. **Specificity**

Specificity or selectivity of the method, an injection of diluent along with system suitability solution and each individual standard solution was injected. No interference peak was observed at the retention time of the main analyte.

5.5.2. **Linearity and range**

Linearity was evaluated by serial dilution of each individual stock and spike to the sample concentration. Under the proposed chromatographic condition, each analyte was found to be linear over the range of LOQ to 150% level of the test concentration. The intercept and slope were calculated to make the linear regression. The coefficient correlation results were found to be ≥0.995. Range data has been reported from linearity, precision and accuracy results met the acceptance criteria.

5.5.3. **Accuracy**

Accuracy testing was evaluated by calculating the percentage recovery. To calculate the percentage recovery, each analyte was spiked to the test solution at various concentration level and calculated the true value against the referance value.

5.5.4. **Precision**

Method repeatability was determined using the six replicates injection at 100% of the test concentration. These was performed in different days. The results of the precision and intermediate parameter was evaluated by its standard deviation and relative standard deviation results.
5.5.5. LOQ and LOD

The quantitation limit is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The detection limit is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value.

5.5.6. Robustness

The robustness of the test procedure was verified by using varied column temperature condition (30±2°C). System suitability solution was injected at varied condition. Results are summarised in Table 5 and 6 which indicate that there were no significant difference between the nominal and robustness results.

Acknowledgement: The authors are thankful to the College of Pharmaceutical Science, Bangalore for providing the necessary support to carry out the research work.

Conflicts Of Interest: The authors declare that there are no conflicts of interest regarding the publication of this article.

Authors Contribution: Concept - I.S.; Design - I.S.; Supervision - M.V.; Materials - I.S.; Data collection – I.S., M.V.; Literature search - I.S., M.V.; Writing - I.S.; Critical reviews - M.V., I.S.

REFERENCES

[1] Pereyra CE, Dantas RF, Ferreira SB, Gomes LP, Silva-Jr FP. The diverse mechanisms and anticancer potential of naphthoquinones. Cancer Cell Int. 2019; 19: 207. [CrossRef]

[2] Pretty JR, Connor TH, Spasojevic I, Kurtz KS, McLaurin JL, B’Hymer C, Debord DG. Sampling and mass spectrometric analytical methods for five antineoplastic agents in healthcare environment. J Onco Phar Pract. 2012; 18(1): 22-26. [CrossRef]

[3] Kung-Tien L, Chien-Hsin C. Determination of impurities in pharmaceuticals: Why and How. Quality Management and quality Control- New Trends and Developments. 2019. [CrossRef]

[4] Shivakumar G, Dwivedi J. Identification of degradation products in cyclophosphamide api by LC-QTOF mass spectrometry. J Liq Chromarogr Relat Technol. 2016; 38(2): 190-195. [CrossRef]

[5] Arzamastev A, Goizman M, Grishina. A TLC determination of cyclophosphamide degradation products in parent substance and drug preparations. Pharm Chem J. 2004; 37(4): 210-216.

[6] Zhou J, Gao S, Zhang F, Jiang B, Zhan Q, Cai F, Li J, Chen W. Liquid- chromatography tandem mass spectroscopy method for simultaneous determination of seven commonly used anticancer drugs in human plasma. J Chromatogr B. 2012; 906: 1-8. [CrossRef]

[7] Sottani C, Rinaldi P, Leoni E, Poggi G, Teragni C, Delmonte A, Minoia C. Simultaneous determination of cyclophosphamide, ifosfamide, doxorubicin, epirubicin and daunorubicin in human urine using HPLC/electrospray ionization tandem mass spectroscopy. Rapid Commun Mass sp. 2008; 22(17): 2645-2649. [CrossRef]

[8] Banks CT. The development and applications of coupled HPLC-TLC for pharmaceutical analysis. J Pharm Biomed Anal. 1993; 11(8): 705-710. [CrossRef]

[9] Zolezzi C, Ferrari S, Bacci G, Fasano MC, Sormani G, Pizzoferato, A. Determination of ifosfamide by HPLC using on-Line sample preparation. J Chemother. 1999; 11(1): 69-73. [CrossRef]
Ahmed M, Usman M, Madni A, Zubai M. A fast and simple HPLC-UV method for simultaneous determination of three anti-cancer agents in plasma of breast cancer patients and its application to clinical pharmacokinetics. Afr J Pharm. 2011; 5(7): 915-922.

Larson RR, Khazaeli MB, Dillon HK. Development of an HPLC Method for simultaneous analysis of five antineoplastic agents. Appl Occup Environ Hyg. 2003; 18(2): 109-119. [CrossRef]

Rizk M, Taha EA, Mowaka S, Abdallah YM. Validated stability indicating HPLC method for the determination of mesna in presence of its degradation products. J Chromatogr Sci. 2015; 53(5): 742-748. [CrossRef]

Sarita M, Jeferson O, Ananda L. Simultaneous determination of cyclophosphamide and ifosfamide in plasma using SPE-HPLC-UV method. Lat Am J Pharm. 2009; 41(6): 41-46.

Gibelin N, Dupont D, Imbert S, Rozet E. Use of Total Error concept in the validation of viral activity in cell culture. J Chromatogr B. 2009; 23(1): 2407-2411. [CrossRef]

Hoffman D, Kringle R. A total error Approach for the validation of quantitative analysis analytical methods. Pharmaceut Res. 2007; 24(6): 1157-1164. [CrossRef]

Traple MAL, Saviano AM, Francisco FL, Lourenço FR. Measurement uncertainty in pharmaceutical analysis and its application. J Pharm Ana. 2014; 4(1): 1-5. [CrossRef]

International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), Geneva, Switzerland, 2005, 1–13.

This is an open access article which is publicly available on our journal's website under Institutional Repository at http://dspace.marmara.edu.tr.