Optimization of the simultaneous determination of imatinib and its major metabolite, CGP74588, in human plasma by a rapid HPLC method using D-optimal experimental design

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ABSTRACT

A simple, rapid and specific HPLC method has been developed and validated for the simultaneous determination of imatinib, a tyrosine kinase inhibitor, and its major metabolite, CGP74588, in human plasma. The optimization of the HPLC procedure involved several variables, of which the influences of each was studied. After a series of preliminary-screening experiments, the composition of the mobile phase and the pH of the added buffer solution were set as the investigated variables, while the resolution between imatinib and CGP74588 peaks, the retention time and the imatinib peak width were chosen as the dependent variables. Applying D-optimal design, the optimal chromatographic conditions for the separation were defined. The method proved to show good agreement between the experimental data and predictive values throughout the studied parameter range.

The optimum assay conditions were achieved with a Chromolith™ Performance RP-8e 100 mm × 4.6 mm column and a mixture of methanol/acetonitrile/triethylamine/diammonium hydrogen phosphate (pH 6.25, 0.048 mol L^{-1}) (20:20:0.1:59.9, v/v/v/v) as the mobile phase at a flow rate of 2 mL min^{-1} and detection wavelength of 261 nm. The run time was less than 5 min, which is much shorter than the previously optimized methods. The optimized method was validated according to FDA guidelines to confirm specificity, linearity, accuracy and precision.

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1. Introduction

Imatinib mesylate (Gleevec®), 4-[(4-methyl-1-piperazinyl)methyl]-N-[4-[1–3]-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-phenyl] benzamide methane sulfonate, acts as a tyrosine kinase inhibitor [1]. Imatinib has been approved for use in BCR/ABL-positive chronic myeloid leukemia (CML) [2] and gastrointestinal stromal (GIST) tumors [3]. Imatinib is metabolized mainly by the human cytochrome P450 enzymes CYP3A4 and CYP3A5 and, to a lesser extent, by CYP1A1/2, 1B1, 2C8/9 and 2D6 [4–6]. The cytochrome P4503A4 has a large inter-individual variability and is susceptible to induction or inhibition by numerous co-medications [7–10]. The major imatinib metabolite, N-desmethyl imatinib (CGP74588), is a product of CYP3A4 and

CYP3A5 that is identified in plasma samples of healthy individuals and patients receiving imatinib. This compound has comparable activity to the parent drug and represents approximately 20% of the parent drug plasma level in patients [11–14]. There are certain cases of suboptimal responses and treatment failures with imatinib. Two major categories of resistance to imatinib are characterized as leukemia cell-based or host-based [15,16], so the monitoring of imatinib plasma levels has been reported to be very useful for the management of patients with CML [13,17].

To conduct further population pharmacokinetic studies of the drug and its major metabolite, we developed and validated a simple and selective bioanalytical high performance liquid chromatography with ultraviolet detector (HPLC–UV) assay for imatinib and CGP74588 in the plasma of CML patients. Approximately 60 patients have participated in our study thus far.

The reported HPLC–UV methods used to determine imatinib and CGP74588 in biological matrices to date [18–22] are time-consuming to the extent that the long analysis times of these

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methods may limit their routine use. The method reported by Oostendorp et al. [18] suffers from a very long run time and unacceptable resolution between the imatinib and CGP74588 peaks. A gradient elution program with 45 min run times has been reported by Widmer et al. [19]. We were unable to resolve the imatinib and CGP74588 peaks with the method reported by Velpandian et al. [20] in two different laboratories. Additionally, the analysis time of the method reported by Schleyer et al. [21] was about 40 min, and the newly published HPLC method by Davies et al. [22] for the determination of nilotinib, imatinib and CGP74588 uses solid phase extraction (SPE) for sample preparation.

Because HPLC utilizes a wide selection of chromatographic factors, e.g., the type of column, the type and concentration of organic modifier, pH, buffer molarity, temperature and flow rate, the optimization of experimental conditions can be quite a complicated process. Therefore, a systematic approach, such as experimental design [23] to optimize the HPLC method, is essential. D-optimal design is a very efficient experimental design for mixture and mixture-process experiments and is commonly used to reveal the main effects and interaction effects between the independent variables of the experiment in the least possible number of experiments [24]. Here, we have used this design to develop and optimize a reproducible HPLC method with a short run time and acceptable resolution between imatinib and its metabolites that could be used in more practical conditions. The large number of patients tested here demonstrates that our HPLC–UV method has sufficient selectivity, as the peak purities of the patients' samples were checked and confirmed with a photo diode array detector. This is important because HPLC–UV is readily available in many labs and is much more inexpensive than mass spectrometers.

2. Materials and methods

2.1. Materials

The pure substances of imatinib, CGP74588 and olanzapine (as internal standard) were kindly supplied by Osveh Pharmaceutical Co. (Tehran, Iran). HPLC grade acetonitrile and methanol and analytical grade diammonium hydrogen phosphate and triethylamine were supplied by Merck (Darmstadt, Germany).

2.2. Apparatus and chromatographic condition

The chromatographic apparatus consisted of a low pressure gradient HPLC pump, a photo diode array detector and an online degasser, all from Knauer (Berlin, Germany). A Rheodyne model 7725i injector with a 100 µL loop was used. The data were acquired and processed by means of Chromgate chromatography software (Agilent Technologies, United States). Chromatographic separation was achieved using a Chromolith[TM] Performance RP-8e 100 mm × 4.6 mm column (protected by a Chromolith[TM] Guard Cartridge RP-18e 5 mm × 4.6 mm, Merck, Darmastadt, Germany). For the mobile phase, a mixture of methanol/acetonitrile/triethylamine/diammonium hydrogen phosphate (pH 6.25, 0.048 mol L^{-1}) (20:20:0.1:59.9, v/v/v/v) was delivered in isocratic mode at a flow rate of 2 mL min^{-1} at a detection wavelength of 261 nm.

2.3. Preparation of standard solutions

Stock solutions of imatinib and CGP74588 (1 mg mL^{-1}) were prepared in methanol and were stable for at least 6 months when stored at 4 °C. Working solutions were prepared daily from these stock solutions by dilution with the mobile phase.

2.4. Sample preparation

The plasma samples were prepared using a liquid–liquid extraction method (LLE). Three hundred microliters of plasma were spiked with 50 µL of internal standard (olanzapine, 4 µg mL^{-1} in methanol) and 50 µL of sodium hydroxide (1 N). A total of 1.5 mL of hexane/ethyl acetate (30:70, v/v) was added to the mixture in a 2 mL Eppendorf polypropylene tube. After horizontal agitation (10 min) and centrifugation (14,000 × g for 10 min), 1.2 mL of the upper organic layer was transferred into a conical glass tube. The organic layer was evaporated under a gentle stream of nitrogen. The residue was then reconstituted in 150 µL of the mobile phase. One hundred microliters of the reconstituted sample were then injected into the HPLC system.

2.5. Experimental design

Statistical parameter evaluation and experimental design are two major tools for optimization techniques. It is beneficial to evaluate and identify the most important parameters with a minimum number of runs while using an appropriate model. The choice of the proper parameter levels through trial-and-error experiments is a time-consuming process, from which the optimal parameter settings may not readily be obtained. We selected a D-optimal design to determine the best experimental conditions in reversed-phase chromatography (RP-HPLC). One of the most advantageous properties of this design is to use the points that minimize the variance associated with the estimates of specified model coefficients. A D-optimal design minimizes the determinants of the $(X'X)^{-1}$ matrix. This design is built algorithmically to provide the most accurate estimates of the model coefficients. Design-Expert (version 7.1, Stat-Ease Inc., Minneapolis, USA) software was used for the construction of the experimental design matrix and to construct the best model that fit to the data by D-optimal design. This design provided an empirical model to describe the effects of mobile ingredients on the separation efficiency in RP-HPLC. A stepwise regression model was used to fit the polynomial model to the data. For the detection of outliers, a normal probability plot and Cook's distance were used. A lack of fit test with the ANOVA model, leverage, a plot of the residuals vs. predicted values, and a graphical demonstration of the experimental vs. predicted values revealed the adequacy of the model. Response surfaces and contour plots were constructed to evaluate the optimum conditions for the response variables.

2.6. Validation of final chromatographic condition

Validation studies were conducted using the optimized assay conditions, based on the principles of validation described in the FDA guidance for bioanalytical method validation [25]. Key analytical parameters, including specificity, accuracy, precision, linearity, detection limit and quantification limits, were evaluated.

3. Results and discussion

3.1. Preliminary studies

The development of an optimized method requires plenty of experiments that increase exponentially with the number of independent variables. To decrease the number of experiments, a decrease in dimensions of independent variables was considered in a series of preliminary-screening experiments. A relatively simple and feasible system that included a separation mode and stationary phase was selected first. Some controllable parameters and their ranges, needing further optimization, were then selected

Table 1
Independent and dependent variables and corresponding constraints.

Independent variables	Symbol	Constraints	Level[a]	
			Low	High
Mixture components				
Acetonitrile	A	In range	20	30
Methanol	B	Maximize	0	20
Isopropanol	C	Minimize	0	10
Triethylamine	D	Minimize	0	0.5
Buffer	E	In range	39.5	80
Numeric factor				
pH	F	Minimize	5	7
Dependent variables				
Resolution	Y_1	In range	1.7	5.7
Retention time	Y_2	In range	3	10
Peak width	Y_3	Minimize	0.1	2.5

[a] $A + B + C + D + E = 100\%$.

on the basis of preliminary experiments. A series of experiments were performed with C_8 and C_{18} columns because C_8 and C_{18} HPLC columns are among the most widely used and most available columns. The resolution between imatinib and the CGP74588 peaks with the C_8 column was higher than with the C_{18} column (same dimensions), with a much better peak symmetry. We checked chromatograms at three temperatures, which were 10, 25 and 45 °C, respectively. Although higher temperature caused a greater sharpness of the peak shape, it also might reduce the column life. Therefore, room temperature (25 °C) was chosen to establish a method that could be transferable from one laboratory to another. The pH adjustment was performed with two different acids, ortho-phosphoric acid and trifluoroacetic acid (TFA), and it was concluded that ortho-phosphoric acid gave higher resolution. Three different buffers, ammonium acetate, potassium dihydrogen phosphate and diammonium hydrogen phosphate, were used, and it was found that diammonium hydrogen phosphate improved the peak shape of imatinib and produced the best resolution. Different molarities of the buffer were also tested, and the best results were obtained with $0.048\,mol\,L^{-1}$ of diammonium hydrogen phosphate.

3.2. D-optimal design

Six independent variables, including the percent of acetonitrile, methanol, isopropanol, triethylamine and buffer in the mobile phase and the pH of the buffer solution, along with three response variables consisting of resolution efficiency, retention time and peak width, were selected for the final optimization studies. Levels of the mixture components and numeric factor are presented in Table 1.

The D-optimal mixture design was used to construct the experiments. This design is a powerful tool for the optimization of mixtures when limitations and restrictions regarding the experimental designs are high. It needs fewer optimization trials compared to other optimization techniques. Sixty experiments were designed and then experimentally performed for the optimization of the mobile phase composition at a constant flow rate ($1.5\,mL\,min^{-1}$) (Table 2). Forty-five points were chosen for the model, 5 points were chosen for an estimation of lack of fit, 5 points were chosen for replication and 5 points were chosen for additional center points. Three responses were analyzed using the Design-Expert software. The stepwise regression was used to describe and fit the obtained data. Hierarchical terms were added after each stepwise regression. Table 2 shows the design and results of the experiments carried out by the D-optimal design. For all of the reduced models, p-values <0.05 were obtained, as shown in Table 3, implying that these models were significant.

3.2.1. Resolution

Resolution (R) was defined according to USP32 with Eq. (1):

$$R = \frac{2(t_2 - t_1)}{w_2 + w_1} \tag{1}$$

in which t_2 and t_1 are the retention times of the imatinib and CGP74588, and w_2 and w_1 are the corresponding widths at the bases of the peaks that were obtained by extrapolating the relatively straight sides of the peaks to the baseline. The data, involving the dependence of the relative resolution of imatinib with CGP74588 on the volume percent of the organic phases and pH of the mobile phase, are tabulated in Table 2. The peak resolutions (R) for all of the 60 experiments were fitted to polynomial models, with no detectable outliers according to the Cook's distances (data not shown). No transformation was used. The F-value of the model implied that the model was significant. In addition, the lack of fit test showed that it was not significant relative to the pure error. The low standard deviation (CV% = 12.37) and high adjusted R-squared (0.9900) values indicated a good relationship between the experimental data and those of the fitted models. The predicted R-squared of 0.9806 was in acceptable concordance with the adjusted R-squared of 0.9900. Adequate precision was defined as a signal-to-noise ratio greater than 4, and the obtained ratio of 64.170 indicated an adequate signal. This model was then used to pilot the design space. The final equation, in terms of actual components and factors, is as follows:

$$
\begin{aligned}
Y_1 = {} & -1.34886 * A - 0.16314 * B + 2.47033 * C - 90.45661 * D - 0.036425 \\
& * E + 0.021890 * A * B - 0.013714 * A * C + 0.43772 * A * D + 0.024407 \\
& * A * E + 0.32778 * A * F - 9.59659E - 003 * B * C + 8.98216E - 003 * B \\
& * E - 0.063676 * B * F - 0.027485 * C * E - 0.22922 * C * F + 1.57331 * D \\
& * E + 29.27294 * D * F - 0.025412 * E * F - 4.65079E - 003 * A * B * F - 0.075250 \\
& * A * D * F - 5.58860E - 003 * A * E * F - 1.54014E - 003 * B * E * F + 2.50397E - 003 \\
& * C * E * F - 0.53808 * D * E * F - 5.52403E - 003 * A * F^2 + 0.014764 * B \\
& * F^2 - 5.52403E - 003 * C * F^2 - 2.33700 * D * F^2 + 8.46086E - 003 * E \\
& * F^2 + 0.045179 * D * E * F^2
\end{aligned}
$$

where A, B, C, D and E are the percentage of components in the mixture, F is pH of the buffer solution and Y_1 is the resolution factor presented in Table 1.

Optimization in RP-HPLC is usually applied to reduce the analysis time without losing the resolution between the peaks. When the resolution is higher than l.7, two peaks are considered to be resolved at the baseline. The effects of the composition and pH of the mobile phase on resolution are shown in Fig. 1. A higher

Table 2
Experimental design matrix and results of D-optimal design.

Run no.	Independent variables						Dependent variables			Run no.	Independent variables						Dependent variables		
	A	B	C	D	E	F	Y_1	Y_2	Y_3		A	B	C	D	E	F	Y_1	Y_2	Y_3
1	20	0	0	0.25	79.75	7	5.78	36.85	2.50	31	30	0	10	0.25	59.75	5	0.00	1.50	0.20
2	30	0	0	0	70.00	5	0.42	2.42	0.20	32	30	0	0	0	70.00	7	3.38	5.52	0.60
3	30	20	10	0	40.00	7	1.00	1.52	0.20	33	20	0	5	0.5	74.50	6	1.20	6.33	0.54
4	30	0	0	0.5	69.50	5.5	0.53	2.32	0.20	34	20	20	0	0.5	59.50	7	3.46	6.55	0.62
5	20	0	10	0.5	69.50	7	3.24	4.52	0.50	35	20	0	10	0	70.00	5	0.00	2.98	0.30
6	30	20	0	0.25	49.75	7	1.92	2.67	0.35	36	25	10	5	0.25	59.75	6	0.30	1.98	0.25
7	25	0	5	0.25	69.75	6.5	1.55	3.35	0.32	37	20	20	0	0.5	59.50	5	0.00	3.48	0.43
8	25	10	5	0.25	59.75	6	0.33	2.07	0.25	38	20	20	10	0.5	49.50	6	0.00	1.53	0.20
9	20	20	5	0	55.00	5	0.00	2.07	0.30	39	20	0	10	0.5	69.50	5	0.00	2.60	0.30
10	25	20	0	0	55.00	7	2.70	3.72	0.40	40	25	0	0	0	75.00	6	2.24	6.97	0.66
11	20	0	0	0.25	79.75	6	2.81	21.60	1.35	41	20	20	0	0.5	59.50	7	3.39	7.23	0.71
12	20	20	5	0	55.00	7	2.36	3.73	0.44	42	30	20	0	0	50.00	5.5	0.11	1.98	0.25
13	20	20	0	0	60.00	6.5	2.34	5.67	0.56	43	30	10	10	0.5	49.50	6.5	0.22	1.45	0.11
14	25	10	5	0.25	59.75	6	0.52	2.10	0.22	44	30	20	10	0	40.00	5	0.00	1.27	0.13
15	30	0	0	0.5	69.50	7	2.83	3.77	0.41	45	30	0	10	0.25	59.75	6	0.20	1.60	0.13
16	20	20	0	0.5	59.50	5.5	0.29	3.67	0.38	46	20	20	5	0	55.00	6	0.61	2.37	0.23
17	25	10	5	0.25	59.75	6	0.06	2.02	0.20	47	20	0	10	0	70.00	7	3.12	7.10	0.80
18	25	10	5	0.25	59.75	6	0.00	1.88	0.20	48	30	20	0	0.25	49.75	5	0.00	1.58	0.15
19	30	20	10	0.5	39.50	5	0.00	1.25	0.15	49	20	20	10	0.25	49.75	5	0.00	1.45	0.11
20	30	0	10	0.25	59.75	7	1.38	1.90	0.25	50	20	0	0	0	80.00	5.5	2.03	20.28	1.30
21	30	0	10	0.25	59.75	5	0.00	1.48	0.12	51	30	20	10	0.5	39.50	6	0.00	1.28	0.10
22	20	0	10	0.5	69.50	7	3.09	4.72	0.50	52	20	20	10	0.25	49.75	7	1.11	1.83	0.16
23	20	0	10	0.5	69.50	5	0.00	2.72	0.30	53	25	10	5	0.25	59.75	7	1.71	2.90	0.28
24	25	0	10	0.5	64.50	6	0.14	1.83	0.20	54	20	10	0	0	70.00	7	4.81	19.58	1.70
25	30	20	10	0	40.00	5	0.00	1.25	0.20	55	20	10	0	0	70.00	5	0.92	8.63	0.62
26	25	20	0	0	55.00	5	0.00	2.03	0.20	56	30	20	10	0	40.00	6	0.00	1.33	0.10
27	20	0	0	0.25	79.75	5	1.20	15.33	0.85	57	30	0	0	0.5	69.50	5	0.51	2.27	0.25
28	30	20	10	0.5	39.50	7	0.25	1.42	0.10	58	20	10	0	0	70.00	6	2.16	14.08	1.20
29	25	10	5	0.25	59.75	6	0.10	1.98	0.28	59	20	0	10	0	70.00	6	0.90	3.62	0.42
30	25	10	5	0.25	59.75	6	0.10	1.98	0.28	60	25	10	10	0.25	54.75	5.5	0.00	1.47	0.13

Table 3
Summary of the ANOVA for model fitted for the three responses.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F value	p-value	
Resolution						
Model	112.65	27	4.17	216.39	<0.0001	Significant
Residual	0.62	32	0.019			Not significant
Lack of fit	0.44	22	0.020	1.14	0.4354	
Pure error	0.18	10	0.018			
Cor total	113.27	59				
Retention time						
Model	2.33	25	0.093	236.49	<0.0001	Significant
Residual	0.013	34	0.00039			Significant
Lack of fit	0.013	24	0.00053	7.18	0.0013	
Pure error	0.000736	10	7.4E−05			
Cor total	2.35	59				
Width						
Model	5.80	11	0.527	69.07	<0.0001	Significant
Residual	0.37	48	0.00763			Not significant
Lack of fit	0.30	38	0.00795	1.24	0.3751	
Pure error	0.06	10	0.0064			
Cor total	6.16	59				

buffer percentage caused an improvement in the resolution (Fig. 1b compared to Fig. 1c), but also caused a longer retention time and a widening of the imatinib peak. Although higher pH increased the resolution (Fig. 1a), it led to a longer retention time and a widening of the imatinib peak. The presence of methanol in the mobile phase improved the resolution in comparison with isopropanol and acetonitrile alone (Fig. 1).

3.2.2. Retention time

The resolution and retention time both changed after varying the composition and pH of the mobile phase, so optimization involved the simultaneous adjustment of both the composition and pH of the mobile phase. To improve the fitting ability of the model, a reciprocal square root was used for the transformation according to a Box–Cox plot (data not shown). The model coefficients were calculated by stepwise multiple regression. The model's F-value of 236.49 implied that the model was significant. A low standard deviation (CV% = 3.26) and high adjusted R-squared value (0.9901) indicated a good relationship between the experimental data and the fitted model. The predicted R-squared of 0.9721 was in reasonable agreement with the adjusted R-squared value of 0.9901. Accordingly, this model was used to navigate the design space as
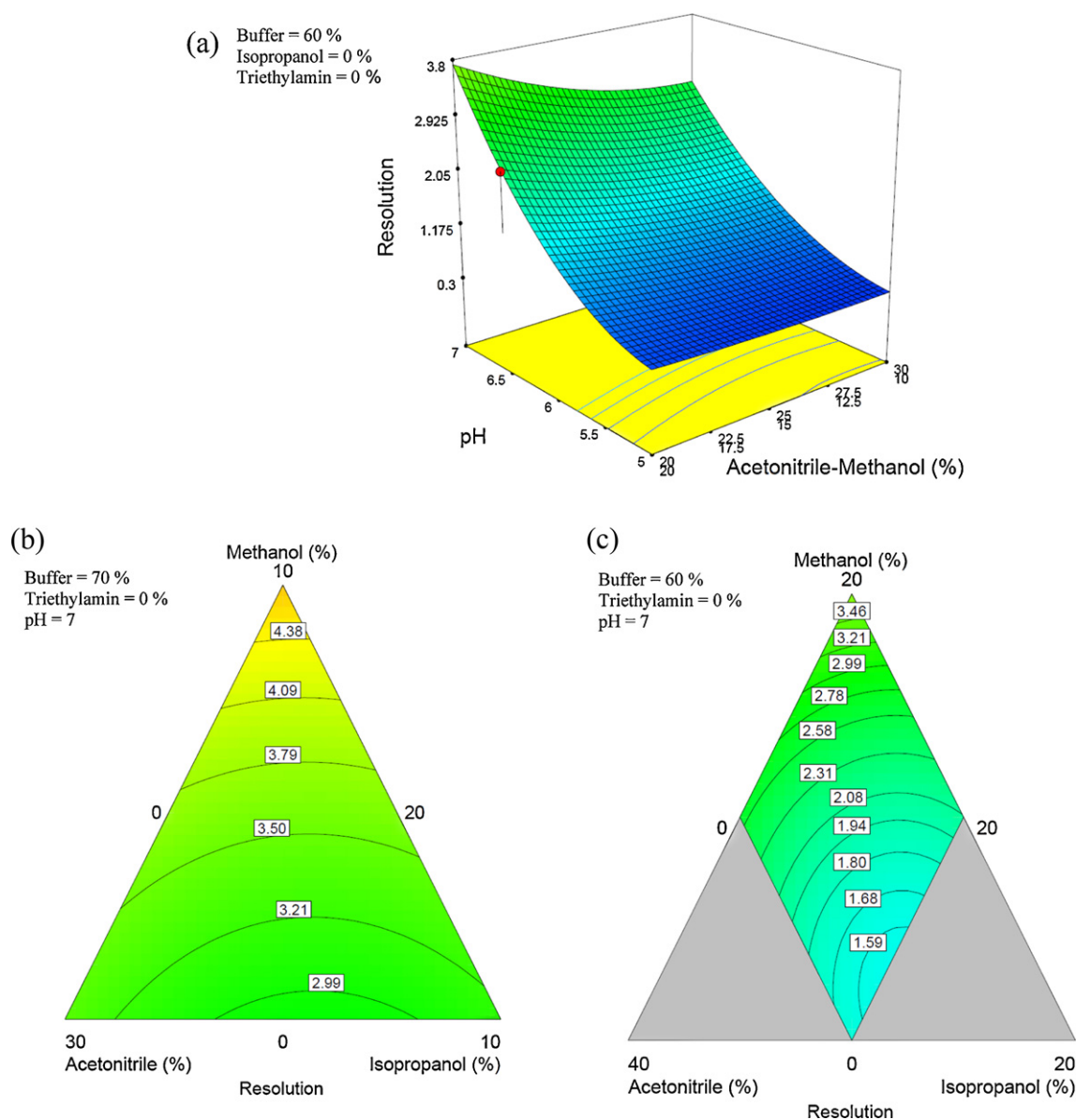
Fig. 1. Contour plot and three-dimensional graph showing the effect of variables and prediction of optimum space for resolution.

follows:

$$\frac{1}{\sqrt{Y_2}} = -0.16427 * A + 0.33903 * B - 0.21736$$

$$* C + 0.78385 * D - 0.026125 * E + 1.80573E - 003 * A$$

$$* B + 1.45418E - 003 * A * C + 3.79386E - 003 * A$$

$$* E + 7.85161E - 003 * A * F - 7.41887E - 003 * B * D - 6.24082E - 003$$

$$B * E - 003 * B * E * F - 3.65464E - 004 * C * E * F + 1.43672E - 003$$

$$* D * E * F + 6.94390E - 003 * B * F^2 - 3.41658E - 003 * C$$

$$* F^2 + 3.00399E - 005 * E * F^2 - 1.46212E - 004 * B * E * F^2$$

An increase in buffer volume and pH of the mobile phase increased the retention times of the analytes also increase of pH increased the retention time of the eluting peaks (Fig. 2).

3.2.3. Peak width

There were variations in peak width as the organic modifier content and pH changed. While an increase in pH resulted in peak tailing, the presence of methanol in the mobile phase decreased the fronting of the peak shape (Fig. 3). However, a decrease in pH and an increase of the triethylamine content resulted in sharper peaks, as presented in Fig. 4. It should be mentioned that when the pH was decreased to less than six, the resolution was almost lost (Fig. 1a). A base 10 log root was used for the transformation according to a

Box–Cox plot (data not shown). The model coefficients were calculated by stepwise multiple regression. The model's F-value of 69.07 implied that the model was significant. A low standard deviation (CV% = 17.13) and high adjusted R-squared value (0.9270) indicated a good relationship between the experimental data and
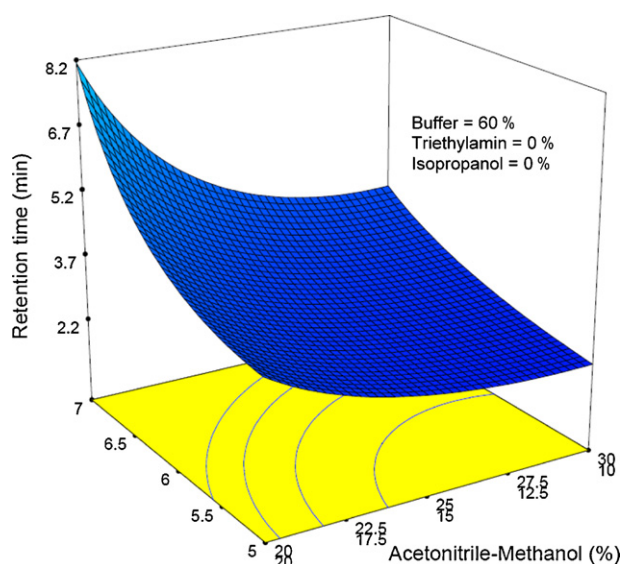
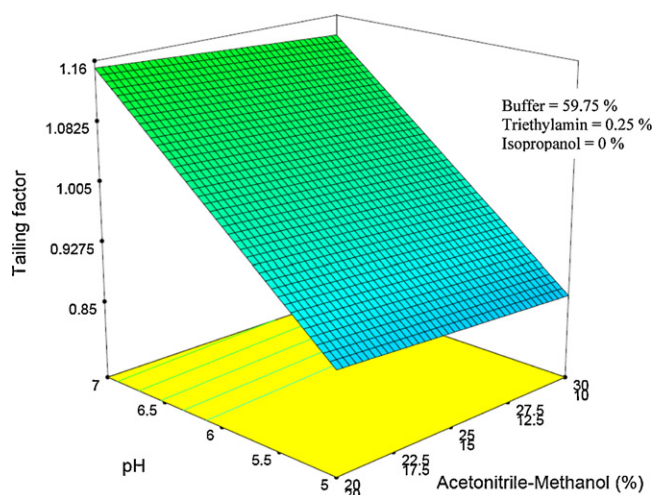**Fig. 2.** Three-dimensional graph showing the effect of components and pH on retention time.

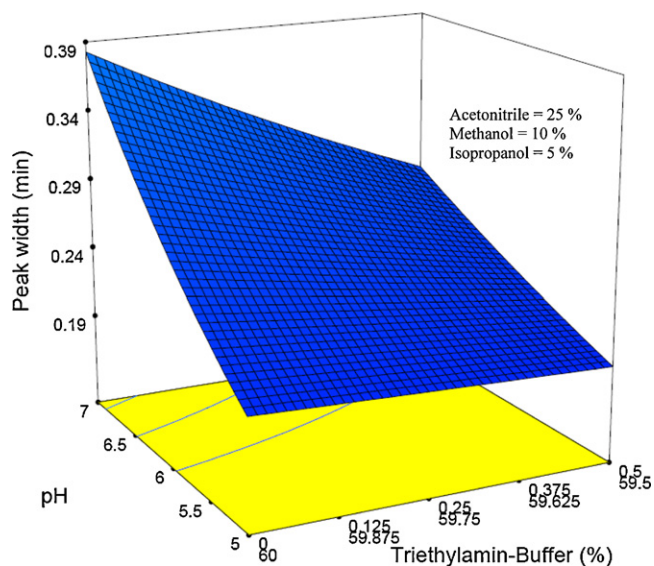**Fig. 3.** Effect of pH and organic phase on tailing factor of imatinib.

Fig. 4. Three-dimensional graph showing the effect of components and the pH of mobile phase on the peak width of imatinib.

the fitted model. The predicted R-squared of 0.9048 was in reasonable concordance with the adjusted R-squared value of 0.9270. Accordingly, this model was used to navigate the design space as follows:

$$
\begin{aligned}
\text{Log } 10(Y_3) = \ & +0.14240 * A - 1.92579E - 003 * B - 0.079135 \\
& * C + 0.037163 * D + 0.012217 * E - 1.69297E - 003 * A \\
& * B - 3.23003E - 003 * A * E - 2.52823E \\
& -003 \\
& * B * F + 0.098366C * D - 9.59477E - 004 * C * F - 0.041054 * D \\
& * F + 3.87236E - 003 * E * F - 0.016650 * C * D * F
\end{aligned}
$$

3.2.4. Multiple criteria optimization

Obtaining an optimal procedure required different dependent and independent variables to be simultaneously set, which is called multiple criteria optimization (MCO). Derringer's desirability function was first used in chromatography by Deming [26]. The Derringer's desirability [27], D, is defined as the geometric mean, weighted or otherwise, of the individual desirability functions. The expression that defines the Derringer's desirability function is:

$$
D = \left[d_1^{p_1} \times d_2^{p_2} \times d_3^{p_3} \times \cdots \times d_n^{p_n} \right]^{1/n} \tag{2}
$$

where p_i is the weight of the response, n is the number of responses and d_i is the individual desirability function of each response obtained from the transformation of the individual response of each experiment. The scale of the individual desirability function ranges between $d_i = 0$ for a completely undesired response and $d_i = 1$ for a fully desired response. For a value of D close to 1, response values are near the target values.

The following constraints in this study that were imposed on the responses included a resolution greater than 1.7 and retention times less than 10 min, in addition to the minimization of peak width. MCO was used, based on the desirability index of Derringer and performed with the aid of the computer program Design Expert version 7.1. It appeared that the most significant factors that influenced resolution, retention time and width of the chromatographic peaks were the acetonitrile content, methanol content and pH of the mobile phase and, less significantly, the ionic strength (i.e., the concentration of the phosphate buffer). The interaction between the organic content and the pH of the mobile phase was significant. Separation of the ionizable analytes (acids and bases) depends on the pH of the mobile phase. Imatinib is a weak base, and its relative percentages of cationic, neutral forms in the solution depend upon the pH of the solution. The retention times of imatinib and its major metabolite were increased on a nonpolar column by increasing the pH (5–7) because these analytes were separated in their non-ionized forms, and the interactions between the analytes and the hydrophobic surface of the column packing that cause poor peak shape were increased and resulted in peak widening (Figs. 3 and 4). Also by lowering the pH to 5, the resolution between the peaks decreased (Fig. 1a), as the difference in polarity between imatinib and its major metabolite was reduced (Fig. 5). The optimization of resolution, width and retention time showed that suitable responses were situated between pH 6 and 7. These constraints (summarized in Table 1) were used to achieve the best chromatographic separation of imatinib, CGP74588 and the internal standard from the soluble plasma constituents. The comparison between the experimental and predicted values under the optimum conditions was performed, and the confidence interval estimates of the slopes and intercepts of the linear regression equation between the actual and predicted values for three responses were 1 and zero, respectively (data not shown). These findings indicated the best agreement between
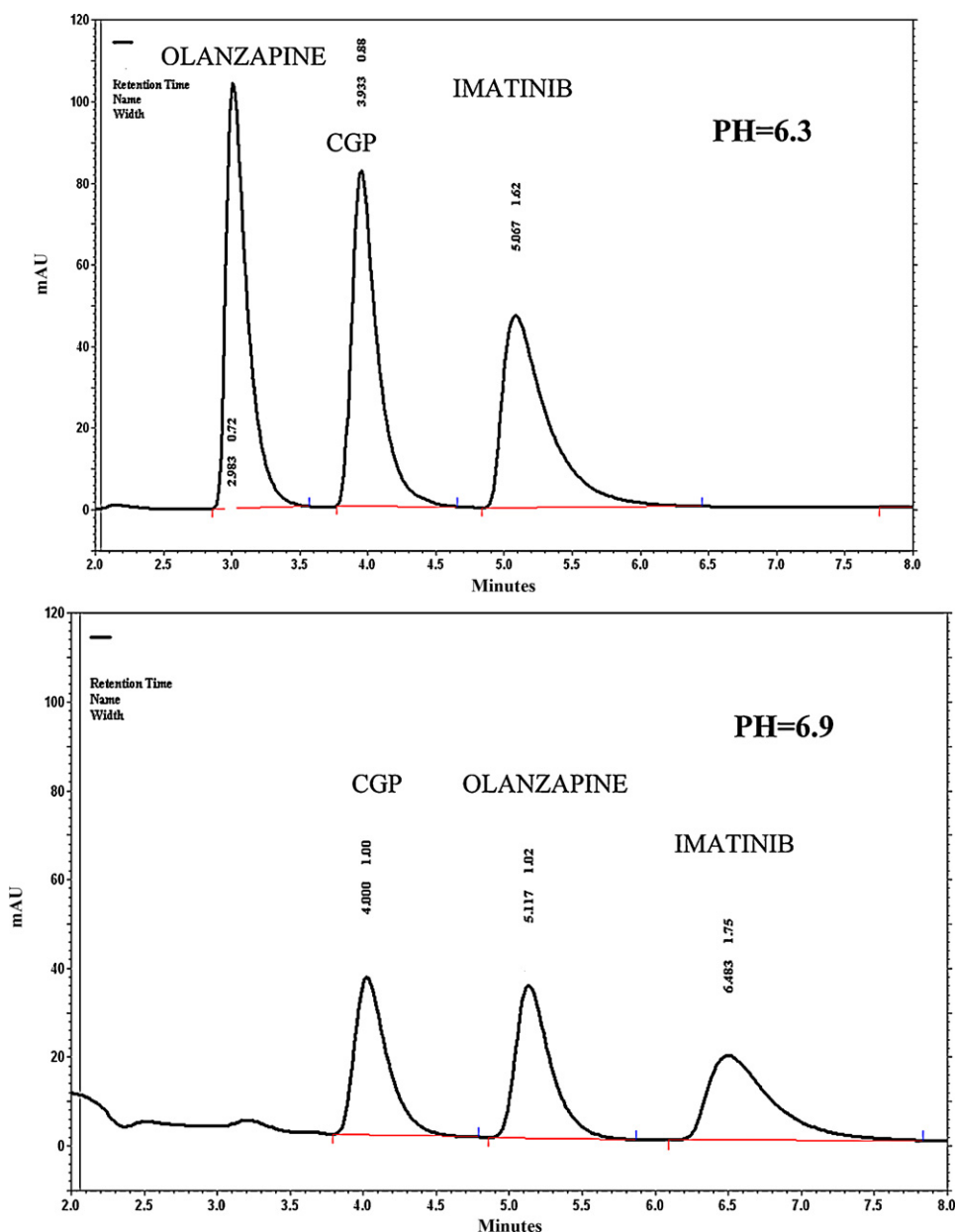
Fig. 5. Chromatographic profile of the plasma spiked with imatinib, CGP74588 and olanzapine at two different pH values (6.3 and 6.9) and identical compositions of mobile phase (methanol/acetonitrile/triethylamine-buffer (0.048 M) (20:20:0.1:59.9, v/v/v/v)) at a flow rate of 1.5 mL min^{-1}.

the model and experimental data for the range of experimental values assessed. Consequently, the coordinates producing the maximum desirability value ($D = 0.76$) were 20% methanol, 20% acetonitrile, 0.1% triethylamine and pH 6.25 (data not shown). While using these optimum conditions, a baseline separation with resolution higher than 1.7 and analysis time less than 8 min (with a flow rate of 1.5 mL min^{-1}) was achieved. The effect of flow rate with optimized mobile phase composition was studied at 1.5 and 2 mL min^{-1}. When the flow rate was increased, there was a decrease in the peak width and retention time of imatinib, without a significant decrease in the resolution of the peaks between the studied compounds. Hence, a flow rate of 2 mL min^{-1} was selected as the optimum flow rate because it yielded good peak shapes without endogenous peak interference at the retention times of the analytes. Therefore, the analysis time, about five min, was optimized without losing resolution.

3.3. Method validation

3.3.1. Selectivity

The separation achieved, using the optimized conditions of the assay for imatinib and its main metabolites, are presented in Fig. 6. The absence of interfering endogenous components at the retention times of all analytes in blank plasma shows the high selectivity of the developed chromatographic method. Retention times for imatinib, CGP74588 and the IS (olanzapine) in plasma were 3, 2.4 and 2 min, respectively. A sample chromatogram from a patient receiving 400 mg imatinib daily (24 h after the last dose was taken) is shown in Fig. 7.

3.3.2. Linearity

Starting from a stock solution of imatinib and CGP74588 (1 mg mL^{-1} in methanol), standards were prepared using pooled
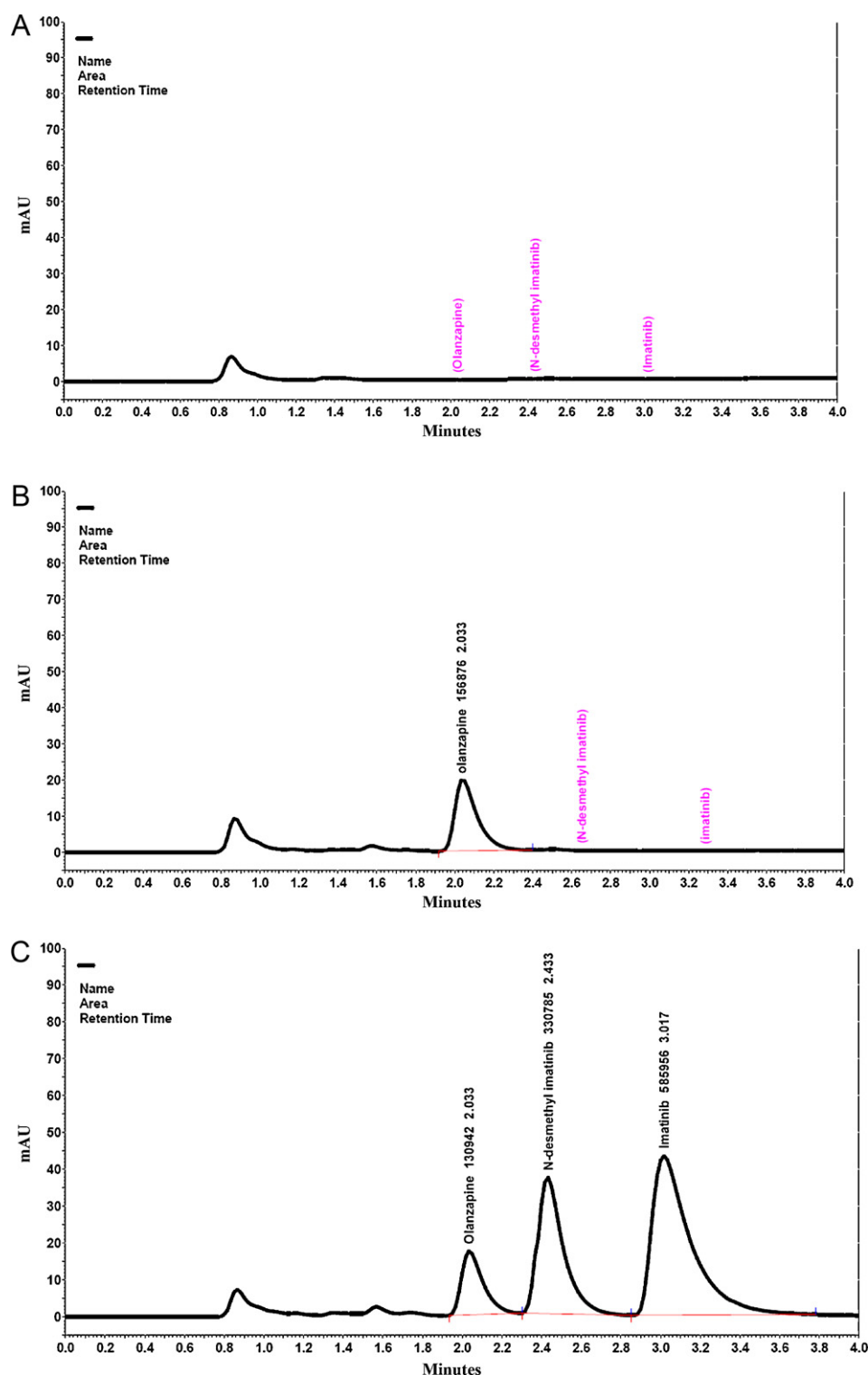
Fig. 6. Chromatograms of imatinib, CGP74588 and olanzapine (IS) in human plasma using the liquid–liquid extraction method. (a) Blank human plasma; (b) plasma spiked with olanzapine (IS); and (c) plasma spiked with olanzapine, CGP74588 and imatinib (4 μg mL^{-1}).

human drug-free plasma obtained from healthy volunteers as the diluent. The calibration curve was performed with final concentrations of the standards (imatinib and CGP74588) of 62.5, 125, 250, 500, 1000, 2000, 4000 and 6000 ng mL^{-1} in human plasma. Calibration curves were determined by least squares linear regression analysis. The peak area ratio of each analyte to olanzapine (Y) vs. the corresponding concentration (X = concentration of the standard in μg mL^{-1}) was plotted. The linearity of the relationship between

peak area ratios and corresponding concentrations, which were needed in this study, was demonstrated by the correlation coefficients obtained for the regression lines. The correlation coefficients of all standard curves were more than 0.965 using the liquid–liquid extraction method (data not shown). The common slope and intercept for imatinib and CGP74588 over three days was calculated. The equations were: for imatinib, $Y = 2.044X + 0.056$ and for CGP74588, $Y = 0.923X + 0.024$. Because the F-value showed a lack of significance
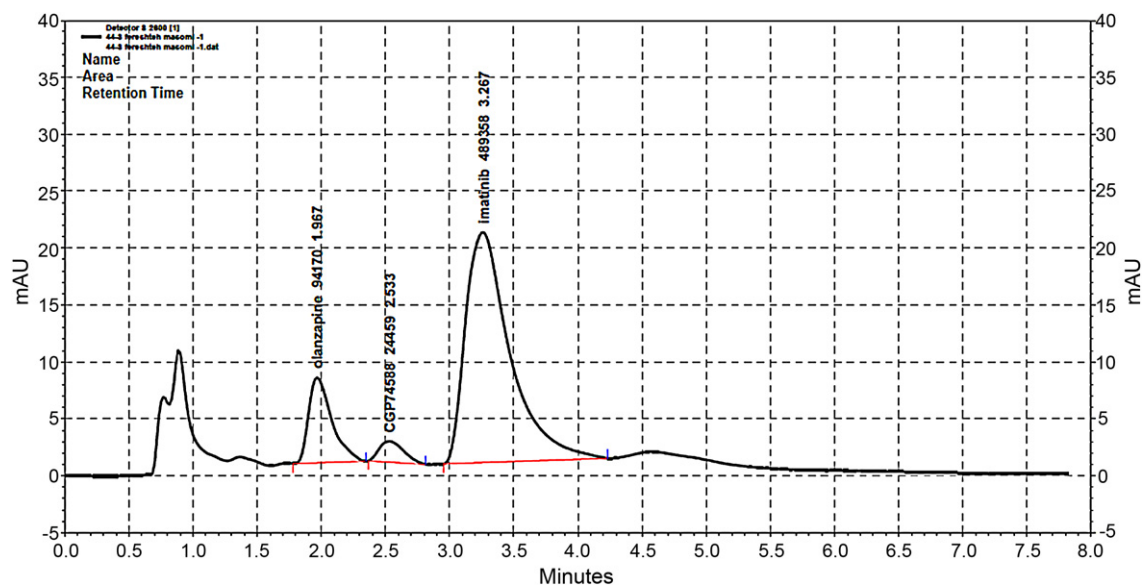
Fig. 7. A sample chromatogram from a patient (24 h after receiving 4×100 mg Glivec tablets in steady state).

at the 5% level for comparing calibration curve equations for imatinib and CGP74588 over three days, we concluded that the lines appeared to be the same on different days.

3.3.3. Accuracy and precision

Five replicates of spiked samples were assayed inter- and intraday at eight different concentrations in plasma (62.5, 125, 250, 500, 1000, 2000, 4000 and 6000 ng mL^{-1}) for imatinib and CGP74588. Accuracy was calculated as the deviation of the mean from the nominal concentration. Inter- and intra-day precisions were expressed as the relative standard deviation of each calculated concentration. The results from the validation of the method in human plasma are listed in Table 4. The method proved to be accurate and precise according to the FDA guidance for bioanalytical method validation [25]. Precision and accuracy studies in plasma showed an acceptable RSD value ($\leq$14.6%), and accuracy ranged from 80.2 to 102.4% for both inter- and intra-day studies ($n = 5$).

Table 4

Inter- and intra-day accuracy, precision and recovery of the HPLC method for the determination of imatinib and CGP74588 in human plasma.

Concentration Plasma (ng mL^{-1})	Within-day R.S.D.	Within-day Accuracy	Between-day R.S.D.	Between-day Accuracy	Recovery %	Recovery R.S.D.
Imatinib						
62.5	12.4	82.5	7.1	80.2	98.4	6.4
125	8.9	89.5	9.6	90.1	97.5	5.9
250	4.4	97.2	5.6	96.4	99.3	6
500	5.2	100.1	5.7	99.3	98.2	5.4
1000	4.8	100.8	5.1	102.4	96.2	5.3
2000	4.3	99.7	4.3	100.7	96.2	4.6
4000	4.1	102.3	4.3	100.3	94.2	4.3
6000	4.9	99.5	5.1	100.3	92.8	5.2
N-desmethyl imatinib						
62.5	13.5	83.2	14.6	81.2	70.8	6.9
125	12.2	86.3	13.6	85.9	70.2	6.7
250	8.2	95.2	9.1	97.2	68.2	5.3
500	4.6	98.2	5.6	99.5	66.4	5.6
1000	4.6	99.4	4.2	99.2	65.3	4.2
2000	4.3	98.5	4.4	99.5	64.7	4.9
4000	5.1	102.2	4.9	100.5	64.8	5.3
6000	5.7	98.1	6.1	97.3	62	6.7

3.3.4. Recovery

The absolute recovery of imatinib and CGP74588 from plasma was obtained from the peak area response of imatinib and CGP74588 in the processed samples, expressed as a percentage of the response of the same amount of imatinib and CGP74588 in standard aqueous solutions (contained in the 100 μL injection volume) that were directly injected onto the HPLC column. The absolute recoveries of all analytes are shown in Table 4. With the liquid–liquid extraction method, the absolute recoveries ranged from 92.8% to 99.3% and 62% to 70.8% for imatinib and CGP74588, respectively. The mean recovery was determined by calculating the ratio of the slopes of the processed vs. unprocessed calibration curves. The mean recovery was 95.76% for imatinib and 64.29% for CGP74588 using the liquid–liquid extraction method.

3.3.5. Lower limit of quantification

The lower limit of quantification (LLOQ) was defined as the lowest analyte concentration that could be determined with an accuracy and precision of <20% [25]. The LLOQ values for imatinib and CGP74588 were 62.5 ng mL^{-1}.

3.3.6. Stability

The stability of plasma samples stored at room temperature was determined up to 72 h. At concentrations of 0.5, 1 and 2.0 μg mL^{-1} of imatinib and CGP74588, the variations of imatinib and CGP74588 levels over time were always less than ±5%, indicating that the plasma samples were stable for at least 72 h at room temperature, and three freeze–thaw cycles had no effect on the imatinib or CGP74588 levels. The long-term stability of imatinib and CGP74588 levels in human plasma under storage conditions of −70 °C for up to 6 months was also acceptable. The stability of imatinib and CGP74588 in blood samples stored at room temperature for 24 h was also investigated. The variations of the levels at concentrations of 0.5, 1 and 2.0 μg mL^{-1} of imatinib and CGP74588 were always less than ±5% over 24 h, irrespective of the anticoagulant used (heparin or EDTA). The stability of imatinib and CGP74588 in hemolysis samples produced by three freeze–thaw cycles of whole blood samples was also checked. The results showed no effect on the recovery of analytes in hemolysed samples.
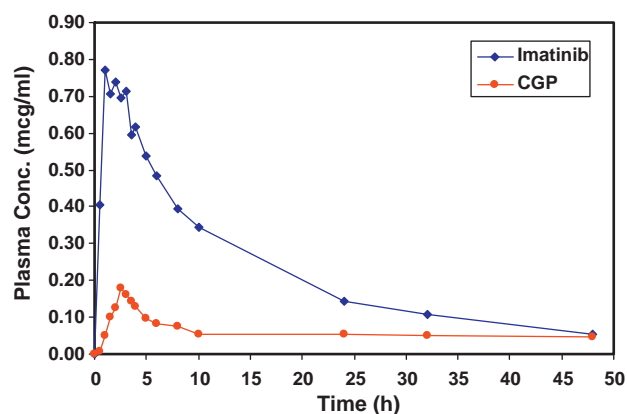
Fig. 8. Plasma profiles of imatinib and its metabolite in a healthy volunteer after receiving 2 × 100 mg Glivec tablets.

4. Application of the HPLC method

This method is currently used in research protocol for assessing the inter-individual and residual intra-individual variability of imatinib in CML patients using a population pharmacokinetics approach. It is used to determine the relationship between imatinib plasma concentration and the clinical outcome (e.g., relapses, initial/secondary failure or side effects). The plasma concentration profile of imatinib and its metabolite in a healthy volunteer who received 200 mg imatinib is presented in Fig. 8.

5. Conclusions

A reversed-phase HPLC method for the determination of imatinib and CGP74588 in human plasma was developed and optimized using a D-optimal design. The objective responses of resolution, width and analysis time were simultaneously optimized by the use of Derringer's desirability function, and the method was validated. The validation study supported the selected conditions by confirming that the assays were specific, accurate, linear and precise. This method has a very short run time, it determines both imatinib and CGP74588 very selectively with baseline resolution and it could be applied to pharmacokinetic studies.

Acknowledgement

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