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Abstract

A new method using high performance liquid chromatography coupled with electrospray mass spectrometry is described for the quantification of PBMC concentration of tyrosine kinase inhibitors imatinib, dasatinib and nilotinib. A simple PBMC isolation and extraction procedure were applied on 10-14 mL of blood aliquots. Chromatographic separation of drugs and Internal Standard (quinoxaline) was achieved with a gradient (acetonitrile and water + formic acid 0.05%) on a C18 reverse phase analytical column with 25 min of analytical run, at flow rate of 0.25 mL/min. Mean intra- and inter-day precision for all compounds were 8.76 and 12.20%; mean accuracy was -3.86%; extraction recovery ranged within 79 and 91%. Calibration curves ranged from 50.0 to 0.25 ng. The limit of quantification was set at 0.25 ng for all the analyzed drugs.

This novel developed methodology allows a specific, sensitive and reliable simultaneous intracellular determination of the three tyrosine kinase inhibitors imatinib, dasatinib and nilotinib in a single chromatographic run, useful for drugs estimation in PBMC of patients affected by chronic myeloid leukemia.

1. Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder, characterized by the presence of the Philadelphia chromosome, consequence of a translocation 9-22, producing a fusion oncogene referred to as BCR-ABL, encoding for a BCR-ABL kinase [1]. Thus, the use of BCR-ABL-targeted therapy is the standard of care for this disease. Current frontline therapy for CML is imatinib (Gleevec™, STI-571), a 2-phenylaminopyrimidine-type inhibitor of the BCR-ABL kinase [2], that competitively inhibits the binding of ATP to the ATP binding pocket of BCR-ABL [3-4]. In the International Randomized Study of Interferon and STI571 (IRIS; ClinicalTrials.gov number, NCT00006343), imatinib has been associated with a superior response rate and improved progression-free survival, as compared with the previous standard therapy, interferon alfa plus low-dose cytarabine [5-7]. Eight-year follow-up of IRIS revealed that responses to imatinib were durable and had an acceptable adverse-event profile, with an estimated rate of overall survival of 85% [8]. Although most patients show excellent responses to imatinib treatment, nearly 20% of patients who take the drug do not have a complete cytogenetic response, and others may have intolerable side effects or drug resistance over time [8]. Resistance, mainly caused by point mutations, leads to a reduced affinity of imatinib for the ATP binding domain of the BCR-ABL protein and to a reactivation of the BCR-ABL kinase activity [9-10]. Loss of response and transformation to advanced disease occur mainly in the first 3 years of imatinib therapy, inducing a rate of overall survival often poor in these patients [8]. To overcome imatinib resistance and intolerance, more potent tyrosine kinase inhibitors (TKIs), such as dasatinib and nilotinib, have been developed for the treatment of CML. Dasatinib (Sprycel™, BMS-354825) is a structurally distinct drug which has a more potent activity than imatinib [11-12]. It also inhibits Src kinases, proteins that play a critical role in the development, growth, progression, and metastasis of a number of human cancers [13]. Dasatinib offers a new treatment option for patients with CML or Ph-positive acute lymphoblastic leukemia who are either unable to tolerate or resistant to previous therapy, including imatinib. Dasatinib has been found to be more effective in eliciting a cytogenetic

or hematologic response and better tolerated than high-dose imatinib. However, many factors play a role in determining whether dasatinib may provide benefit to patients. Some of these factors are known, such as BCR-ABL-sensitive mutations, whereas other mechanisms of CML not related to BCR-ABL are unknown or unclear [14]. Another second generation TKI is Nilotinib (Tasigna™, AMN107). It is a close analog of imatinib with higher potency and selectivity for BCR-ABL kinase inhibition *in-vitro* and *in-vivo* than imatinib [15-16]. Nilotinib was first approved in the United States and elsewhere in 2007 for patients with CML in the chronic or accelerated phase who had resistance to or could not tolerate imatinib [17-18]. In a phase 3 study, randomized, open-label, multicenter trial, authors compared the efficacy and safety of nilotinib with that of imatinib. Analysis was done for patients with newly diagnosed Philadelphia chromosome–positive CML in the chronic phase, with the rate of major molecular response at 12 months as the primary end point. Study showed more efficacy for nilotinib [19].

Due to the large TKIs proposal in CML treatment, imatinib-resistant patients may find the right path to reach clinical effectiveness. A good help may be also offered to this purpose by employing of therapeutic drug monitoring (TDM), essential tool, today, for the management of CML patients. Sub-inhibitory drug concentrations and sequential treatment with multiple TKIs can promote, in fact, the selection of BCR-ABL kinase domain mutations in CML patients. Measurement of antileukemia drugs plasma concentrations reached by treated patients, then, can be useful to monitor CML patients over time, leading the evaluation of patient adherence to daily oral therapy, potential drug–drug interactions, treatment efficacy, and severe drug-related adverse events [20-21]. In recent years, numerous laboratories have reported the use of high-throughput bioanalytical procedures for the single and simultaneous quantification of plasma concentrations of antileukemia drugs [22-35]. Based on validated high performance liquid chromatography (HPLC) methods, the plasma pharmacokinetics (PK) of imatinib has been well investigated, while only few paper on the PK of dasatinib and nilotinib are available to date [11-21-36-39]. If data miss about plasmatic PK, no literature exist at all related to intracellular quantification of all TKIs, included imatinib. Topic

that should be deeply investigated, instead, in order to have a better indication about ongoing treatment efficacy. Measurement of TKIs plasma concentrations, in fact, is a reliable tool to perform TDM; however, only the drugs fraction reaching the intracellular compartment is expected to exert action. Then a convenient correlation should be done, also, between clinical outcome and intracellular drug levels reached in treated patients. As variability in drug PK and inadequate patient compliance, also poor penetration of drugs into body compartments, particularly in leukocytes or peripheral blood mononuclear cell (PBMC), may contribute to the occurrence of sub-therapeutic drug level, leading loss of treatment efficacy. By the way the mechanisms by which TKIs drugs accumulate within cells remain generally unknown and it should be further investigated, because very few data are published to date [26-40-42]. Moreover, a number of transmembrane transport proteins, such as P-glycoprotein, the gene product of *ABCB*1 (*MDR*1), and related ABC (ATP binding cassette) B1 (ABCB1), ABCG2, such as solute carrier 22A1 (SLC22A1), solute carrier organic anion transporter family members 1B1 (SLCO1B1) and SLCO1B3 transporters are known to actively mediate the efflux and uptake of drugs from cells. The genes coding for these transport and regulatory proteins are polymorphic in humans, with consequences on the expression and function, potentially influencing the intracellular levels of TKIs drugs. In particular was observed in CML patients an association of SLCO1B3 polymorphism with intracellular accumulation of imatinib in leukocytes [42]. Thus, as intracellular concentrations of TKIs drugs are influenced by both their physico-chemical properties and host genetic factors, an assay enabling the monitoring of TKIs levels at the site of their pharmacological action, appears to be an essential tool for the ongoing investigations aimed at preventing TKIs therapy failure or toxicity.

Herein, aim of our study was to develop and validate an HPLC method coupled with electrospray mass spectrometry (HPLC–MS) detection for the simultaneous quantification of imatinib, dasatinib and nilotinib in human PBMC.

2. Experimental

2.1. Chemicals

Imatinib (Glivec™, STI-571) and nilotinib (Tasigna™, AMN-107) were kindly supplied by Novartis Pharma AG (Basel, Switzerland); dasatinib (Sprycel™, BMS-354825) was purchased from Sequoia Research (Pangbourne, United Kingdom). Acetonitrile HPLC grade and methanol HPLC grade were purchased from J.T. Baker (Deventer, Holland). HPLC grade water was produced with Milli-DI system coupled with a Synergy 185 system by Millipore (Milan, Italy). Quinoxaline (QX) and formic acid were obtained from Sigma–Aldrich (Milan, Italy). Lymphoprep was purchased from Sentinel Diagnostics (Milan, Italy). Blank cells (PBMCs) were isolated from the blood of healthy donors, kindly supplied by the Blood Bank of the Maria Vittoria Hospital (Turin, Italy).

2.2. Stock solutions, standards (STD) and quality controls (QC)

The stock solutions of imatinib, dasatinib and nilotinib were prepared by dissolving an accurately weighed amount of drug in methanol to obtain a final concentration of 1 mg/mL; all stock solutions were then stored at -20°C. The stock solutions were stored maximum 3 months.

The Internal Standars (IS) working solution was prepared with QX [1 μg/mL] in methanol and HPLC grade water (50:50 v/v) and stored at 4°C until use. The six calibration standards and three quality controls (QCs) were prepared adding a determined volume of stock solutions, or diluted stock solution, to each blank PBMCs aliquots before storage at –80°C, during no more than three months, in a manner similar to that described in other publications [43-44].

Calibration ranges, from STD 6 to STD 1, and QC amount for all drugs are listed in Table 1.

2.3 PBMC isolation.

Clinical samples were collected, after obtaining written informed consent according to local Ethics Committee indications, from patients treated with TKIs. Blood samples were collected in two EDTA tubes (2x7 mL).

PBMCs were isolated from 10 to 14 mL of blood using lymphoprep density gradient centrifugation (700 *g*, 25 min, 4°C with a Jouan Centrifuge [Model BR4i, Saint-Herblain, France]) at each sampling, as described previously [44]. PBMCs were then fast washed twice in 40 mL cold-ice phosphate-buffered saline and centrifuged (750 *g*, 6 min, 4°C).

The resulting pellet of washed PBMCs was dissolved with 1 mL extraction solution (methanol:water, 70:30 vol/vol), switched in two criovials (500 μl each) and then stored at –80°C until analyses, and for no longer than three months.

The time taken to process PBMCs from phlebotomy to methanol extraction solution was less than 1 hour, ensuring that sampling conditions were ice cold to prevent drug loss [45-46]. Blank PBMCs

isolated from the blood of healthy donors, as previously described, were stored in aliquots of around $5x10^6$ cells.

The cell number were afterwards determined by absorbance with a new procedure (paper in progress). This method used 10 microliters of stored unknown number PBMC aliquots versus 10 microliters of known numbers of PBMC reference aliquots. The known number of the reference aliquots were obtained by a Bekman Coulter Z2 (Instrumentation Laboratory, Milan, Italy), and managed by Z2 AccuComp Software (Version 3.01), without the use of the trypan blue exclusion.

The cellular volume used to calculate intracellular concentrations of TKIs was 285 femto-Liters, as described previously [47].

2.4. STD, QC and samples preparation

The stored aliquots of STDs, QCs and patient samples PBMCs were defrosted at room temperature. Fifty μl of IS working solution were added to each tube and the samples were vortexed for 10 seconds. STDs, QCs and patient tube samples were sonicated in an ice-water bath three time (Cycle 0,75; Amplitude 80%), to fully lyse PBMCs, using a sonicator UP-50 H (Dr Hielscher GmbH, Teltow, Germany). After a centrifugation at (7.000 *g*, 10 minutes at 4°C), 10 μl of supernatant were used for the cell count, and the remainder supernatant were collected into glass tubes. The remaining pellets were then washed by vortex for 10 seconds with 200 μl of acetonitrile:metanol solution (50:50, vol/vol), centrifuged (7.000 *g*, 10 minutes at 4°C) and each supernatant was collected in the indicated glass tubes to be treated by vortex-vacuum evaporation to dryness at 60°C.

Each extract was reconstituted with 60 μl of HPLC-grade water and acetonitrile solution (60:40, vol/vol) and 20 μl were injected into the column. For validation purposes, all samples were extracted and analyzed in duplicate. All procedure steps were carried out at room temperature, excluding that of sonication.

2.5. Chromatographic and MS conditions

The HPLC-MS instrument used was a Waters system (Milan, Italy), with binary pump model 1525, AF degaser, 717-plus autosampler, and Micromass ZQ mass detector. LC-MS Empower Pro software (version year 2002, Waters; Milan, Italy) was used.

The chromatographic separation was performed at 35°C using a column oven, on Atlantis T3 C-18 3μ column (150x2.1 mm I.D.) (Waters; Milan, Italy), protected by a Security Guard with C18 (4.0x3.0mm I.D.) pre-column (Phenomenex; CA, USA). The chromatographic run was performed with a gradient (Table 2), and the mobile phase was composed by HPLC grade water containing 0.05% formic acid, for mobile phase A, and HPLC grade acetonitrile containing 0.05% formic acid, for mobile phase B.

Detector settings were ESI, positive polarity ionization; capillary voltage 3.5kV; source temperature 110°C; desolvation temperature 350°C; nitrogen desolvation flow 800 l/h; nitrogen cone flow 100 l/h. Ions detected, in single ion recording (SIR) mode, were *m/z* 493.8 with a cone voltage of 45V for imatinib, *m/z* 487.5 with a cone voltage of 35V for dasatinib, *m/z* 529.5 with a cone voltage of 35V for nilotinib and *m/z* 313.0 with a cone voltage of 50V for QX (IS).

2.6. Specificity and selectivity

Interference from endogenous compounds was investigated by analysis of six different blank PBMC samples. Moreover interference from some potentially co-administered drugs were evaluated for: zidovudine, didanosine, stavudine, lamivudine, abacavir, tenofovir, emtricitabine, ethambutol, isoniazid, ribavirin, voriconazole, posaconazole and itraconazole (some of many drugs routinely analyzed in our laboratory). An “interfering drug” has been considered as a molecule

which exhibits a retention time close to 0.3 min from the analytes, and with the potential capability to cause ion suppression.

2.7. Matrix effect

"Matrix effect" was investigated using six different blank PBMC samples and comparing peak areas obtained from standard solutions of a solution of water and acetonitrile (60:40), containing all our analytes at three different concentrations, and peak areas obtained from blanks post-extraction solution with the same amount of analytes, as described by Taylor [48]. Possible "matrix effect" was calculated, as deviation %, comparing the peak area obtained from the PBMC extract with the peak area obtained from the standard solution.

2.8. Accuracy, precision, calibration and limit of quantification

Intra-day and inter-day accuracy and precision were determined by assaying ten spiked PBMC samples at three different concentrations (QCs) for each drug. Accuracy was calculated as the percent deviation from the nominal concentration. Inter-day and intra-day precision were expressed as the standard deviation at each QC concentration. Each calibration curve was obtained using six calibration points in duplicate, and the ranges are listed in Table 1. Calibration curves were created by plotting the peak area ratios of each drugs relative to the IS against the various drugs concentrations in the spiked PBMC standards. A quadratic regression was used for all curves in order to obtain the best fit for all calibration points. The limit of detection (LOD) in PBMC was defined as the concentration that yields a signal-to-noise ratio of 3/1. The lowest concentration levels that could determined with a percent deviation from the nominal concentration and relative standard deviation < 20%, was considered the lowest limit of quantification (LOQ), as requested by international guidelines [49].

2.9. Recovery

Recovery from PBMC, using the extraction procedures, was assessed by comparing the peak area obtained from multiple analyses (n=3) of spiked samples (QCs) with the peak area from standard solution of all analytes in a solution of water and acetonitrile (60:40) at the same concentrations, as described by international guidelines [49].

2.10. Stability

The stability of antileukemia drugs at different conditions has been previously assayed in many articles [3-25-32-34-50]. For this reason stability assays were not performed.

2.11 Patients PBMC samples

Patients receiving standard dosing of imatinib, dasatinib or nilotinib, underwent blood sampling after obtaining their informed consent for the measurement of PBMC drugs concentrations. Blood samples were collected in EDTA tube (2x7 mL) and processed as described above (see section 2.3).

3. Results

Time of analytical run was chosen as 25 min, according to the retention times of substances, their good separation and with the use of a wash column step and its re-equilibration, that allows to reduce potential ghost-peak interferences highlighted without the washing step. Our analyte retention times were 9.6±0.2 minutes for imatinib, 10.6±0.2 for dasatinib, 12.0±0.2 for nilotinib, 14.8±0.2 for QX. Representative chromatograms of a blank PBMC extracted and STD1 of imatinib, dasatinib and nilotinib are shown in Figure 1A, 1B and 1C, respectively. Representative chromatogram of a imatinib, dasatinib and nilotinib STD6 PBMC extracted and QX is shown in Figure 2. Mean regression coefficient (r^2) of all calibration curves was higher than 0.99 for all analytes.

3.1. Specificity and selectivity

The assay did not show any significant interference with other potentially concomitant drugs (see section 2.6). The tested six blank PBMC samples did not show any interference in the retention times analytes windows for each specified ion detected (Figure 1A, 1B and 1C).

3.2. Accuracy, precision, limit of quantification

Results of the validation of the method are listed in Table 3 for all analytes. All observed data (intra-day and inter-day precision [R.S.D.%]) were all below 15.0%, as request for FDA guidelines [49]. LOQ and LOD are listed in Table 1.

3.3. Recovery

Multiple aliquots (n=3) at each of the three QCs amounts were assayed and mean recovery of drugs were 79% for imatinib, 82% for nilotinib and 91% for dasatinib (mean CV, for all drugs, 6.8%). Mean IS recovery was 89% (mean CV 2.0%).

3.4. Matrix effect

The deviation % of the peak area at the three amounts for all analytes is comparable, ranging from –13.5% to -1.4% (mean -6.4%), showing the absence of the "matrix effect".

3.5. Analysis of plasma samples from treated patients

Method developed was applied for assaying of 52 PBMC samples, corresponding to different points of time–concentration curve, obtained from 40 patients treated with TKI drugs (28 with imatinib, 4 with dasatinib, 8 with nilotinib). Values obtained resulted in the expected range of concentrations, previously defined in our laboratory (unpublished data) and close to published results by different procedure and kind of analyzed cell [26-40-42]. The mean ratios (PBMC/plasma) of Ctrough concentrations were 7.8, 35.3 and 5.7 for imatinib, dasatinib and nilotinib, respectively.

4. Discussion and Conclusion

Targeted therapies using imatinib, dasatinib and nilotinib, based on the inhibition of protein tyrosine kinases, represent currently the therapeutic strategies for treatment of CML. TDM, recently, has become an essential tool for the management of CML patients, particularly for patients taking imatinib [21-39], which efficacy threshold in terms of plasma concentrations is clearly defined. In order to manage primary or acquired resistance to imatinib, clinical studies using dasatinib or nilotinib as second line therapy or combination of therapies with different TKIs, in sequential or simultaneous administration, are currently under evaluation [21-39]. Sub-inhibitory intracellular drug concentrations, probably, and sequential treatment with multiple tyrosine kinase inhibitors promote the selection of BCR-ABL kinase domain mutations in CML patients [51-52]. Poor penetration of drugs into leukocytes or PBMC, inadequate treatment adherence, and variability in drug PK may also contribute to the occurrence of sub-therapeutic drug level. In fact, only the fraction reaching the intracellular compartment is reasonably expected to exert a therapeutic action. Very few data are published to date [26-40-42], probably due to the difficult to develop, validate and use, a reliable method to quantify intracellular drug concentration. In this context, aim of our study, an HPLC–MS method setting for the simultaneous quantification of TKIs in human PBMC, has encountered current need, because no methods for this simultaneous intracellular determination of these drugs, are available up to now.

The method we've developed and validated, based on a very close PBMC isolation and drug extraction procedure published by our group for antiretroviral drugs [44]. This method can be used everywhere, because require instruments available in all laboratories. These procedures were then coupled to HPLC instrument with single mass detector.

For this purpose we have chosen as our internal standard quinoxaline, as it is easy to purchase, inexpensive (compared with deuterated drug isotopes) and reliable in its chemical behavior. This xenobiotic was fully used as IS in our other validated method [25-44-53-60], with UV and mass detectors. Moreover it is not a potential coadministred drug as was the case in other studies [41-42].

The method developed is suitable to be used in clinical studies due to its high extraction efficiency, its high sensitivity, its good reproducibility and due to the simultaneous quantification of the three drugs using a small volumes of blood (10-14 mL).

In fact, since no therapeutic window has been clearly defined for the imatinib, dasatinib and nilotinib in PBMC, as in plasma (excluding imatinib [36-38-39]), it could be interesting try to quantify intracellular concentrations of the three drugs and assess at first potential correlation with plasma concentrations, then a concentration-response relationship. At present no reports showed data related to intracellular levels. Moreover, measurement of antileukemia intracellular concentrations, even if more elaborated than TKIs plasma determination, can be useful, as plasma concentration, to evaluate patient adherence to daily oral therapy, treatment efficacy, severe drug-related adverse events, and potential drug–drug interactions, especially in patients who must take drugs interfering with cytochrome P450 [20].

Moreover, TKIs are substrate of transmembrane transport proteins encoded by ABCB1, SLCO1B1 and SLCO1B3 gene [42]. The genes coding for these transport are polymorphic with consequences on the expression and function, potentially influencing the intracellular levels of TKI drugs.

The LOQ (0.25 ng) and calibration curves included a wide range of imatinib, dasatinib and nilotinib concentrations/amounts, optimized according to expected and founded ranges of drug concentrations in PBMC patients. Choice of limits of these ranges was based on the values available in the clinical reports, too [26-40-42].

Data of the developed assay makes our method suitable, then, to perform imatinib, dasatinib and nilotinib quantification in PBMC CML patients. Reliability of our method has been demonstrated for all drug concentrations; relative error at QCs concentrations, intra-day and inter-day precision (Table 3) indicate the good performances of our method. Absence of interference peaks at the analyte retention times, without a “matrix effect”, coupled with an experienced collection procedure and treatment of PBMC [44] allowed accurate measurement of drugs intracellular levels.

5 Conclusion

We report a new method using HPLC-MS for the simultaneous intracellular determination of imatinib, dasatinib and nilotinib in a single chromatographic run. The selectivity of the assay described could be exploited optimally when monitoring all the three substances in PBMC and/or tissues during animal and clinical PK studies of single or concomitant administration. The described HPLC-MS method allows the rapid, simple, sensitive, and selective simultaneous intracellular quantification of the three major antileukemia drugs. This method could be currently used for the clinical study (or monitoring) of PBMC TKIs concentrations in patients treated with imatinib, dasatinib and/or nilotinib.

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Table 1. Detected mass (Dalton), cone voltage used (Volts) and retention time (RT, in minutes) used to quantify Internal Standard and each drug, and absolute amounts from STD6 to STD1 (LOQ), QCs (QC high, QC medium and QC low) and LOD.

Drugs Data				Amounts (ng)									
Drugs	**RT (minutes)**	**Mass (dalton)**	**Cone Voltage (volts)**	**STD 6**	**STD 5**	**STD 4**	**STD 3**	**STD 2**	**LOQ / STD 1**	**QC High**	**QC Medium**	**QC Low**	**LOD**
Imatinib	9.6	493.80	45	50	10	5	2	0.5	0.25	8	4	1	0.06
Dasatinib	10.6	487.50	35	50	10	5	2	0.5	0.25	8	4	1	0.06
Nilotinib	12.0	529.50	35	50	10	5	2	0.5	0.25	8	4	1	0.06
IS	14.8	313.30	50	-	-	-	-	-	-	-	-	-	-

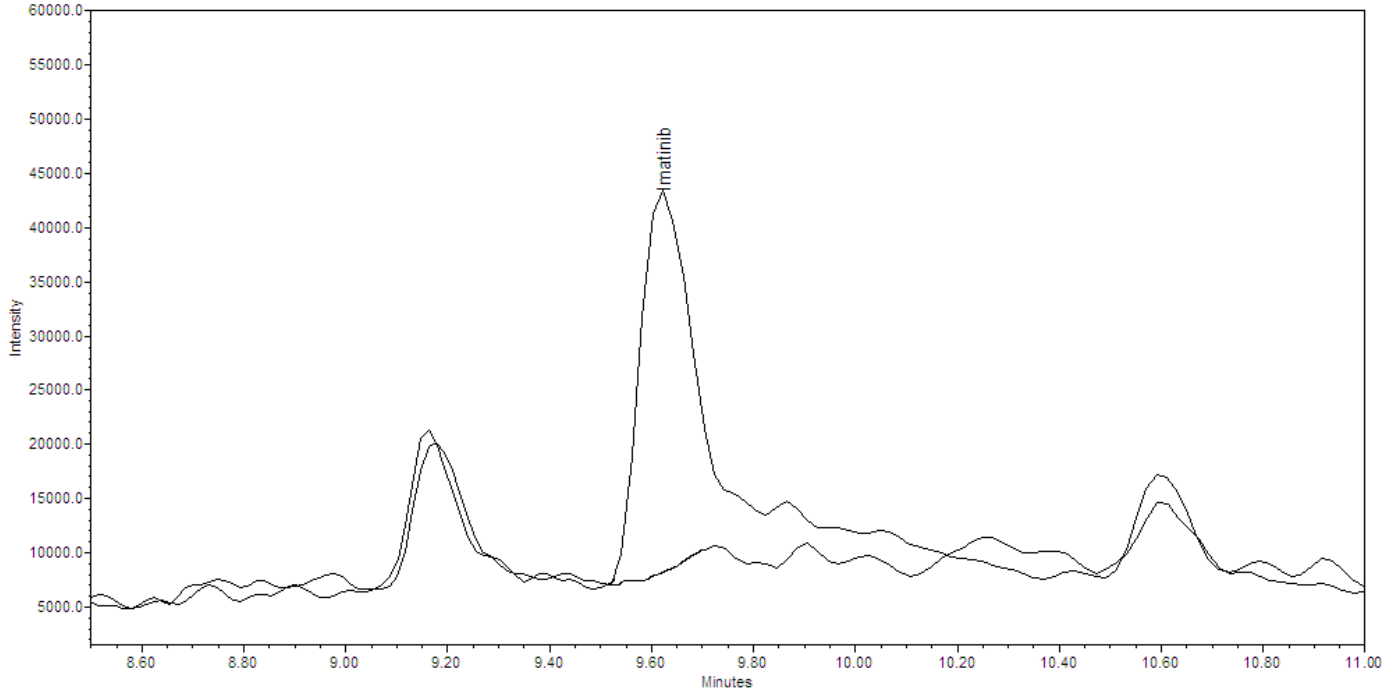
Table 2. Chromatographic condition (gradient): Mobile Phase A (HPLC grade water + 0.05% formic acid) and Mobile Phase B (HPLC grade acetonitrile + 0.05% formic acid). The flow was 0.25 ml/min.

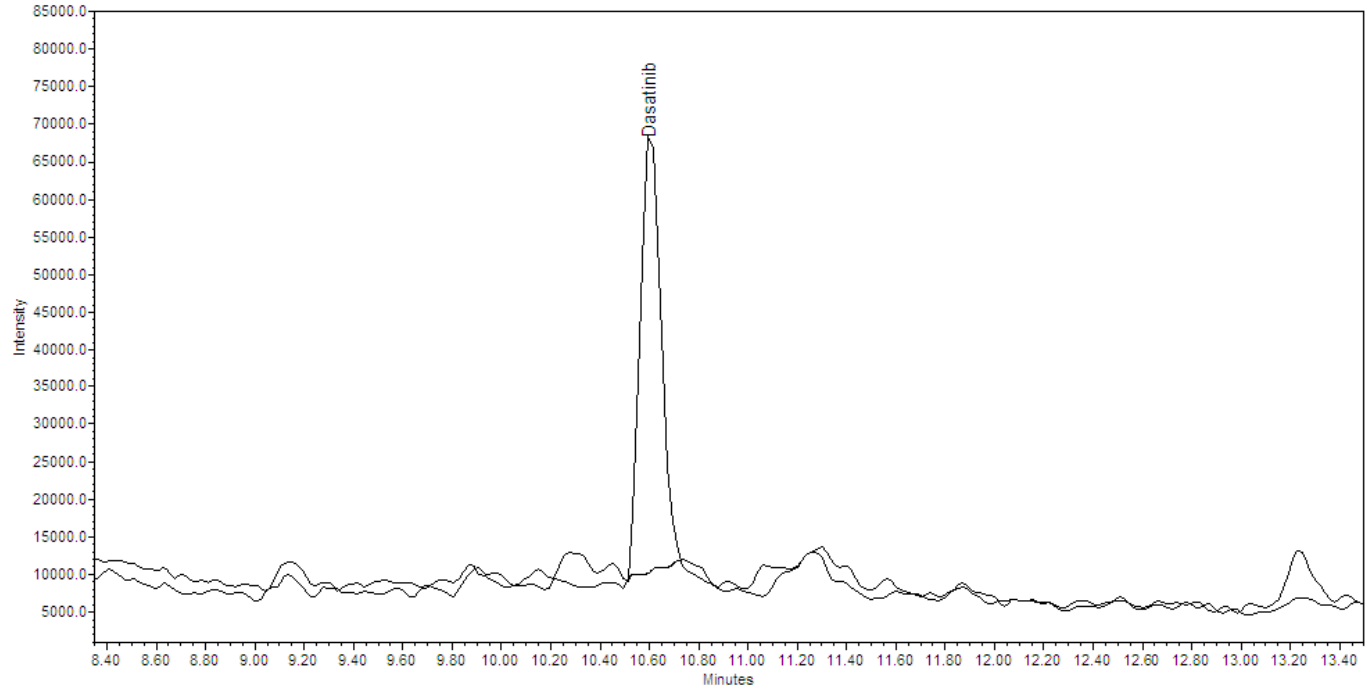
TIME (min.)	% Mobile Phase A	% Mobile Phase B
0.0	74	26
0.1	73	27
2.0	55	45
4.0	50	50
9.0	40	60
9.5	30	70
9.6	5	95
14.0	5	95
14.1	95	5
15.1	95	5
25.0	75	25

Table 3. Accuracy (CV%), Intraday and Interday Precision, as relative standard deviation (RSD%), assayed for all drugs (n=10).

Drugs	QC high			QC medium			QC low		
	Accuracy %	Precision R.S.D. %		Accuracy %	Precision R.S.D. %		Accuracy %	Precision R.S.D. %	
		Intra-day	Inter-day		Intra-day	Inter-day		Intra-day	Inter-day
IMATINIB	-5.72%	7.57%	11.04%	-4.56%	8.54%	13.01%	-6.63%	10.69%	14.12%
DASATINIB	2.68%	7.98%	10.73%	-4.22%	8.98%	11.28%	-3.60%	9.53%	12.28%
NILOTINIB	2.05%	7.60%	10.61%	-5.77%	7.99%	12.56%	-8.96%	9.94%	14.18%

Figure 1. Overlapping of STD1 and blank PBMC extracted ions detection (A, Imatinib; B, Dasatinib; C Nilotinib).





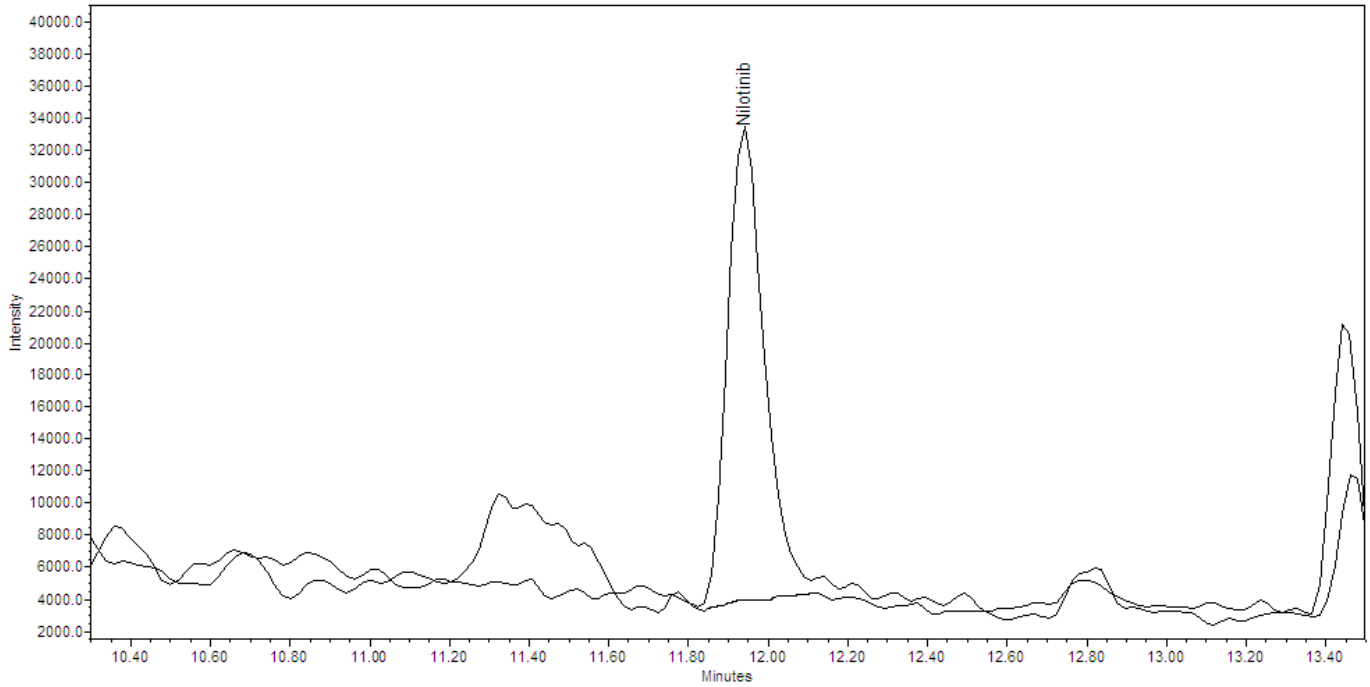

Nilotinib
Intensity
40000.0
38000.0
36000.0
34000.0
32000.0
30000.0
28000.0
26000.0
24000.0
22000.0
20000.0
18000.0
16000.0
14000.0
12000.0
10000.0
8000.0
6000.0
4000.0
2000.0
10.40
10.60
10.80
11.00
11.20
11.40
11.60
11.80
12.00
12.20
12.40
12.60
12.80
13.00
13.20
13.40
Minutes

Figure 2. STD6 extracted chromatogram.