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Method development and validation for the simultaneous determination of imatinib mesylate and *N-desmethyl* imatinib using rapid resolution high performance liquid chromatography coupled with UV-detection

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ABSTRACT

We developed a simple and sensitive method for the simultaneous detection of imatinib mesylate (IM) and its active metabolite, *N-desmethyl* imatinib (M1), in human serum samples. Separation was successfully achieved using an Agilent® ZORBAX *Eclipse plus* C_{18} reversed phase column (50 mm × 2.1 mm, i.d.; 1.8 μm) under isocratic mobile phase conditions consisting of acetonitrile: 0.02 M potassium dihydrogen phosphate with 0.2% triethylamine at pH 3 (25:75, v/v) and ultra-violet detection was achieved at 235 nm. Extraction of the target compounds was completed using 100% cold acetonitrile. Good linearities ($r^2 > 0.99$) for both IM and M1 were achieved for the concentration ranges of 50–1800 ng/mL and 50–360 ng/mL, respectively. The detection limits were 20 ng/mL and 10 ng/mL for M1 and IM, respectively. The intra- and inter-day precisions were less than 1% with percent recoveries of more than 90%. The method was successfully applied to calculate the pharmacokinetic parameters of chronic myeloid leukemia patients receiving imatinib. The method is suitable to be routinely applied for determination of IM and M1 in serum.

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1. Introduction

Imatinib mesylate (IM) is a tyrosine kinase inhibitor therapeutically used in the treatment of chronic myeloid leukemia (CML). It is a synthetic drug that belongs to the 2-phenylamino-pyrimidine (PAP) group and has four pK_a values (1.52, 2.56, 3.73, 8.07) [1]. After oral administration of an IM capsule of 400 mg, IM showed a maximum concentration (C_{max}) at 2–4 h post-dose [2]. The elimination half-lives of IM and its active metabolite, *N-desmethyl* imatinib (M1), were approximately 18 and 40 h, respectively [2].

Resistance to IM is a major problem. Recent studies have shown that the mechanism of IM's resistance is influenced by a patient's genetic background [3] and environmental factors [4]. These factors can lead to variable drug disposition in the human body, which causes a large inter-individual variability in drug concentrations among CML patients. Therefore, knowledge of the serum drug concentration to identify pharmacokinetic variability is important in successful patient management.

Higher plasma IM concentration is usually correlated to a more effective drug response [5,6], which emphasizes the importance of therapeutic drug monitoring (TDM). Picard et al. revealed that the optimum trough plasma IM level, 1002 ng/mL, is able to induce a significant molecular response [6]. Following this discovery, TDM of IM was utilized to individualize the IM dose. TDM is particularly useful for a drug such as IM, which exhibits a large inter-individual variability but also exhibits a consistent concentration–response relationship. Additionally, TDM plays an important role in evaluating a patient's adherence to a daily oral therapy by elucidating potential drug–drug interactions and in determining the drug's efficacy and drug-related adverse events. Therefore, quantification of the plasma IM level is useful in providing a better understanding of treatment failure or determining if there is a suboptimal response in the patient receiving a standard-dose of IM. Quantification of the plasma M1 level will be an added advantage, as it is an active metabolite with equipotent pharmacological activities to the parent drug, IM and is approximately 15–20% of the area under the curve (AUC) of IM [2].

To date, there are many published methods for the detection of IM in pharmaceutical formulations [7–12], IM metabolite profiling [9,13] and different biological specimens, including blood [14], human plasma [4,14–23], cerebrospinal fluid [24], urine [24], animals [25–27], insect models [13,28], cell lines [29] and bacterial lysate [24]. The developed high performance liquid chromatography (HPLC) methods utilize either mass spectrometry (MS) [13,14,17,19,21,25–28], ultra-violet (UV) [4,8–12,15,16,18,22,24,29–31,20] or high-performance thin layer

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chromatography (HPTLC) [7]. In general, the HPLC-UV method is the most widely used and is reported to be the most suitable for trough IM plasma level determination in clinical practice [15]. To date, there have been three studies comparing HPLC-UV and LC–MS/MS methods [13]. Rochat et al. [13] reported that IM levels determined using LC–MS/MS were comparable to those obtained using HPLC-UV, which implies that the HPLC-UV method is equally applicable if not better than the LC–MS/MS method. However, not many researchers have reported on a simultaneous detection method for both IM and M1.

The use of IM-D_8 as an isotopically labeled internal standard can be hazardous to the operator and will increase the cost-per-sample analysis. Some proposed HPLC-UV methods are able to separate IM and M1, but the peaks are not well resolved. Meanwhile, although the methods proposed by Schleyer et al. [24] and Davies et al. [18] managed to separate IM from M1, they involved a long total analytical time of 35 min. Many methods also require gradient elution analysis, which may cause inconsistent retention times of analyte peaks when compared to isocratic methods [32].

Our objective is to develop and validate a new HPLC method that is fast and that allows for the simultaneous analysis of both M1 and IM in a small volume of plasma for a routine application. In this paper, we report a new and sensitive UHPLC method for the simultaneous analysis of IM and M1 in human serum, which was validated based on FDA guidelines [33] and is suitable for routine clinical application.

2. Experimental

2.1. Chemicals and reagents

Drug standards IM and M1 were purchased from TLC Pharmachem., Inc (Ontario, Canada) (Fig. 1). Internal standard candidates such as pyrilamine, theophylline, imipramine, procaine, diphenhydramine, ephedrine, captopril, enalapril, propranolol and ibuprofen were purchased from Sigma (St Louis, MO, USA). Acetonitrile (ACN) and methanol (MeOH) were HPLC-grade and were purchased from Merck (Darmstadt, Germany). Blank plasma samples used for the preparation of calibration and control samples were obtained from expired blood from the hospital blood transfusion units. Ultrapure water for the mobile phase was deionized and purified using a Milli-Q® ultrafiltration-Plus system (Millipore Corp., Burlington, MA, USA).

2.2. Chromatographic conditions

The UHPLC system was an Agilent™ 1200 series Rapid Resolution Liquid Chromatography (RRLC) System (Agilent™ Technologies Inc., Waldbronn, Germany), which consisted of a G1367D High Performance Autosampler SL Plus and a model G1315C Diode-Array SL detector. The controller consisted of DELL® compatible computer equipped with LC Chemstation® software (Agilent™ Technologies Inc., Waldbronn, Germany). Separation was performed using a narrow-bore column Agilent™ *ZORBAX* Eclipse Plus C_{18} (50 mm × 2.1 mm, i.d.; 1.8 μm) (Agilent™ Technologies, Wilmington, DE, USA) and a mobile phase consisting of acetonitrile: potassium dihydrogen phosphate (0.05 M) buffer solution (25:75, v/v). The pH of the buffer was adjusted to 3.0 using 85% orthophosphoric acid followed by the addition of 0.2% triethylamine (TEA). Polytetrafluoroethylene (PTFE) filtration filters (0.22 μm) (Whatman®, England) were used to filter the mobile phase. The flow rate was 0.05 mL/min and the detection wavelength was set at 235 nm.

Imatinib Mesylate

N-desmethyl Imatinib

Pyrilamine Maleate

Fig. 1. Chemical structures of imatinib mesylate (IM), *N-desmethyl* imatinib (M1) and pyrilamine maleate (PM).

2.3. Data processing

LC Chemstation® software (Agilent Technologies, USA) workstation was used for data acquisition, data processing and reporting. Microsoft® Excel (version 2007) and Origin® pro (version 8.0) (OriginLab® corporation, Northampton, MA, USA) were used for data processing, statistical analysis and graphing. Quantitation was performed by integration of the area under the specific chromatographic peaks. The calibration curves were generated using a linear least squares regression analysis of the calibration samples according to the equation $y = a + bx$ (where "y" is the peak area substance/peak area internal standard, "x" is the concentration of analyte in the calibration sample, "a" is the intercept and "b" is the

mAU 0 20 40 60 80 100
200 225 250 275 300 325 350 375 nm
PM IM M1

Fig. 2. UV spectra of IM (10 μg/mL), M1 (10 μg/mL) and PM (20 μg/mL) obtained using a photodiode array (PDA) detector.

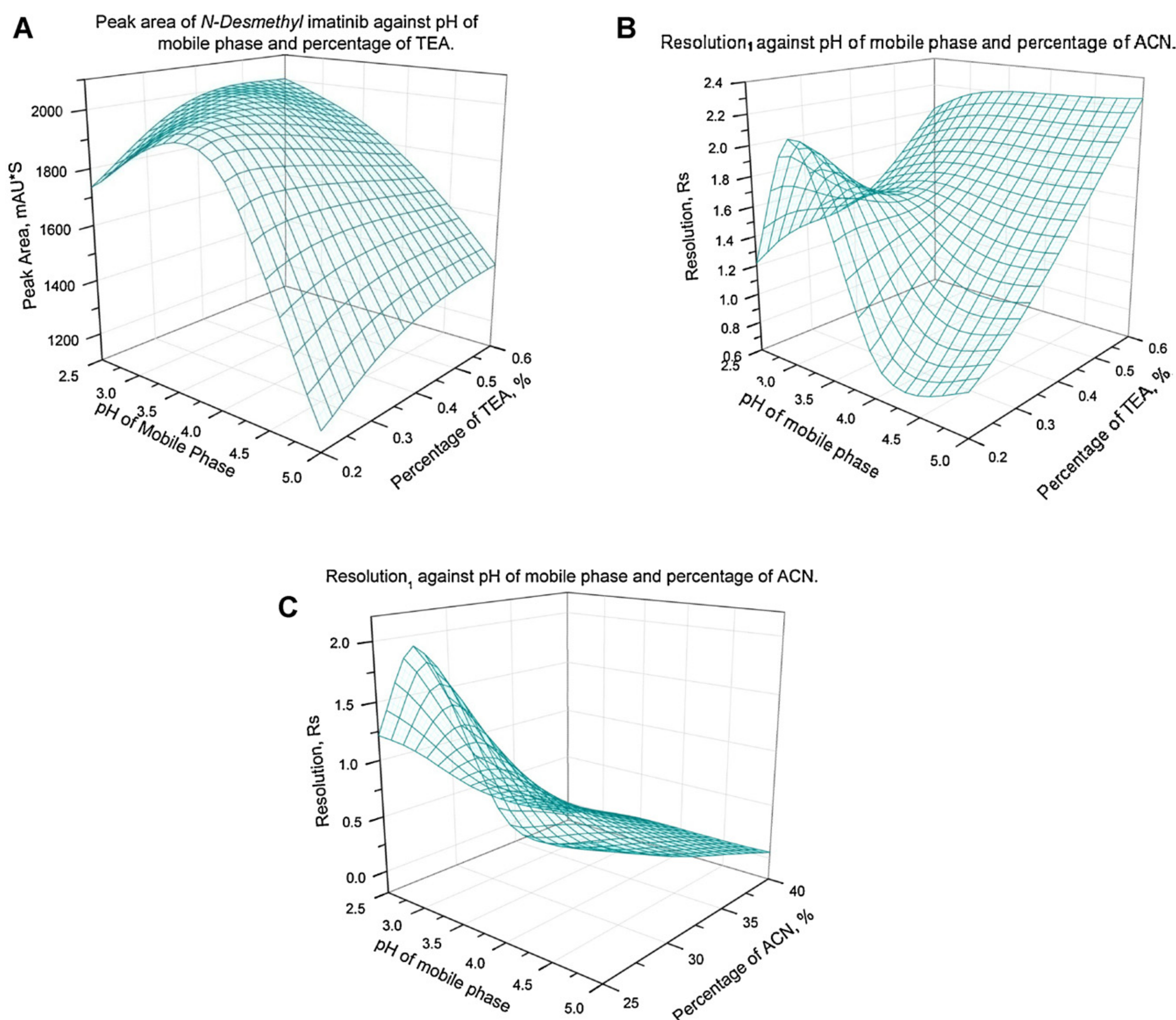


Fig. 3. Representative three-dimensional (3D) surface plots demonstrate the predicted responses and inter-relationship of chromatographic variations. (A) Relationship between the pH of the mobile phase and the percentage of TEA in response to M1's peak area IM's retention time. (B) Relationship between the pH of the mobile phase and the percentage of TEA in response to resolutions (R_1) between peak IM and M1. (C) Relationship between the pH of the mobile phase and the percentage of ACN in response to resolutions (R_1) between peak IM and M1.

slope of the regression line). For pharmacokinetics data analysis, PK Solutions 2.0™ (SummitPK® Research Services, Montrose, CO, USA) was used.

2.4. Stock solutions, standards and quality control samples (QC)

Stock solutions of IM and M1 were prepared by dissolving an accurately weighed quantity of the drug into deionized water to obtain a final concentration of 1 mg/mL. A working solution of the internal standard (IS) was made using pyrilamine maleate (1 mg/mL). All stock solutions were stored at 4 °C in glass volumetric flasks with caps tightly wrapped with Parafilm® and were aluminum-wrapped to protect them from light. Drug standards IM (10 μg/mL), M1 (10 μg/mL) and pyrilamine maleate (20 μg/mL) were injected along with samples during every analytical run.

The calibration standards were prepared daily by spiking an appropriate aliquot of the stock solution into the blank human plasma samples. Six calibration standards (50, 600, 800, 1000, 1500 and 1800 ng/mL for IM and 50, 150, 200, 250, 300 and 360 ng/mL for M1, respectively) as well as three quality control samples (QC) (500, 900 and 1600 ng/mL for IM and 100, 220 and 320 ng/mL for M1, respectively) were prepared by spiking a pre-calculated volume per batch daily. One blank plasma and one zero plasma were also injected each time.

2.5. Plasma collection

The study protocol was approved by the local Institutional Ethics Committee and complies to the Declaration of Helsinki. Patients with CML received 400 mg (po) IM after signing written informed consent forms. The exact time of blood sampling, daily regimen, dose, date and exact time of the last IM administration were recorded. Blood samples (3 mL) were collected at 0, 2 and 4 h by venipuncture at the antecubital fossa during the patients' regular medical visits. The blood was put into plain tubes before being immediately transferred to the lab.

The blood was centrifuged at 2000 × g for 10 min at 4 °C and the serum was transferred into polypropylene test tubes and stored at −20 °C until analysis. The IS working solution was added to each plasma patient sample in a polypropylene microcentrifuge tube before extraction.

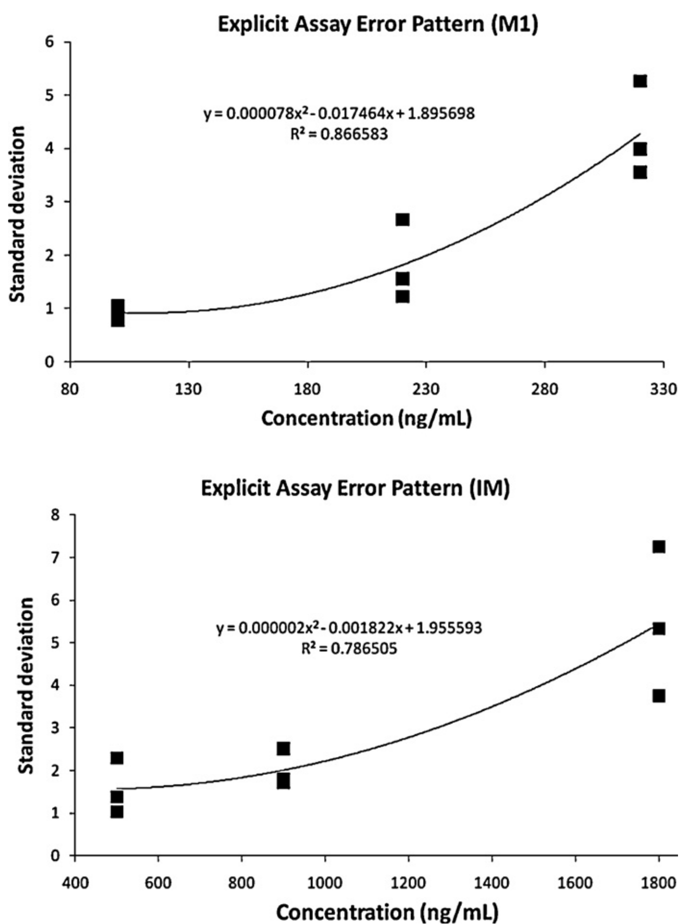


Fig. 4. Explicit assay error pattern of concentrations for both M1 and IM using the proposed UHPLC method.

2.6. Plasma sample extraction using a protein precipitation (PPT) procedure

An appropriate aliquot of drug standard stock solution was spiked into a plasma sample aliquot (200 μL). The resulting sample was subjected to protein precipitation using cold ACN (200 μL) (4 °C) and was vortex-mixed. The mixture was then centrifuged at 4 °C for 15 min at 7000 × *g*. The supernatant was transferred into a 0.22 μm polypropylene microcentrifuge filter tube reservoir and was subsequently centrifuged and filtered at 10,000 × *g* for 15 min at 4 °C. The steps were repeated three times using cold ACN (200 μL) precipitation. The resultant filtrate was dried under nitrogen for 20 min and then reconstituted with 200 μL deionized water. Cloudiness in the sample would suggest undissolved proteins. In this case, the sample should be further centrifuged at 12,000 × *g* until a pellet is formed at the bottom and then the supernatant is separated and transferred to a new microtube. For analysis, 20 μL of the final sample was injected into the HPLC system.

2.7. HPLC method optimization

2.7.1. Chromatography

To establish the suitable wavelength to be used, IM, M1 and the IS were scanned for their maximum UV absorbance using the Diode Array Detector (DAD). We investigated a total of nine possible drugs such as pyrilamine maleate, theophylline, imipramine, procaine, diphenhydramine, ephedrine, captopril, enalapril, propranolol and ibuprofen as potential internal standards.

During the optimization of the sample injection volume, five different injection volumes (1, 3, 5, 10 and 20 μL) were tested. To achieve a peak with good symmetry and large peak area, three different buffer types were tested: potassium dihydrogen phosphate (KH_2PO_4), ammonium acetate (CH_3COONH_3) and ammonium formate (NH_4HCO_2). Three different buffer solution pHs (2.5, 3.0 and 4.0), which were within the range as recommended by the supplier to be suitable for the column, were also investigated. Care must be taken when determining the optimum pH for the mobile phase, as IM has four distinct pK_a values [32]. The buffer pH was adjusted using 85% ortho-phosphoric acid. The suitable buffer type and pH determined above were further tested at three different concentrations (0.01 M, 0.03 M and 0.05 M).

Using the most suitable buffer type, pH and concentration, the appropriate ratio of ACN:KH_2PO_4 was tested at three different combinations (40:60, 30:70 and 25:75, v/v). We also investigated the effects on the peak properties of three different types of organic modifiers: triethylamine, 1-octane sulphonic acid sodium salt and acetic acid. Three different concentrations of TEA (0.01, 0.05 and 0.10%, v/v), which was the selected organic solvent, were tested to determine the optimum concentration to improve peak symmetry.

Three different column oven temperatures (25 °C, 35 °C and 40 °C) were also investigated to examine their effects on the peak area. Then, mobile phase flow rates were varied (0.05, 0.1, 0.15 and 0.2 mL/min) and tested. Finally, system suitability parameters, such as retention factor (k), the number of theoretical plates (N) and resolution (R_s), were calculated.

2.7.2. Robustness testing

Robustness is defined as a measure of a method's capacity to remain unaffected by small but deliberate variations in method parameters (ICH, 2001) [34]. We investigated robustness by using a three-dimensional response surface methodology (RSM). RSM is useful for analyzing problems where several independent variables may influence the dependent variable or response with the goal of determining the most optimal UHPLC-UV chromatographic condition.

2.8. Method validation

2.8.1. Calibration curve and linearity

The linearity of the method was evaluated over the concentration range of 50–1800 ng/mL for IM and 50–360 ng/mL for IM in plasma. The correlation coefficient was calculated based on the peak-area ratio of the drug to the IS versus the concentrations of the respective analytes in each standard sample.

Calibration curves were created by plotting the peak area ratios of each drug relative to the IS against the various drug concentrations in the spiked plasma standards and the samples were injected in duplicates. A linear regression was used to obtain the best fit line for all of the calibration points.

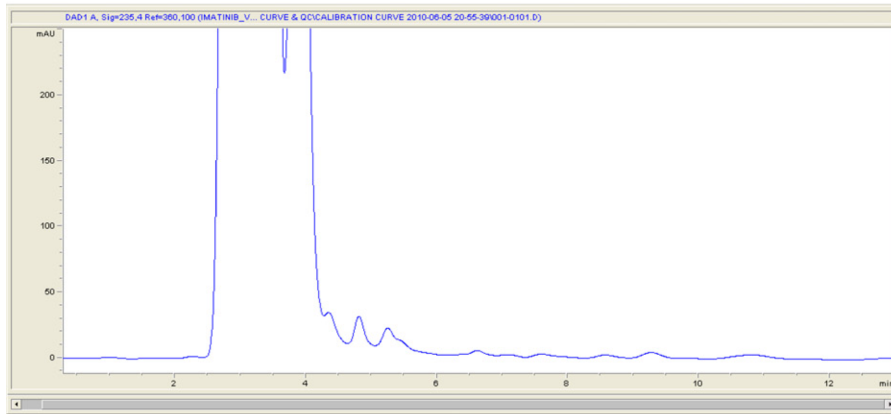
2.8.2. Accuracy and precision

Accuracy and precision were based on three different concentrations of QC samples reflecting low, medium and high concentrations (500, 900 and 1600 ng/mL and 100, 220 and 320 ng/mL for IM and M1, respectively). Accuracy was calculated as the percentage of the measured concentration from the nominal concentration. Precision was calculated as the percentage coefficient of variation (CV %) within a single run (intra-day) and between assays done on different days (inter-day).

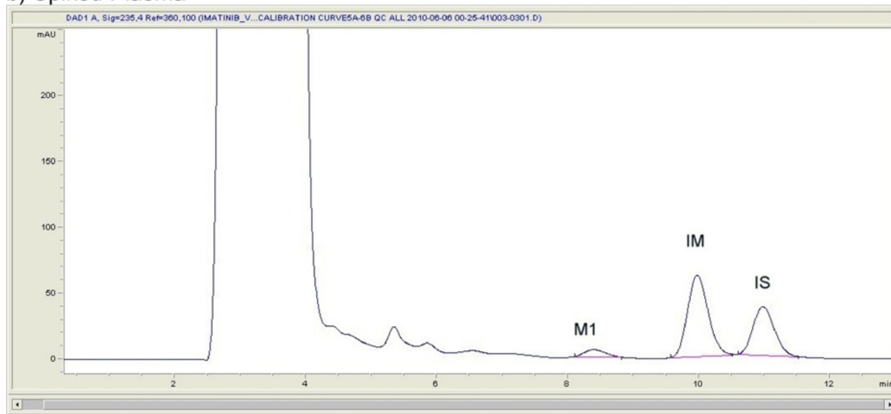
2.8.3. Recovery

The absolute recovery of IM from plasma was obtained as the peak-area response of IM in the extracted serum and was expressed

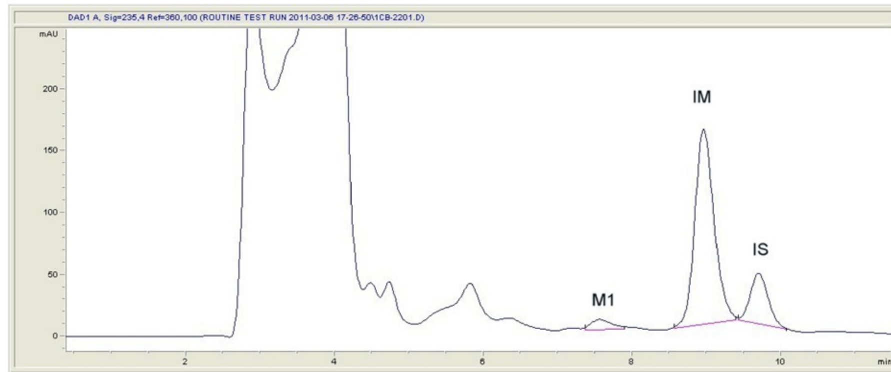
a) Blank Plasma



b) Spiked Plasma



c) Patient's Sample



M1: N-desmethyl imatinib; IM: Imatinib; IS: Internal standard

Fig. 5. Representative chromatograms of blank plasma extract, spiked plasma sample and a patient's sample.

as the percentage of the response of the same concentration in a drug standard.

2.8.4. *Specificity*

To ensure that no endogenous compounds would interfere with the peaks of interest, six different blank plasma samples spiked with commonly co-administered drugs prescribed to CML patients at our hospital: allopurinol, prednisone, diclofenac, ranitidine, acetaminophen and atenolol.

2.8.5. *Sensitivity*

The lowest limit of quantification (LOQ) is the lowest concentration that can be determined with a relative standard deviation <20% from the nominal concentration [4]. The limit of detection (LOD) is considered as the concentration of IM that provides a signal corresponding to three times the HPLC background signal [4].

2.8.6. *Stability*

Short-term (bench top, room temperature) and long-term stabilities for both M1 and IM were studied. Triplicates of the low

Table 1
System suitability parameters.

Compound	Retention time (min)	Capacity factor (k)	Resolution (R_s)	N (plate count)	T (tailing factor)
M1	7.0	5.1	4.2	40,876	1.7
IM	9.2	6.2	2.1	39,295	1.8
IS	10.6	8.1	2.3	62,384	1.7

(150 ng/mL of M1; 750 ng/mL of IM) and high (320 ng/mL of M1; 1700 ng/mL of IM) concentrations of these drugs in plasma samples were kept frozen at −20 °C. For the freeze and thaw stability test, plasma samples were subjected to three freeze–thaw cycles before analysis. For short term temperature stability, plasma samples were frozen for 24 h before analysis. For long term stability tests, samples were stored at −20 °C and analyzed after 1, 3 and 6 months of storage.

2.9. Application of the UHPLC method

The developed and validated method was used to analyze serum samples from three CML patients on oral IM treatment at 400 mg daily. Blood samples (3 mL) were collected from patients at 0, 2 and 4 hrs post-dose.

3. Results and discussion

In this study, we have successfully developed and validated a new UHPLC-UV method for the determination of both IM and M1 in human serum. We demonstrated a robustness test using RSM to predict and select the optimum conditions for separation.

The UV-absorption spectra showed two wavelength maximums at 235 nm and 265 nm (Fig. 2). Although absorbance at 200 nm gave the highest reading, this wavelength is too close to the UV cut-off of most solvents and may create a noisy baseline. Absorbance at 235 nm gave the second most optimal detection for M1, IM and the IS; therefore, this wavelength was selected.

To select the most suitable IS, nine drug standards were tested that were chosen based on their chemical structures and similar physicochemical properties to IM. Of these nine, pyrilamine maleate (PM) was selected because its peak was distinguishable from the baseline, it had a good peak shape and it was well separated from both the IM and M1 peaks. Furthermore, PM is chemically similar (a heterocyclic base) to IM and M1 (Fig. 3).

During the UHPLC optimization, nine parameters were varied for investigation: (1) injection volume, (2) pH buffer, (3) type of buffer, (4) buffer concentration, (5) percentage of organic solvent, (6) the effect of adding organic modifiers, (7) percentage of organic modifier and (8) flow rate.

It was observed that as the injection volume increased, the peaks tended to become taller. An injection volume of 20 μL, which produced a larger peak area was finally selected.

The pH of the buffer is also critical. Because both IM and M1 are basic drugs [1], they are able to gain a proton and become ionized (less hydrophobic) in low pH conditions. Thus, lowering the pH increased the peak area and decreased the retention times. It was concluded that pH 3.0 successfully separated IM and M1, giving the best area count, and peak symmetry as well as showing a good resolution (R_s = 2.1).

The type of buffer solution also plays a role in producing optimized separations. In this experiment, we have selected KH_2PO_4 as the buffer because it gave the largest area, best peak symmetry and shape.

The buffer solution concentration also plays an important role. Retention times of the peaks tend to decrease with an increase

Table 2a
Linearity data for IM calibration standard response values.

Calibration	Set 1	Set 2	SD	Mean
Day 1				
Intercept	0.01164	0.00941	0.00166	0.01053
Slope	0.00101	0.00103	0.00001	0.00102
Correlation coefficient (r^2)	0.997	0.997	0	0.999
Day 2				
Intercept	0.03452	0.03016	0.00206	0.03234
Slope	0.00097	0.00098	0.00001	0.00098
Correlation coefficient (r^2)	0.999	0.998	0.00071	0.999
Day 3				
Intercept	0.02967	0.04063	0.00775	0.03515
Slope	0.00094	0.00093	0.00001	0.00094
Correlation coefficient (r^2)	0.997	0.997	0	0.997

Table 2b
Linearity data for M1 calibration standard response values.

Calibration	Set 1	Set 2	SD	Mean
Day 1				
Intercept	0.00446	0.00211	0.00166	0.00329
Slope	0.00075	0.00076	0.00001	0.00075
Correlation coefficient (r^2)	0.999	0.999	0	0.999
Day 2				
Intercept	0.00376	0.00473	0.00069	0.00425
Slope	0.00074	0.00073	0.00001	0.00074
Correlation coefficient (r^2)	0.999	0.998	0.00071	0.999
Day 3				
Intercept	0.00542	0.00423	0.00084	0.00048
Slope	0.00068	0.00069	0.00001	0.00069
Correlation coefficient (r^2)	0.999	0.999	0	0.999

Table 3
Accuracy and precision for the determination of IM and M1 in human plasma.

Concentration (ng/mL)	Mean	SD	Precision RSD (%)	Accuracy (%)	Percentage difference (%)
Imatinib mesylate					
Intra-day (within batch)					
500	499.83	1.38	0.28	99.97	−0.03
900	900.03	1.71	0.19	100.00	0.00
1800	1802.93	3.76	0.21	100.16	+0.16
Inter-day (between batch)					
500	500.18	1.21	0.25	100.04	+0.04
900	902.95	1.76	0.19	100.33	+0.33
1800	1800.35	4.55	0.26	100.02	+0.02
M1					
Intra-day (within batch)					
100	99.39	0.77	0.77	99.39	−0.61
220	217.63	1.22	0.56	98.92	−1.08
320	319.39	5.27	1.65	99.80	−0.02
Inter-day (between batch)					
100	99.00	0.91	0.92	99.00	−1.00
220	220.39	1.95	0.88	99.82	−0.18
320	321.20	4.41	1.38	100.38	+0.38

in buffer concentration. There was also improvement in the peak area, which is best explained by the increased ionic strength of the mobile phase. Finally, KH_2PO_4 at 0.02 M was selected to preserve the column life as too concentrated buffer solutions may cause salt precipitation.

The percentage of organic solvent is an important factor in influencing separation. In our study, although a higher percentage of ACN gives bigger peak areas, they also tend to reduce the retention times of the drugs. However, at more than 30% ACN, the peaks were not properly resolved. The composition of a 25% ACN and 75% KH_2PO_4 buffer combination was finally chosen as it gave a faster retention time, well-resolved peaks and the least band tailing.

Organic modifiers can reduce peak tailing, which can be caused by secondary interactions occurring between a protonated base (M1 and IM) and an acidic silanol on the surface of the silica stationary phases. Several changes can be made to solve this problem: (1) mobile phase pH can be made more acidic to allow the protonation of silanol groups and (2) organic modifiers can be added to improve peak symmetry. In our experiment, TEA was selected based on these reasons.

During optimization of the percentage of TEA, 0.2% TEA concentrations (v/v) gave the best peak symmetry and the earliest retention time with good resolution (>2.0), which fulfills FDA guidelines [33]. Therefore, TEA was used at this percentage.

The use of low particle size columns such as sub-2 μm, which was used in our study, can lead to cost reduction because less volume of mobile phase will be used. Additionally, using a narrow-bore column allows for slower flow rates, which increase the resolution and improve LC performance by shortening the analytical time [35]. Our observation was that slower flow rates tend to increase the peak area. Therefore, a flow rate at 0.05 mL/min was chosen.

Table 4
Short- and long-term stability studies.

Stability sample	Low concentration M1 (150 ng/mL)	IM (750 ng/mL)	High concentration M1 (320 ng/mL)	IM (1700 ng/mL)
(A) Short-term stability (1 day)				
Replicate 1	145.34	739.01	328.61	1581.73
Replicate 2	145.07	745.04	315.08	1604.13
Replicate 3	157.44	742.00	315.19	1573.77
Mean	145.20	742.02	321.85	1592.93
SD	0.19	4.26	9.57	15.84
CV (%)	0.12	0.57	2.97	0.99
(B) Freeze–thaw cycle				
Replicate 1	152.42	320.08	759.71	1696.39
Replicate 2	150.80	322.72	756.26	1697.30
Replicate 3	148.10	320.14	758.79	1683.31
Mean	151.62	321.40	757.99	1696.84
SD	1.14	1.86	2.44	0.64
CV (%)	0.75	0.58	0.32	0.04
(C) One week long-term stability				
Replicate 1	147.34	320.12	751.15	1674.41
Replicate 2	154.82	328.35	745.04	1707.73
Replicate 3	153.66	323.63	745.48	1714.00
Mean	151.08	324.24	748.09	1691.07
SD	5.28	5.82	4.32	23.56
CV (%)	3.5	1.79	0.58	1.39
(D) One month long-term stability				
Replicate 1	146.66	312.18	752.63	1692.21
Replicate 2	147.66	313.81	747.55	1709.03
Replicate 3	145.73	317.40	752.75	1690.84
Mean	146.68	314.46	750.09	1700.62
SD	0.71	1.15	3.59	11.88
CV (%)	0.48	0.36	0.48	0.70

Table 5
Pharmacokinetic parameters of IM and M1 in three patients (P1, P2 and P3).

Pharmacokinetic parameters (unit)	P1	P2	P3	Mean
(a) IM				
K_a (h^{-1})	0.21	0.25	0.26	0.24 ± 0.03
V_d (L)	152.71	66.61	120.65	113.32 ± 43.52
K_{el} (h^{-1})	0.024	0.08	0.049	0.05 ± 0.03
CL ($L h^{-1}$)	3.66	5.46	5.91	5.01 ± 1.19
$t_{1/2}$ (h)	28.83	8.45	14.14	17.14 ± 10.52
C_{max} (ng/mL)	3 08	6400	3750	3415.00 ± 473.76
t_{max} (h)	1.80	2.40	2.80	2.33 ± 0.50
$AUC_{0-24 h}$ ($ng h L^{-1}$)	53,780	73,260	61,825	62953.33 ± 9791.38
(b) M1				
K_a (h^{-1})	0.12	0.157	0.036	0.104 ± 0.06
V_d (L)	384.30	203.61	657.90	415.27 ± 228.72
K_{el} (h^{-1})	0.005	0.02	0.014	0.013 ± 0.01
CL ($L h^{-1}$)	1.92	3.40	9.21	4.84 ± 3.85
$t_{1/2}$ (h)	138.60	34.65	49.50	74.25 ± 56.22
C_{max} (ng/mL)	250.00	445.00	184.00	293.00 ± 135.71
t_{max} (h)	3.60	2.00	4.20	3.27 ± 1.14
$AUC_{0-24 h}$ ($ng h L^{-1}$)	4969.00	8795.00	2991.46	5585.15 ± 2950.42

In summary, optimal chromatographic separation was achieved using an isocratic flow rate of 0.05 mL/min (25:75 acetonitrile:potassium dihydrogen phosphate + triethylamine 1.0%) on an Agilent® ZORBAX *Eclipse plus* a C18 reversed phase column (50 mm × 2.1 mm I.D., 1.8 μm) at 30 °C with a total run time of 13 min. The pH of the buffer–triethylamine combination was adjusted to 3.0 using 85% ortho-phosphoric acid and analyte detection was performed at 235 nm. Pyrilamine maleate was used as the internal standard.

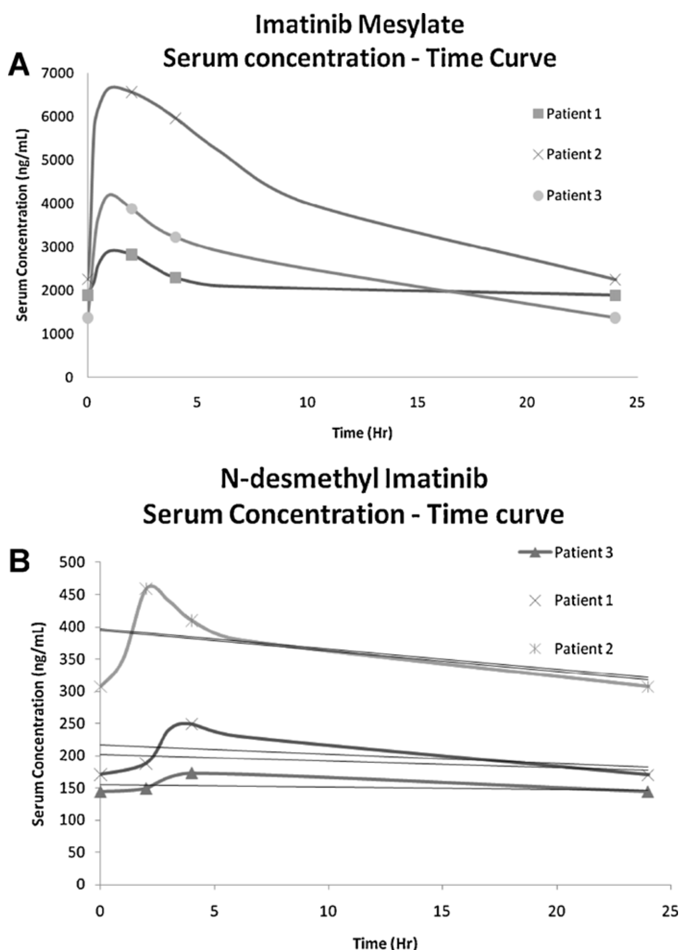


Fig. 6. Mean concentration–time profiles for IM and M1 in three patients following a single 400 mg (po) dose of IM.

The average retention times for M1, IM, and IS were 7.1 ± 0.1, 8.3 ± 0.1 and 9.6 ± 0.1 min, respectively. Using the optimized parameters, system suitability parameters were calculated (Table 1) and were found to be within the recommended FDA guidelines [33].

For validation, calibration curves over the ranges of 50–1800 ng/mL for IM and 50–360 ng/mL for M1 were linear as described by a linear regression analysis (Tables 2a and 2b). Over the range, the regression coefficient, r^2, of the calibration samples was greater than 0.99. All observed data (intra-day and inter-day precisions) were below 15% (Table 3). The accuracy ranged between 85 and 115%, which fulfills the FDA requirement. The recovery calculated from the three QCs concentration for both IM and M1 ranged between 90 and 105%.

The stability of the plasma samples indicated that at room temperature the plasma samples were stable for at least 24 h (Table 4). No significant loss of either IM or M1 was observed after up to three freeze–thaw cycles. No evidence of IM decomposition was found when plasma samples were stored in the freezer at −20 °C for up to 1 month, which indicates that the sample is stable for up to one month when stored at the abovementioned temperature.

Sample preparation is also a critical factor in the method development and validation. The PPT method is rapid and simple, but samples may contain partially dissolved proteins. Therefore, extreme care is needed to prevent blockage in the small narrow-bore pore size (95 Å) column used for analysis. The use of microscale 100% cold organic solvent as in our method is able to shorten the time for drying the samples and reduce chemical exposure to the researchers. The method can easily be applied in any laboratory as opposed to using an automated solid-phase extraction or a semi-automated high-throughput precipitation procedure.

To date, seven HPLC-UV methods for IM quantification in human plasma have been proposed [4,15,19,21,22,24,31]. Our proposed method is advantageous because it is simple, uses a PPT as a pre-treatment step, yields a high recovery (90% for IM and 95% for M1), shows high accuracy and precision, has a lower cost of analysis and utilizes a shorter analysis time of 13 min. Previously described methods require sample enrichment [24], a solid-phase extraction or a sample pre-treatment involving a liquid–liquid extraction. Furthermore, there were methods requiring the use of 500 μL plasma [19], 750 μL [4] of plasma (versus only 200 μL of serum required in our method). Our proposed UHPLC method is suitable for monitoring trough IM plasma levels in CML patients. The PPT extracted

IM and M1 were found to be successfully free from interferences in the samples as observed in the specificity test when six potentially co-administered drugs in CML patients were added to the samples.

The assay error pattern (Fig. 4) and coefficient of variation derived from validation data will be helpful in both population pharmacokinetic studies and therapeutic drug monitoring. A representative relationship between the measured concentration and assay error patterns, which were demonstrated by a polynomial equation, is usually of second-order formulae (parabolic-shaped) [36]. Using this equation, it is easy to calculate the probable standard deviation with any subsequent single serum concentration that is measured within that range [36].

The representative chromatograms of blank plasma extract, spiked plasma sample and a patient's sample is shown in Fig. 5.

3.1. The serum concentration–time profile of IM and M1 in human serum

The method was successfully applied to measure the serum concentration–time profile of three CML patients in IM and M1 in serum (Fig. 6). The mean elimination half-lives of IM was 13.8 h^{-1} and is comparable with that 18 h^{-1} previously reported [37] (Table 5).

4. Conclusion

In conclusion, a simple, cheap and sensitive method validated according to the FDA's guidelines [33] has been successfully developed and used to calculate patients' pharmacokinetic profiles. To our knowledge, this is the first UHPLC-UV method that simultaneously separates M1 and IM from human serum. Our method is suitable to be used in routine clinical analysis due to its high extraction efficiency, good reproducibility and due to the simultaneous quantification of the three drugs using small volumes of plasma (200 μL).

Future studies should focus on determining the effect of IM and M1 on clinical response. The proposed method will be used to correlate the steady-state trough serum concentrations and to study the concentration–time profile of CML cases.

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References

[1] J. Zimmermann, G. Caravatti, H. Mett, T. Meyer, M. Muller, N.B. Lydon, D. Fabbro, Arch. Pharm. (Weinheim) 329 (1996) 7.
[2] S. Sweetman, Martindale: The Complete Drug Reference, 36 ed., Pharmaceutical Press, London, 2009.
[3] R. Nimmanapalli, K. Bhalla, Curr. Opin. Oncol. 14 (2002) 6.
[4] N. Widmer, A. Béguin, B. Rochat, T. Buclin, T. Kovacsovics, M.A. Duchosal, S. Leyvraz, A. Rosselet, J. Biollaz, L.A. Decosterd, J. Chromatogr. B 803 (2004) 7.
[5] R.A. Larson, B.J. Druker, F. Guilhot, S.G. O'Brien, G.J. Riviere, T. Krahnke, I. Gathmann, Y. Wang, Blood 111 (2008) 8.
[6] S. Picard, K. Titier, G. Etienne, E. Teilhet, D. Ducint, M.A. Bernard, R. Lassalle, G. Marit, J. Reiffers, B. Begaud, N. Moore, M. Molimard, F.X. Mahon, Blood 109 (2007) 8.
[7] N. Vadera, G. Subramanian, P. Musmade, J. Pharm. Biomed. Anal. 43 (2007) 6.
[8] B. Girish, K. Sivacharan, K. Venugopal, S. Ranendra, Chromatographia 66 (2007) 11.
[9] M.A. Rosasco, M.A. Moyano, M.T. Pizzorno, A.I. Segall, J. Liquid Chromatogr. Relat. Technol. 28 (2005) 20.
[10] M. Medenica, B. Jancic, D. Ivanovic, A. Malenovic, J. Chromatogr. A 1031 (2004) 1.
[11] D. Ivanovic, M. Medenica, B. Jancic, A. Malenovic, J. Chromatogr. B 800 (2004) 2.
[12] V.V. Vivekanand, D.S. Rao, G. Vaidyanathan, N.M. Sekhar, S.A. Kelkar, P.P. Ramachandra, J. Pharm. Biomed. Anal. 33 (2003) 4.
[13] B. Rochat, A.L. Fayet, N. Widmer, S.L. Lahrichi, B.T. Pesse, L.A. Decosterd, J.R.M. Biollaz, J. Mass Spectrom. 43 (2008) 1.
[14] G. Guetens, G.D. Boeck, M. Highley, H. Dumez, A.T.V. Oosterom, E.A.D. Bruijn, J. Chromatogr. A 1020 (2003) 1.
[15] O. Roth, O. Spreux-Varoquaux, S. Bouchet, P. Rousselot, S. Castaigne, S. Rigaudeau, V. Raggueneau, P. Therond, P. Devillier, M. Molimard, B. Maneglier, Clin. Chim. Acta 411 (2010) 3.
[16] D.F. Silvia, D.A. Antonio, D.M. Francesca, P. Elisa, B. Lorena, S. Marco, S. Marco, R. Silvia, S. Giuseppe, D.C. Francesco, D.P. Giovanni, J. Chromatogr. B 877 (2009) 3.
[17] A. Haouala, B. Zanolari, B. Rochat, M. Montemurro, K. Zaman, M.A. Duchosal, H.B. Ris, S. Leyvraz, N. Widmer, L.A. Decosterd, J. Chromatogr. B 877 (2009) 22.
[18] A. Davies, A.K. Hayes, K. Knight, S.J. Watmough, M. Pirmohamed, R.E. Clark, Leuk. Res. (2009).
[19] A. Awidi, I.I. Salem, N. Najib, R. Mefleh, B. Tarawneh, Leuk. Res. 34 (2009) 6.
[20] I. Solassol, F. Bressolle, L. Philibert, V. Charasson, C. Astre, F. Pinguet, J. Liquid Chromatogr. Relat. Technol. 29 (2006) 20.
[21] K. Titier, S. Picard, D. Ducint, E. Teilhet, N. Moore, P. Berthaud, F.X. Mahon, M. Molimard, Ther. Drug Monit. 27 (2005) 5.
[22] T. Velpandian, R. Mathur, N.K. Agarwal, B. Arora, L. Kumar, S.K. Gupta, J. Chromatogr. B 804 (2004).
[23] R.A. Parise, R.K. Ramanathan, M.J. Hayes, M.J. Egorin, J Chromatogr. B Analyt. Technol. Biomed. Life Sci. 791 (2003) 1.
[24] E. Schleyer, S. Pursche, C.H. Köhne, U. Schuler, U.U. Renner, H. Gschaidmeier, J. Freiberg-Richter, T. Leopold, A. Jenke, M. Bonin, T. Bergemann, P. le Coutre, M. Gruner, M. Bornhäuser, O.G. Ottmann, G. Ehninger, J. Chromatogr. B 799 (2004) 1.
[25] Y. Hsieh, G. Galviz, Q. Zhou, C. Duncan, Rapid Commun. Mass Spectrom. 23 (2009) 9.
[26] R. Bakhtiar, J. Lohne, L. Ramos, L. Khemani, M. Hayes, F. Tse, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 768 (2002) 2.
[27] R. Bakhtiar, L. Khemani, M. Hayes, T. Bedman, F. Tse, J. Pharm. Biomed. Anal. 28 (2002).
[28] M. Marull, B. Rochat, J. Mass Spectrom. 41 (2006) 3.
[29] G. Guetens, H. Prenen, G. De Boeck, A. van Oosterom, P. Schoffski, M. Highley, E.A. De Bruijn, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 846 (2007) 1.
[30] W.J. Szczepek, B. Kosmacinska, A. Bielejewska, W. Luniewski, M. Skarzynski, D. Rozmarynowska, J. Pharm. Biomed. Anal. 43 (2007) 5.
[31] R.L. Oostendorp, J.H. Beijnen, J.H.M. Schellens, O. Tellingen, Biomed. Chromatogr. 21 (2007).
[32] L.R. Snyder, J.J. Kirkland, J.L. Glajch, Practical HPLC Method Development, 2nd ed., Wiley-Interscience, New York, 1997.
[33] Guidance for Industry: Bioanalytical Method Validation, Center for Drug Evaluation and Research (CDER), Rockville, MD, USA, 2001, http://www.fda.gov/cder/guidance/index.htm.
[34] International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human use: Validation of Analytical Procedures, November, 2005 www.ich.org/.
[35] J. Schappler, R. Nicoli, D. Nguyen, S. Rudaz, J.L. Veuthey, D. Guillarme, Talanta 78 (2009) 2.
[36] R. Jelliffe, R. Schumitzky, A. Van Guilder, M. Liu, M. Hu, L. Maire, P. Gomis, P. Barbaut, X.B. Tahani, Ther. Drug Monit. 15 (1993) 380.
[37] A. Petain, D. Kattygnarath, J. Azard, E. Chatelut, C. Delbaldo, B. Geoerger, M. Barrois, S. Seronie-Vivien, A. LeCesne, G. Vassal, Clin. Cancer Res. 14 (2008) 21.