Simultaneous Determination of Two Tyrosine Kinase Inhibitors in Tablets by HPLC-MS analysis

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ABSTRACT: The class of tyrosine kinase inhibitors (TKIs) is represented by a group of compounds which are currently used in the treatment of different types of cancer. These oral medicines present a narrow therapeutic index and a large inter-and intra-individual variability. Within this work, a simple, accurate and rapid reversed phase ultra-high-performance liquid chromatographic (RP-UHPLC) method with mass spectrometric (MS) detection for simultaneous analysis of two TKIs, ibrutinib and ruxolitinib, using pentoxifylline as internal standard (IS) in tablet dosage forms is presented. The separation was carried out on a Waters (Milford, Massachusetts, USA) Arc System coupled with a Waters QDa mass detector. The column used was a Waters CORTECS C18 (4.6×50mm, 2.7μm); a gradient elution was carried out using a mixture of ammonium formate 10 mM aqueous solution and acetonitrile. The flow rate of the mobile phase was set to 0.5mL/min. The column temperature was equilibrated to 40°C. The injected volume was 5μL. All samples were kept at 20°C during the entire analysis. Mass spectra were recorded in positive ionization mode in the range of m/z 100-400 for ruxolitinib and m/z 100-500 for ibrutinib. Quantification was established in single ion recording (SIR) mode for each compound, using pentoxifylline as internal standard. The method was validated according to International Guidelines in terms of stability, limit of detection, limit of quantitation, linearity, precision and accuracy. The validated method can be successfully applied for simultaneous determination of TKIs in tablet dosage forms.

KEYWORDS: Ibrutinib, Ruxolitinib, Simultaneous Analysis, Liquid Chromatography, Mass Spectrometry, Tablets.

Introduction

Clinical use of tyrosine kinase inhibitors (TKIs) has grown significantly in recent years [1-8].

TKIs, analyzed in different studies, are used in the treatment of various, usually hematological malignancies: imatinib, dasatinib, ibrutinib, nilotinib; they are also used in solid tumors including gastro-intestinal tumors (imatinib) or renal and breast forms of cancer (sorafenib and palbociclib) or hepatocellular carcinoma (sorafenib) [9].

Moreover, ruxolitinib treatment has been used in patients with myelofibrosis [10].

Most TKIs are anti-androgen medicines used for eliminating prostate metastatic cancer.

Both drugs inhibit tumor growth effects of androgens.

Abiraterone lowers cells from producing adrenal androgens, meanwhile enzalutamide acts as an androgen-like receptor signaling inhibitor [11].

Ibrutinib is a relatively new drug. FDA approved its medical use in 2013.

The drug provides a strong chemical affinity to a cysteine amino acid in the receptor site, following to prolonged inhibition regarding receptor’s activity.

Receptors represented by BTK are part of the signaling sites for B-cells, which imply a major role in pathogenic processes of most B-cell cancers.

Preclinical investigations demonstrated ibrutinib efficiency for the inhibition of the survival and multiplication in B-cells “in vitro” and also the migration and adhesion processes which occur “in vitro” [12].

Ruxolitinib takes part of the class of Janus Kinase inhibitors (JAK) which possess large affinity to JAK1/2 subgroups.

Ruxolitinib blocks the JAK induced myeloid fibrosis.

Both JAK1 and JAK2 stop “signal transducers and activators of transcription” to receptors for cytokines, followed by the modification in the expression of genes [13].

Chemical structures of ibrutinib and ruxolitinib are presented in Figure 1.
Analytical determination of these compounds is, nowadays, in increasing interest [14].

With this paper, a fully validated method for the qualitative and quantitative determination of these two drugs is presented.

Validation was achieved in terms of limit of quantitation/detection, stability, linearity, accuracy and precision for both compounds.

The validated method was successfully used for quantitative determination in tablets containing the drugs.

**Material and Methods**

**Chemicals and Reagents**

Ibrutinib and ruxolitinib were purchased from Biomedica Medizinprodukte (Romania).

Pentoxifylline was achieved from Athos Chemicals (India).

Ammonium formate, and MS grade ultrapure LC solvents (water, acetonitrile, methanol) came from Merck.

**Chromatographic System and Conditions**

RP-UHPLC determination was achieved using a Waters instrument (Massachusetts, Milford, USA) Arc system coupled with a Waters QDa mass detector.

The UHPLC column used was a Waters CORTECS C18 (4.6mm diameter×50mm column length, 2.7µm particle size).

The gradient elution implied a mixture of two solvents: solvent A (ammonium formate 10mM) and solvent B (acetonitrile).

The gradient was as follows: 0-9 minutes gradually increasing eluent B from 25% to 75% B, 9-10 min gradually decreasing eluent B back to 25%.

The flow rate of the mobile phase was finally established to be 0.5mL/min.

Temperature for the analytical column was set to 40°C during separation process.

The final sample volume injection was 5µL.

All samples were kept in the autosampler at 20°C during the entire analysis.

Eluted compounds were analyzed using a QDa mass detector preceded by an electrospray ionization interface (ESI).

Capillary voltage was adjusted to 0.8kV, cone voltage was set to 25V and the mass spectrometric acquisition was performed in positive ionization mode in the m/z range 100-400 for ruxolitinib and pentoxifylline, and m/z 100-500 for ibrutinib.

Quantification was established in SIR mode for each compound, using pentoxifylline as internal standard.

The ions used for quantification were m/z 441, 307 and 279 for ibrutinib, ruxolitinib and internal standard, respectively.

HC-MS equipment was controlled using the EmPower 3 software package.
Preparation of Standard Solutions
Ibrutinib, ruxolitinib and the internal standard were dissolved in methanol, resulting 1mg/mL stock solutions.
Solutions were kept at-20°C and replaced every 30 days.
Working solutions (1 and 20µg/mL) were prepared by diluting stock solutions using the same solvent; working solutions were also kept at 4°C and renewed every 30 days, also.
Final standard solutions for calibration curves (CC) were obtained also by dilution, resulting concentrations of 2, 5, 10, 20, 50 and 100ng/mL ibrutinib and ruxolitinib, respectively.
Quality control standards (QC) used for determining accuracy and precision, implied concentrations of 5, 50 and 80ng/mL, for both compounds, corresponding to low, medium and high concentration levels.
The internal standard was added in CC and QC samples to get a final level of 100ng/mL in all standards.

Preparation of Sample Solutions
Ten tablets from each compound (ibrutinib 420mg and ruxolitinib 5mg, according to prospects) were weighed and triturated in separate flasks.
Tablets powder corresponding to 5mg for each compound was weighed and dissolved in a 50mL flask using pure methanol as solvent, obtaining sample solutions of ibrutinib and ruxolitinib at 100µg/mL concentration level.
One mL of the sample stock solution was first filtered, then put into another flask and diluted to the target volume (100mL) with the initial gradient ratio of the mobile phase (acetonitrile-ammonium formate, 25:75, v/v) to obtain working sample solution of the two analytes at the concentration of 1µg/mL.

Method Validation
The developed method was validated according to international guidelines regarding stability, linearity, limit of detection and quantification, robustness, precision and accuracy.
To assess stability of both analytes and internal standard, evaluation implied the investigation of the stock solutions (1mg/mL) stored at-20°C for 30 days and by investigation of working solutions (1 and 20µg/mL) stored at 4°C for 30 days and at room temperature for 6 hours.
Stability was evaluated by comparing with fresh prepared solutions.

Regarding limit of detection, a ratio of at least 3 to 1 between peak areas of the analytes and baseline noise was considered satisfactory.
Regarding limit of quantitation, a ratio signal-to-noise of 10:1 was considered for a proper precision of the method.
Moreover, the limit of quantitation must also accomplish the condition for the lowest calibration curve point for precision (expressed as relative standard deviation) to be less than 15%.
Accuracy was determined to be the percentage ratio between mean found concentration and spiked concentration. Precision represents relative standard deviation (RSD) and it is calculated is the percentage ratio between standard deviation and mean found concentration.
Both accuracy and precision were assessed for 5, 50 and 80ng/mL concentration for both compounds.
In terms of linearity for validation data, investigation was carried out from 2-100ng/mL concentration level.
The calibration curves were designed by calculating the peak area ratio of analytes and internal standard plotted to concentration of analytes.
Slope, intercept and correlation coefficient were investigated to assess a proper linearity.
Precision in tablets analysis was determined by using the procedure previously presented in five replicates of commercially available capsules (Imbruvica 420mg and Jakavi 5mg).
Robustness of the method was assessed by analyzing working solutions after small changes of LC and MS parameters.

Results
Stability Tests
All stability tests (room temperature, -20°C and 4°C) were within the range 96-103%.

Limit of Detection and Quantitation
Limits of detection and quantitation were obtained by decreasing gradually the quantities of injected analytes.
As described in method validation paragraph, the results were 0.5ng/mL and 2ng/mL for LOD and LOQ, respectively.
Chromatograms in SIR mode for ibrutinib (m/z 441) and ruxolitinib (m/z 307) are presented in Figure 2.
Figure 2. Single Ion Recording Chromatograms for (A) ibrutinib m/z 441 and (B) ruxolitinib m/z 307.

Linearity
Calibration curves representing peak area ratios analytes/internal standard plotted to concentration of each analyte were all linear in the investigated concentration range. Figure 3 reveals the calibration curves obtained for both compounds, the linear regression equation and the correlation coefficient, larger than 0.99, expressing a high linearity in both cases.

Figure 3. Calibration curves, regression equations and correlation coefficients for ibrutinib and ruxolitinib in concentration range from 2-100ng/mL.
Precision and Accuracy
The values for precision and accuracy indicate the presented method is precise and accurate.

Statistical calculus was carried out at 5% significance level.

Results for precision and accuracy, both intra-day and inter-day are presented in Table 1. The accuracy (expressed by recovery test) was determined as the percentage ratio between the average found concentration (obtained by calculus from the calibration curves) and the spiked concentration and it was found to be more than 85%.

Precision (expressed as relative standard deviation) was calculated as a percentage ratio between the standard deviation and the average found concentration.

All values were less than 15%, including lowest calibration points.

| Analyte  | Target concentration (ng/mL) | Accuracy % | Precision % |
|----------|-----------------------------|------------|-------------|
|          |                             | Intra-day  | Inter-day   | Intra-day  | Inter-day   |
| Ibrutinib| 5                           | 88.36      | 88.02       | 13.59      | 13.79       |
|          | 50                          | 95.67      | 94.98       | 9.35       | 9.65        |
|          | 80                          | 97.69      | 96.37       | 6.59       | 7.01        |
| Ruxolitinib| 5                       | 86.69      | 85.56       | 12.56      | 12.22       |
|          | 50                          | 93.36      | 94.25       | 9.89       | 9.58        |
|          | 80                          | 96.54      | 95.64       | 5.97       | 5.91        |

Robustness
The robustness of the method was investigated, because small changes in chromatographic parameters analysis, like mobile phase composition and/or pump rate could generate important changes in selectivity or quantification of TKIs.

None of these changes did not affect the analysis.

Discussion
Regarding method assessment, several parameters were modified for the selection of adequate working values regarding both LC separation (composition, aqueous/organic ratio, gradient scale, buffer pH for mobile phase) or MS instrument (capillary voltage, cone voltage and m/z ions used for quantification).

Good selectivity in chromatographic separation was achieved by using a mobile phase consisting in ammonium formate 10mM and acetonitrile.

The gradient was obtained by increasing the ratio of acetonitrile from 25%-75% in the 0-9 minutes interval, then decreasing it back to 25% within only 1 minute.

The optimum pump rate providing a proper separation selectivity was established to be 0.5mL/min.

The intensity of the signal is more than two times larger for ruxolitinib than ibrutinib.

To our knowledge, two papers in the literature describe RP-HPLC methods for ruxolitinib and ibrutinib determination in tablets [15,16].

The authors used a C18 analytical column and an isocratic mobile phase elution with UV detection at 227 [15] and 258nm [16]. LOD and LOQ presented were 50 and 160ng/mL, respectively.

Using UHPLC coupled to MS detection we improved both LOD and LOQ data to 0.5 and 2ng/mL.

Moreover, MS detection also provided an increased selectivity because working in SIR mode, with specified m/z values, eliminates the possibility to quantify coeluted compounds which may have the same retention times as the target compounds.

Conclusion
Within this work, a fully validated and precise RP-UHPLC-MS method has been developed for qualitative and quantitative analysis of two tyrosine kinase inhibitors in pharmaceutical forms.

The method is properly sensitive to be used to quantify TKI drugs from tablets.

The next step will be to investigate the suitability of the method to be applied in human plasma samples from patients to whom ibrutinib and ruxolitinib were previously administered.

Conflict of interests
None to declare.
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