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Development and validation of an HPLC method for simultaneous determination of imatinib mesylate and rifampicin

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ABSTRACT: Imatinib mesylate is used to treat various cancerous diseases. Lately, investigations have focused on the enhancement of chemotherapeutic agents. Thus, rifampicin is a promising candidate due to its chemosensitizing potential beyond its anti-infectious effects. In this study, a reliable separation method for imatinib mesylate and rifampicin have been developed. The HPLC analysis were performed on a C18 column (150 x 4.6 mm, 3 µm particle size) at 25 °C. The best UV decetection was observed at 254 nm. The mobile phase was set as acetonitrile and TEA/phosphate buffer (pH: 7.04; 0.1 M) (50:50, v/v) with isocratic elution. The flow rate was set as 0.8 mL/min. The method validation was performed according to the international guidelines with respect to selectivity, linearity, precision and accuracy, recovery and sensitivity. The detection and quantification limit of the method were 0.63 µg/mL and 1.90 µg/mL, respectively for imatinib mesylate, and 3.04 µg/mL and 9.22 µg/mL for rifampicin. The method was linear in the range of 10–90 µg/mL with determination coefficients (r2≥0.99) for both drugs. Precision, accuracy and recovery values (RSD<3%) of the method was convincing. Considering the various usage of imatinib mesylate and rifampicin, the developed method is applicable to different dosage forms.

KEYWORDS: Imatinib mesylate, rifampicin, HPLC, validation

1. INTRODUCTION

Protein tyrosine kinases are important macromolecules that are responsible for cell-growth, differentiation, metabolism and apoptosis (1). As these macromolecules complete a wide biological process in the cells; tyrosine kinase inhibitors are widely used compounds in the treatment of cancer. They bind to tyrosine kinases and inhibit the cell cycle. Following the inhibition on the receptor, the tumor growth is kept controlled. As tyrosine kinase inhibitors are studied as a new target-directed therapies; many studies have been introduced to the literature (2). Human genome studies revealed that tyrosine kinases include 58 receptor types. Among them; epidermal growth factor receptor (EGFR), platelet derived growth factor receptors, fibroblast growth factor receptors (FGFRs), vascular endothelial growth factor receptors (VGFRs), Met (hepatocyte growth factor/ scatter factor [HGF/SF] receptor), the insulin receptor, etc. (3; 4) are well known. Therefore, researchers focused on developing new tyrosine kinase inhibitors; as well as developing new pharmaceutical dosage forms from existing tyrosine kinases (5).

Among the marketed tyrosine kinase inhibitors, imatinib, a 2-phenylaminopyrimidine-type inhibitor, is widely used in the treatment of chronic myeloid leukemia (CML), gastrointestinal stromal tumors (GISTs), melanoma, mast cell tumors, fibrosarcoma, and squamous cell carcinoma. The mechanism of action of imatinib is explained as binding of tyrosine kinase ATP binding site of BCR-ABL and therefore obstructing the phosphorilation of the receptors (6). An eight-year study on Interferon and STI571 (IRIS, clinicalTrials.gov number, NCT0006343) showed that imatinib improved survival on cancer patients comparing cytarabine therapy (7; 8). In the begining of 2000s imatinib like tyrosine kinase inhibitors registered to the market by FDA

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as oral dosage forms. However, there is still and urgent need for impored oral dosage form of imatinib and other tyrosine kinase inhibitors as they are non-selective and overcosting (5).

Rifampicin (3-([(4-Methyl-1-piperazinyl)imino]methyl)rifamycin) (RF) is the first-line antimicrobial drug used in the treatment of tuberculosis, and also used for the treatment of various bacterial infections. It has anti-microbial effects as well as anti-viral activity (9; 10). Beyond its conventional anti-infectious usage, rifampicin exerts neuroprotective, anti-angiogenic, antioxidative, and anti-apoptotic effects (11; 12; 13; 14; 15).

RF contains two condensed aromatic moieties, which are connected by a long aliphatic chain in a basket-like manner of molecular architecture (**Figure 1**).

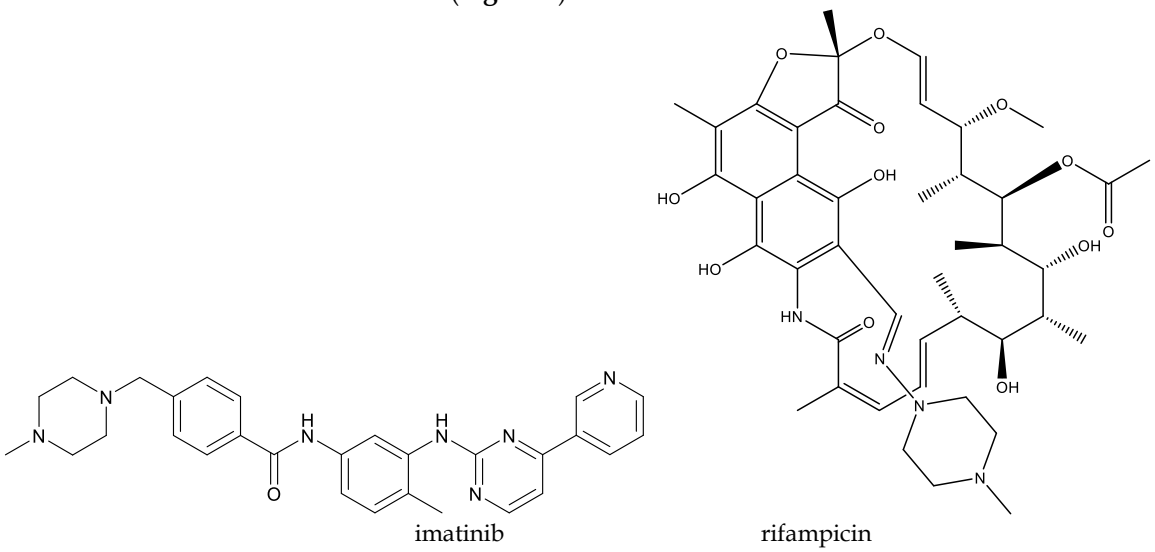

Figure 1 Chemical structure of Imatinib and Rifampicin

For more than two decades, the effects of rifampicin on drug accumulation and/or interaction have been investigated. Fardel et.al elucidated the chemosensitizing potential of rifampicin. This study revealed that rifampicin strongly enhances anti-cancer drug (vinblastine) accumulation and activity in tumor cells via an inhibition of cellular vinblastine efflux. Moreover, rifampicin inhibits p-glycoprotein in a dose-dependent manner (200 µM). Since it increases the sensitivity of resistant cells to vinblastine and doxorubicin, it can be a new tool for overcoming multidrug resistance without major toxicity (16). Although p-glycoprotein (P-gp) overexpression, which is one the mechanisms behind multi-drug resistance (17), is controlled by a highly complex mechanism, it was ascribed that 80 µM of rifampin treatment for 24 h increased P-gp expression (18). On the other hand, rifampicin, at higher concentrations, prominently suppressed the growth and metastases of a variety of human cancer cells that expressed the dose-dependent effect. The effectiveness of rifampicin on cancer therapy associated with its antiangiogenic properties (19). The suppressing effects of rifampicin on endothelial growth- and angiogenesis related factors were rapid and potent, when used at <40 µg/ml (49 µM). Moreover, rifampicin suppressed a protein tyrosine kinase only at 100 µg/ml (122 µM). Leading to potent inhibition of endothelial proliferation make rifampicin favorable as an adjunct anticancer regimen (20). As combination therapy with rifampicin, the effects of co-administration with linezolid were investigated. The rifampicin pretreatment had no effect on linezolid after intravenous administration, although multiple doses of rifampicin decreased the pharmacokinetics of orally administered linezolid (21). Recently, a new iron-rifampicin complex was produced, characterized and its antitumor activity was investigated in vitro and in vivo. In comparison with cisplatin, this novel complex was found to be a bit better antitumor (22).

The addition of rifampicin to cancer therapies ended with promising results. However, to date, there is no combination therapy for rifampicin and imatinib mesylate in cancer treatment. Novel pharmaceutical formulation for imatinib mesylate and rifampicin in a single system, could be beneficial for cancer therapy. In this study, our motivation for the novel drug development of the two drugs, lead us investigate their simultaneous analysis. In this study, we aimed to develop a simple, highly sensitive and validated HPLC method for determination of rifampicin in combination with imatinib mesylate. This study was designed to choose the optimum chromatographic and analytical parameters in line with the analytical method validation procedure for future formulation development studies. The method was validated as to linearity, accuracy, precision, specificity, sensitivity, and stability parameters.

2. RESULTS AND DISCUSSION

2.1. Preparation of standart solutions

Imatinib mesylate and rifampicin were soluble in methanol. However, different ionisable groups caused peak tailing in first separation attempts. Normally, the acidic functional groups can be better separated in acidic mobile phases and basic groups can be separated better in basic mobile phases. Here in this study, while imatinib mesylate is carrying basic groups, rifampicin is carrying weak acidic functional groups (**Figure 1**). Therefore, a stable pH (7.04) was prepared in order to get a better separation for the compounds. Since there was no difficulty in methanol solubility for both compounds, the stock solutions were prepared as 1000 µg/mL for both of the compounds. The stock solutions were then diluted with methanol within the concentration range of 10-90 µg/mL.

2.2. Chromatographic conditions

The chromatographic conditions were optimized by considering the compositions of the solvents, mobile phase, flow rate, and detector wavelength that can affect the quality of separation. Several attempts were made in order to identify the suitable mobile phase for separation. As the compounds do not consist of similar functional groups in terms of acidity, adjusted pH was assumed to be necessary (see section 2.4 for details). During the previous attempts for finding the best mobile phases, rifampicin peak was observed as duplicate and tailed. However, there seems to be no problem with imatinib peak shape even with the first attempts. When the optimum mobile phase was determined as acetonitrile/TEA-phosphate buffer (50:50, v/v), the elution system was set. The gradient elution system was not needed, as the retention times of the drugs were already acceptable (resolution value is greater than 2). Comparing multiple datas in the literature, solvent peaks of rifampicin is a challange in accordance with the imatinib mesylate seperation. Therefore, the difference in the retention times of both drugs could favor the seperation. Even though there were several gradient elution systems reported in the literature, peak shape, overlapping of the peaks could be observed with the gradient elution system. The isocratic elution system was adapted with the dimensions of the column used in our study (23). Eventually, the optimum resolution was obtained with acetonitrile-TEA/phosphate buffer pH: 7.04; 0.1 M (50:50 v/v) mixture, after several attempts.

Selecting the suitable wavelength was performed by scanning the wavelength of these compounds separately at first. It was determined that imatinib mesylate and rifampicin had a maximum absorbance in 254 nm wavelengths. In the present study, the separation of imatinib mesylate and rifampicin with sharp peaks was obtained using the C18 column (150 x 4.6 mm, 3 µm particle size) with a flow rate of 0.8 mL/min at room temperature. The optimum chromatographic condition was given in **Table 1**.

Table 1. The optimum condition for imatinib and rifampicin separation through the HPLC

Wavelength	254 nm
Column	C18 (150 x 4.6 mm, 3 µm particle size)
Mobile phase	acetonitrile-TEA/phosphate buffer pH: 7.04 (1:1 v/v)
Flow rate	0.8 mL/min
Temperature	25 ^{0}C

2.3. Validation of the method

2.3.1. Selectivity

Selectivity is defined as the ability of the analytical method to accurately determine the analyte in the presence of other components (24). In order to verify the selectivity and the separation quality of the method, the samples of imatinib mesylate, rifampicin and their mixture were analyzed. No interferences in the mixture were observed and individual peaks for both compounds were obtained (**Figure 2, 3** and **4**).

With the help of the chromatogram, the resolution and the separation factor were calculated. The resolution was 12.77 while the separation factor was found 1.75 which also greater than 1; meaning that the compounds were separated successfully.

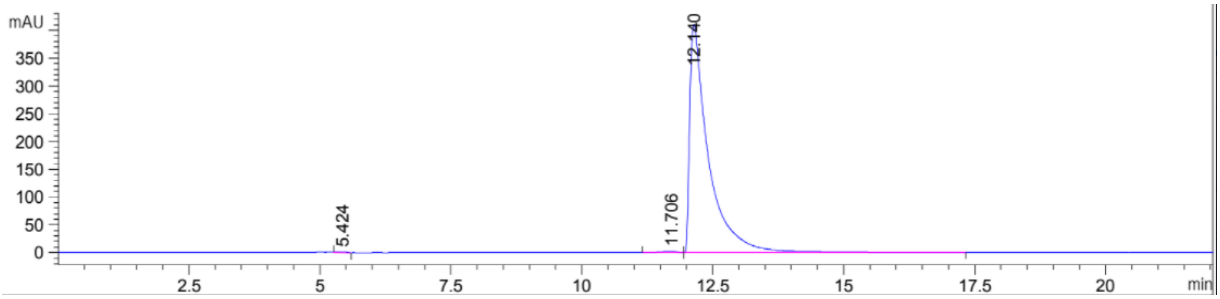

Figure 2. The chromatogram of imatinib mesylate

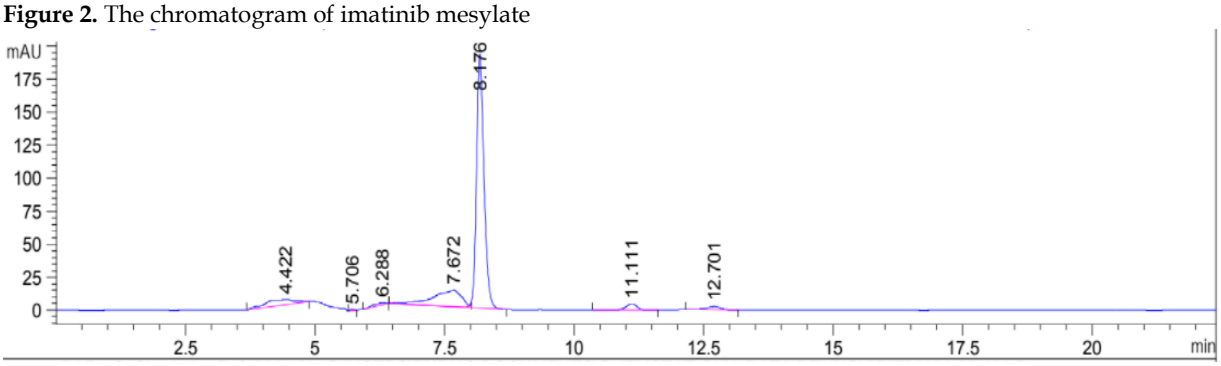

Figure 3. The chromatograms of rifampicin at 254 nm

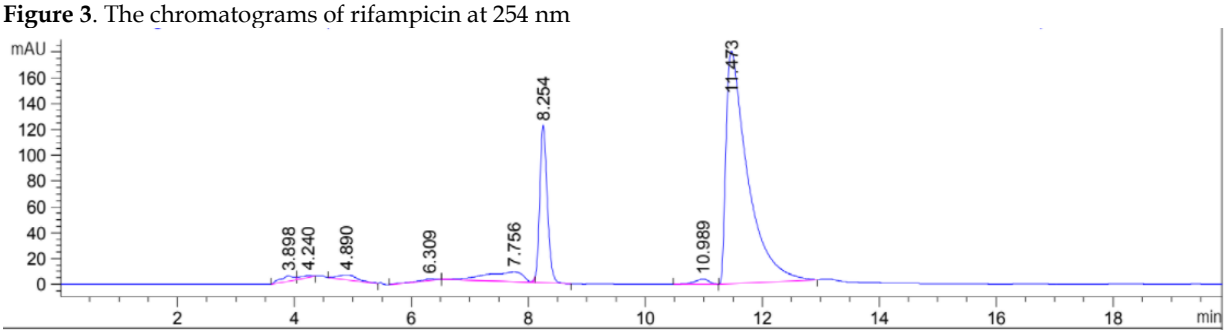

Figure 4. The chromatograms of the mixture. Retention time of rifampicin is 8.254 min, while that of imatinib mesylate is 11.473 min.

2.3.2. Linearity

The linearity describes the relationship between concentration and response (25). Calibration curves were constructed by least-squares linear regression analysis of the peak areas versus the nominal concentrations (10–90 µg/mL). Linearity was evaluated via the calibration equation (**Table 2**), after analyzing eight different concentrations of each sample (n=6). Peak areas versus concentrations were depicted in **Figure 5** a and b. The method was linear up to 90 µg/mL of analytes. The correlation coefficients, as an indication of linearity, were 0.997 for imatinib mesylate, and 0.989 for rifampicin.

Table 2. The linearity data of the developed method

Parameter	Imatinib mesylate	Rifampicin
Calibration equation*	y = 52.63x – 149.93	y = 10.83x – 26.46
Determination coefficient (r2)	0.997	0.989
Linearity range (µg/mL)	10-90	10-90
Number of points	8	8

*where x is the concentration in µg/mL, and y is the peak area.

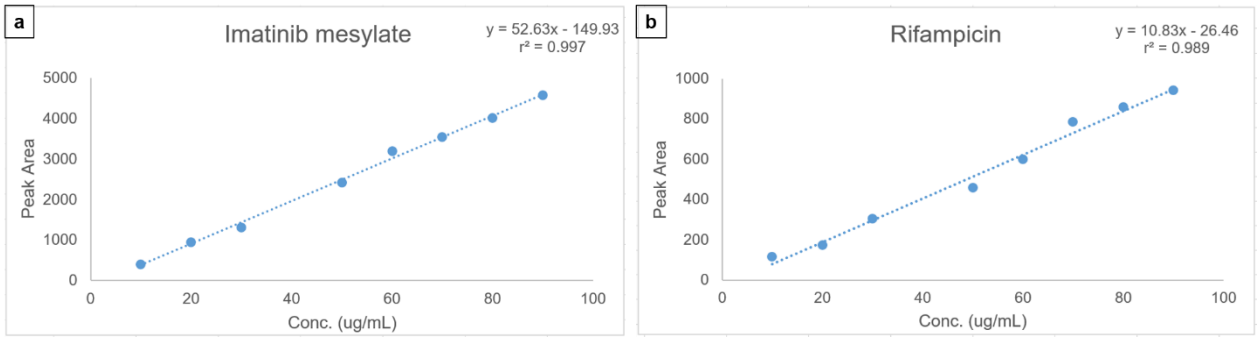

Figure 5. Calibration curve graphs for linearity assessment of; a: imatinib mesylate, and b: rifampicin

2.3.3. Precision and accuracy

The precision of the method in terms of absolute concentrations, recoveries and within-day repeatability were assessed on ten independent mixtures of drug substances at 50 µg/mL concentration. Results were given as mean, standard deviation (SD) and relative standard deviation (RSD %) in **Table 3** after statistical evaluation. Relative errors were also calculated for each measurement according to the below formula (26):

Relative error (%) = determined value-theoretical value (added amount)/theoretical value x 100

Table 3. Intra-day precision (repeatability) results

Sample Number	Concentration (µg/mL)	Imatinib mesylate	Relative error (%)	Rifampicin	Relative error (%)
1	50	49.22	-1.57	49.90	-0.21
2	50	50.92	1.84	49.30	-1.40
3	50	48.57	-2.85	50.82	1.63
4	50	50.85	1.69	50.49	0.98
5	50	51.30	2.60	50.43	0.87
6	50	50.74	1.49	50.55	1.09
7	50	48.98	-2.03	49.51	-0.99
8	50	50.45	0.90	50.76	1.51
9	50	50.96	1.93	49.36	-1.28
10	50	50.54	1.08	49.55	-0.91
Mean	50	50.25	0.44	50.07	0.25
SD	0	0.91	-	0.57	-
RSD (%)	0	1.81	-	1.15	-

Standard deviation (SD) and relative standard deviation (RSD) values for repeatability were less than 1 and 2 %, respectively. Relative error (inaccuracy) values were found in the range of -3.1 to 4 %. These findings indicated that the method is working with the required precision.

The precision and accuracy were also evaluated for day-to-day variation (also known as reproducibility). Samples with the three different levels (low, medium and high: 20, 50 and 70 µg/mL) concentrations were analyzed triplicate over a period of 3 consecutive days. Three replicate determinations were performed for every single day. The results for inter-day precision and accuracy, illustrated in **Table 4**, were expressed as mean, SD, RSD.

Table 4. Inter-day precision and accuracy results

	Imatinib mesylate			**Rifampicin**		
Actual drug conc. (µg/mL)	**20**	**50**	**70**	**20**	**50**	**70**
1st day	20.23	49.78	70.43	20.30	50.11	70.15
2nd day	20.61	50.48	69.94	19.97	49.92	69.26
3rd day	19.80	49.20	71.42	19.70	48.83	71.17
Mean	20.21	49.82	70.60	19.99	49.62	70.19
SD	0.33	0.52	0.75	0.25	0.56	0.96
RSD (%)	1.64	1.05	1.07	1.23	1.14	1.36

The agreement between nominal and measured concentrations of samples was evaluated, and accordingly, the accuracy of the method was determined. The measured concentrations at three different levels on 1st, 2nd, and 3rd days had RSD values less than 2%. These findings indicated the sufficient accuracy of the developed method.

2.2.4. Recovery

In order to evaluate recoveries of the method, the samples at three different concentrations (40, 50 and 60 µg/mL, representing 80%, 100%, and 120%) were prepared and analyzed for repeated measurements. Recoveries were shown in **Table 5**.

Table 5. Recovery results

Concentration of samples	Imatinib mesylate			Rifampicin		
	Mean∓SD	RSD	Mean %	Mean∓SD	RSD	Mean %
40 µg/mL	40.59∓0.97	2.38	101.5	40.19∓1.17	2.91	100.5
50 µg/mL	50.41∓0.11	0.22	100.8	50.06∓0.61	1.21	100.1
60 µg/mL	60.71∓0.06	0.10	101.2	59.93∓1.37	2.44	99.9

Average recoveries were in the range from 101.5% to 99.9% for both samples with the RSD <3% for each analyte. Besides, no significant differences were observed between the recoveries obtained concentrations and their nominal data ($p>0.05$). It was considered that good and satisfactory recoveries were obtained.

2.2.5. Sensitivity

LOD (signal to noise ratios of 3:1) and LOQ (signal to noise ratios of 10:1) values were calculated with the aid of calibration curve to evaluate the sensitivity of the analytical method. The LOD and LOQ values of imatinib were 0.63 µg/mL and 1.90 µg/mL, respectively (seen in **Table 6**) and they were 3.04 µg/mL and 9.22 µg/mL for rifampicin. LOQ was taken as the lowest concentrations of imatinib and rifampicin that could be quantitatively determined with acceptable accuracy and precision.

Table 6. Sensitivity results

Parameter	Imatinib mesylate	Rifampicin
LOD (µg/mL)	0.63	3.04
LOQ (µg/mL)	1.90	9.22

3. CONCLUSION

A new HPLC method was developed and validated for the separation of imatinib mesylate and rifampicin. The method was successfully validated, all results obtained confirmed selectivity, linearity, sensitivity, precision and accuracy. There was no separation method presented for imatinib mesylate and rifampicin in the literature. Therefore, this study is a first and new separation method for both of the substances. The results presented here will help researchers for further formulation studies containing both imatinib mesylate and rifampicin.

4. MATERIALS AND METHODS

4.1. Materials

Imatinib mesylate, rifampicin, acetonitrile, methanol, triethylamine (TEA), and o-phosphoric acid were purchased from Sigma (USA). Deionized water was obtained from the Sartorius Arium Pro (Germany) water purification system and was used to prepare buffer solutions. All other chemicals used were of analytical grade.

4.2. Preparation of standard solutions

Imatinib mesylate and rifampicin are freely soluble in methanol. The stock solutions were prepared as 1000 µg/mL for both of the compounds. The stock solutions were then diluted with methanol within the concentration range of 10-90 µg/mL.

4.3. Instrumentation and chromatographic conditions

Agilent 1260 Infinity II series of HPLC systems (USA) equipped with a solvent pump, manual injection valve and a diode-array detector was used. The separation of compounds was performed with the C18 column (particle size: 3 µm, pore size: 100A). The column compartment was adjusted to 25ºC. The mobile phase consisted of acetonitrile-TEA/phosphate buffer (pH: 7.04; 0.1 M) (50:50, v/v) mixture and delivered at a flow rate of 0.8 mL/min. The injection volume was 20 µL. The DAD detector was operated at 254 nm.

4.4. Method validation

The developed HPLC method was validated as to linearity, accuracy, precision (intra-assay precision and reproducibility), recovery, specificity, sensitivity and stability. In prestudies; literature evaluation for rifampicin and imatinib mesylate was discussed. Several HPLC conditions were obtained for both drugs. As there are no simultaneous application research study for these drugs, it is needed to investigate the separate conditions for both rifampicin and imanitinib mesylate. The choice of column, as well as the mobile phase system depends mostly on physiochemical properties of the analytes. Imanitib consist of mostly basic functional groups like ring nitrogen. However, rifampicin carries phenol, aromatic amide, ester, ether and ring nitrogen. Single analysis in unique conditions for the drugs gave best peaks in their own chromatographic conditions. For example, acetonitrile/water (80:20, v/v) mobile system with C18 column gave a good result for rifampicin (27). Another study however, needed an acidic adjustment of rifampicin analysis as the gradient elution and acetic acid (0.01%) was used in mobile phases. Gradient system was acetonitrile/0.01 %acetic acid:water 0.01/%acetic acid (from 30 to 60% from 0 to 10 min) (28). For biological samples and their analysis, gradient elution system seemed to be favour for rifampicin analysis. The column choice in a study was C18 with 2 µm particle size and the solvent system was set as 10 mM ammonium formate in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) in gradient elution (29). It seemed gradient system and adjustment of a certain pH value favors for rifampicin analysis.

HPLC studies for imatinib mesylate, however, required different chromatographic conditions than those for rifampicin. A study suggested a gradient mobile phase system with a mixture of 0.06 M KH_2PO_4 and acetonitrile (72/28, v/v) with a C18 column (5 µm particle size) as the stationary phase (30). Another study suggested more complex mobile phase system with a mixture of methanol/acetonitrile/triethylamine/diammonium hydrogen phosphate (pH 6.25, 0.048 mol/L) (20:20:0.1:59.9, v/v/v/v) as isocratic elution (31). Due to the structural complexity of imatinib mesylate, another HPLC study needed a buffer system for both of the solvents that are used. In this study, separation of imatinib mesylate was performed with acetonitrile + 0.05% formic acid/water + 0.05 % formic acid (gradient elution) with a C18 column (32). Among all the evaluations based on the literature review, the first attempt was made by the choice of C18 column instead of C8 column. The particle size of the column was chosen as 3 µm as the best seperations were reported with the column's particle size lower than 5 µm. Thus, the stationary phase was adjusted as C18 column. Among all the studies reported, it was observed that the separation system for rifampicin and imatinib mesylate needed a certain buffer. However, we decided to evaluate the first attempt with acetonitrile/water (60:40, v/v) system because of its simplicity. On the other hand, the peak shapes and the separation parameters were not within the targeted values (33). The target value for seperation parameters is for the resolution to be greater than 2. The retention times of both of the drugs were recorded too low for a separation system. Therefore, the adjustment of the reduction the ratio of organic phase was applied and acetonitrile:water (50:50, v/v) mixture was used. Even though the separation seemed acceptable, the shapes of the peaks (especially rifampicin) were not acceptable. Thus, it was decided to use a buffer condition for their simultaneous determination. As the drug molecules contain different functional groups and different pKa values, a neutral pH was decided to used. The previous reports suggested buffers with K_2HPO_4, ammonium formate, formic acid and acetic acid. However, the separation of the compounds could be acceptable with adjusted pH conditions. We therefore came up with the idea of using trimethylamine and adjusted the pH value with phosphoric acid. Eventually, acetonitrile/trimethylamine-phosphate buffer pH 7.04 (70:30, v/v) was used as a first attempt of buffer conditions. In TEA phosphate buffer/acetonitrile (30:70, v/v) of mobile phase, the retention time was too low, resolution was weak (4 min imatinib mesylate, 1.7 min rifampicin) and the separation was not good. In order to increase the retention time, the ratio of organic phase was decreased in regard to polarity of compounds. Finally, the optimum mobile phase was determined as TEA phosphate buffer/acetonitrile (50:50, v/v).

Later on, an adjustment for the flowrate seemed necessary for the separation. Many HPLC conditions (unlike LCMS studies) suggested 1 ml/min or 1.2 ml/min flow rate. With the adjusted mobile phase, the retention times for both of the drugs were acceptable if the flow rate is adjusted as 0.8 ml/min. When the flow rate is increased to 1 ml/min, the retention times became even shorter and the overlapping of the peaks were observed.

4.4.1. Selectivity

The selectivity parameter was evaluated by comparing the chromatograms of imatinib mesylate, rifampicin and their mixture. To this aspect, imatinib mesylateat 1 mg/1 ml stock concentration and rifampicin at 1 mg/1 ml stock concentration were separately dissolved in methanol, and their individual chromatograms evaluated in terms of peak asymmetry. Besides their separate detections, their mixture was prepared at 70 µg/mL concentration and analyzed. In order to demonstrate peak separation, the resolution factor (Rs) was investigated according to the following equation:

$$Rs = 2\,x\,\frac{t_{r2} - t_{r1}}{W_1 + W_2}$$

In this equation, t_{r2} and t_{r1} are retention times for each peak, while W_1 and W_2 represent width of each peak. In addition to the resolution, the separation factor (α) is another indicator of the separation of two peaks. Therefore, the selectivity was also evaluated by calculating the separation factor according to the equation below;

$$\alpha = \frac{k_2}{k_1} = \frac{t_{r2} - t_0}{t_{r1} - t_0}$$

where α is the selectivity index, tr2 retention time of imatinib mesylate, t r1 retention time of rifampicin, t r0 retention time of non-retained component.

4.4.2. Linearity

The linearity of the method was performed by preparing 8 different concentrations between the range of 10-90 µg/mL. The coefficient, slope and interpretation were determined by using the calibration curve.

4.4.3. Precision and accuracy

Precision and accuracy were evaluated with the 40 µg/mL sample for 10 times within the same day (intra-day; also known as repeatability). In addition to this, samples at three different concentrations (20, 50 and 70 µg/mL) were analyzed 3 consecutive days (inter-day; also known as reproducibility). The peak areas of the samples were converted into concentrations using the linearity equation and the results (actual and obtained) were compared.

4.4.4. Recovery

Three different quantities (low, medium and high) of samples were analyzed for recovery. The results were indicated as percentages. Mean, standard deviation (SD) and relative standard deviation (RSD) were calculated.

4.4.5. Sensitivity

In order to evaluate the sensitivity of the analytical method, limit of detection (LOD) and limit of quantitation (LOQ) of samples were determined using the following equations:

LOD: 3.3x σ /S

LOQ: 10x σ /S

where σ is the standard deviation of the lowest concentration (10 µg/mL) measured and S is the slope of the calibration line.

4.5. Statistical analysis

All data were presented as mean ± SD (n = 3). Data sets were analyzed by one-way analysis of variance (ANOVA) followed by a Dunnett's post hoc test using Instat2 (Graph Pad Inc., San Diego, CA). Statistically significant was defined as $p < 0.05$ (*).

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Author contributions: Concept – G.P.C., G.M.D.; Design – G.P.C.; Supervision – G.P.C.; Resources – G.P.C., G.M.D.; Materials – G.P.C., G.M.D.; Data Collection and/or Processing – G.P.C.; Analysis and/or Interpretation – G.P.C.; Literature Search – G.P.C., G.M.D.; Writing – G.P.C., G.M.D.; Critical Reviews – G.P.C., G.M.D.

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