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New HPLC–MS method for the simultaneous quantification of the antileukemia drugs imatinib, dasatinib, and nilotinib in human plasma

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ABSTRACT

A new method using high performance liquid chromatography coupled with electrospray mass spectrometry is described for the quantification of plasma concentration of tyrosine kinase inhibitors imatinib, dasatinib and nilotinib. A simple protein precipitation extraction procedure was applied on 250 μl of plasma 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 20 min of analytical run, at flow rate of 1 ml/min. Mean intra-day and inter-day precision for all compounds were 4.3 and 11.4%; mean accuracy was 1.5%; extraction recovery ranged within 95 and 114%. Calibration curves ranged from 10,000 to 62.5 ng/ml. The limit of quantification was set at 78.1 ng/ml for imatinib and at 62.5 ng/ml for dasatinib and nilotinib. This novel developed methodology allows a specific, sensitive and reliable simultaneous determination of the three tyrosine kinase inhibitors imatinib, dasatinib and nilotinib in a single chromatographic run, useful for drugs estimation in plasma 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 reciprocal translocation between the long arms of chromosomes 9 and 22, producing a fusion oncogene referred to as BCR–ABL [1]. The current frontline therapy for CML is imatinib (Gleevec™, STI-571, Fig. 1), 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]. Although most patients show excellent responses to imatinib treatment, clinical resistance may occur in approximately 15–20% of cases in chronic phase and in a higher percentage in more advanced phases of the disease. 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 [5,6]. Two potent second-generation Bcr–Abl inhibitors, dasatinib and nilotinib (Fig. 1), have recently been registered for the treatment of CML in imatinib-acquired resistance and intolerance cases. Dasatinib (Sprycel™, BMS-354825) is a structurally distinct drug which has a more potent activity than imatinib [7,8]. It also inhibits Src kinases, proteins that play a critical role in the development, growth, progression, and metastasis of a number of human cancers [9]. Nilotinib (Tasigna™, AMN107) is a close analog of imatinib with higher potency regarding Bcr–Abl kinase inhibition in vitro and in vivo [10,11]. Dasatinib and nilotinib are active against most of the imatinib resistant Bcr–Abl mutants. Imatinib-resistant CML patients who develop resistance against nilotinib may still show a response to dasatinib, and less frequently, patients with resistance against dasatinib may still respond to nilotinib [12].

Therapeutic drug monitoring (TDM) has become an essential tool for the management of CML patients. Measurement of antileukemia plasma concentrations can be useful to evaluate patient adherence to daily oral therapy, potential drug–drug interactions, treatment efficacy, and severe drug-related adverse events [13].

In recent years, numerous laboratories have reported the use of high-throughput bioanalytical procedures for the quantification of antileukemia drugs [14,15]. Based on validated high performance liquid chromatography (HPLC) methods, the pharmacokinetics of imatinib has been meanwhile well investigated, while only limited information on the pharmacokinetics of dasatinib and nilotinib are available to date. These knowledge should be achieved, especially in support of current clinical studies that suggest to use TKIs

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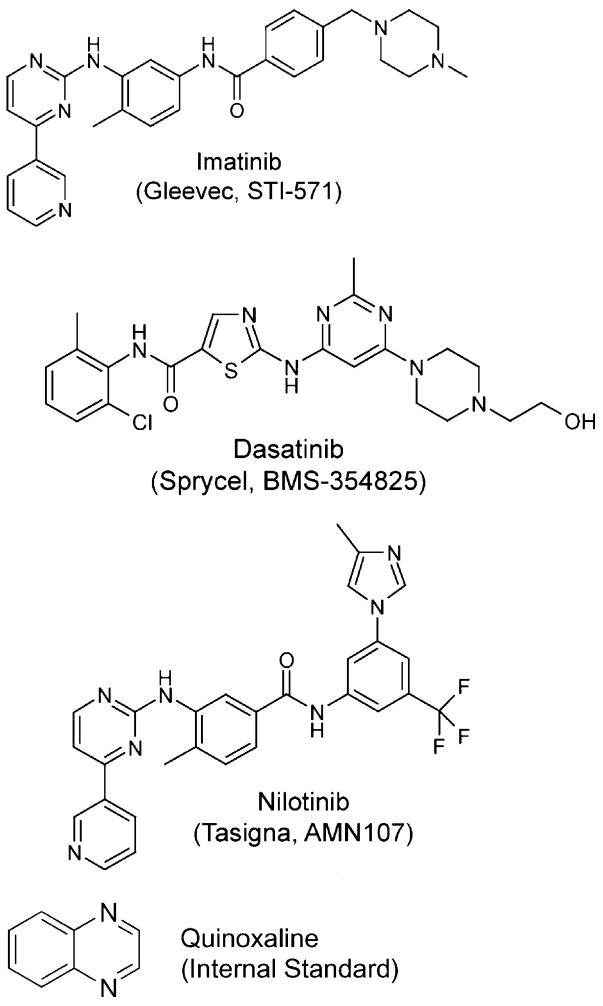

Fig. 1. Structures of imatinib, dasatinib, nilotinib and quinoxaline (internal standard).

(tyrosine kinase inhibitors) in sequential or simultaneous administration [16]. Herein, aim of our study was to develop and validate an high performance liquid chromatography coupled with electrospray mass spectrometry (HPLC–MS) method for the simultaneous quantification of imatinib, dasatinib and nilotinib in human plasma.

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). Blank plasma from healthy donors was kindly supplied by the Blood Bank of San Luigi Hospital (Orbassano, 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. Working solution of internal standard (IS) was made with QX (0.1 μg/ml) in methanol and HPLC grade water (50:50, v/v) and then stored at 4 °C until use. The highest calibration standard (STD8) and three quality controls (QCs) were prepared adding a determined volume of stock solutions to blank plasma; the others calibration standards (STDs) were prepared by serial dilution from STD8 to the lowest calibration standard (STD1) with blank plasma, to obtain eight different spiked concentrations. A blank sample (STD0) was included. Calibration ranges, from STD8 to STD1, and QCs concentrations for all drugs are listed in Table 1. STDs and QCs were then stored at −80 °C until analyses, avoiding more than one freeze–thaw cycle, and not more than 2 months.

Table 1
Calibrations of STD 8 (highest calibration standard), STD 1 (lowest calibration standard) or LOQ (limit of quantification), QCs (quality controls: QC high, QC medium, QC low) and LOD (limit of detection).

Drugs	Concentrations (ng/ml)					
	STD8	STD1 (LOQ)	QC high	QC medium	QC low	LOD
Imatinib	10,000	78.1	8,000	2,000	200	30
Dasatinib	8,000	62.5	6,000	1,500	150	15
Nilotinib	8,000	62.5	6,000	1,500	150	20

2.3. Chromatographic and MS conditions

The HPLC–MS instrument used was a Waters system (Milan, Italy), with binary pump model 1525, AF degasser, 717-plus autosampler, and Micromass ZQ mass detector. LC–MS Empower 2 Pro software (version year 2007, Waters; Milan, Italy) was used. Chromatographic separation was performed at 40 °C using a column oven, on Atlantis T3 C18 5 μm column 150 mm × 4.6 mm (Waters; Milan, Italy), protected by a Security Guard with C18 (4.0 mm × 3.0 mm) pre-column (Phenomenex; CA, USA). Chromatographic run was performed with a gradient (Table 2), and the mobile phase was composed by solvent A (HPLC grade water + 0.05% formic acid) and solvent B (HPLC grade acetonitrile + 0.05% formic acid) with a "T" switch tube only 200 μl/min of total flow (1 ml/min) introduced into the MS detector. Detector settings were ESI, positive polarity ionization; capillary voltage 3.5 kV; 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 45 V for imatinib, *m/z* 487.5 with a cone voltage of 35 V for dasatinib, *m/z* 529.5 with a cone voltage of 35 V for nilotinib and *m/z* 313.0 with a cone voltage of 50 V for QX (IS).

2.4. STD, QC and samples preparation

Extraction method developed was based on protein precipitation: 40 μl of IS working solution was added to 250 μl of plasma sample in a PTFE (polytetrafluoroethylene; Teflon) micro-

Table 2
Chromatographic condition (gradient)—mobile phase: Solvent A (HPLC grade water + 0.05% formic acid) and Solvent B (HPLC grade acetonitrile + 0.05% formic acid).

Time (min)	% Buffer A	% Buffer B	Flow (ml/min)
0.0	75.0	25.0	1.00
0.1	75.0	25.0	1.00
8.0	25.0	75.0	1.00
8.1	2.0	98.0	1.00
15.0	2.0	98.0	1.00
15.1	75.0	25.0	1.00
20.0	75.0	25.0	1.00

centrifuge tube, then 1000 μl of protein precipitation solution (methanol:acetonitrile 50:50, v/v) was added to the sample. The tube was vortexed for 30 s and then centrifuged at 13,000 × *g* (12,000 rpm) for 15 min at 4 °C. 400 μl of supernatant were transferred to a glass vial, diluted with 400 μl of HPLC grade water and briefly vortexed; 50 μl of this solution was injected into the column. In order to develop an accurate methodology, analyses for validation assay were performed in duplicate, and all procedure steps were carried out at room temperature.

2.5. Specificity and selectivity

Interference from endogenous compounds was investigated by analysis of six different blank plasma samples. Moreover interference from potentially co-administered drugs was evaluated for: zidovudine, didanosine, stavudine, lamivudine, abacavir, tenofovir, emtricitabine, ethambutol, isoniazid, ribavirin, amodiaquine. 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.

2.6. Matrix effect

"Matrix effect" was investigated using six different blank plasma and comparing peak areas obtained from standard solutions of a solution of water, methanol and acetonitrile (50:25:25), 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 [17]. Possible "matrix effect" was calculated, as deviation %, comparing the peak area obtained from the plasma extract with the peak area obtained from the standard solution.

2.7. Accuracy, precision, calibration and limit of quantification

Intra-day and inter-day accuracy and precision were determined by assaying twelve spiked plasma 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 was expressed as the standard deviation at each QC concentration. Each calibration curve was obtained using eight 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 plasma 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 plasma 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 [18].

2.8. Recovery

Recovery from plasma, using the extraction procedures, was assessed by comparing the peak area obtained from multiple analyses 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 [18].

2.9. Stability

The stability of antileukemia drugs at different conditions has been previously assayed in many articles [3,15,19,20]. Moreover we have investigated stability on QCs for all the analytes within 2 months of storage at −80 °C, assessed by variation of areas during validation period. A post-extraction short stability (24 h) was also evaluated.

2.10. Patients plasma samples

Patients receiving standard dosing of imatinib, dasatinib or nilotinib, underwent blood sampling after obtaining their informed consent for the measurement of plasma drugs concentrations. Blood samples were collected in lithium heparin tube (7 ml), plasma was obtained after centrifugation at 1400 × *g* (3000 rpm) 10 min at 4 °C. 40 μl of IS working solution was added to 250 μl of each plasma patient sample in a PTFE microcentrifuge tube, then underwent to extraction and were injected into the column, as described above (see Section 2.4).

3. Results

Time of analytical run was chosen as 20 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 3.4 ± 0.1 for imatinib, 5.3 ± 0.1 for dasatinib, 6.6 ± 0.1 for nilotinib, 9.0 ± 0.1 for QX. Representative chromatograms of a blank plasma extracted and STD1 of imatinib, dasatinib and nilotinib are shown in Figs. 2–4. Representative chromatogram of a imatinib, dasatinib and nilotinib STD8 plasma extracted and QX is shown in Fig. 5. 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 2.5). The tested six blank plasma did not show any interference in the retention times analytes windows for each specified ion detected (Figs. 2–4).

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% with the only exception for QC low inter-day of nilotinib that was below 20.0%. LOQ and LOD are listed in Table 1.

3.3. Recovery

Multiple aliquots ($n = 6$) at each of the three QCs concentration were assayed and mean recovery of all drugs plus IS ranged from 95 to 114%.

3.4. Matrix effect

The deviation % of the peak area at the three concentrations for all analytes is comparable, and it never exceeded −15.0%, showing absence of "matrix effect".

3.5. Stability

No remarkable variation was observed for all the analytes from the analysis of stability on QCs within 2 months of storage at −80 °C and from the analysis of post-extraction short stability (24 h, autosampler) at room temperature. Only for the chemical mix with dasatinib, used for the evaluation of recovery, we observed

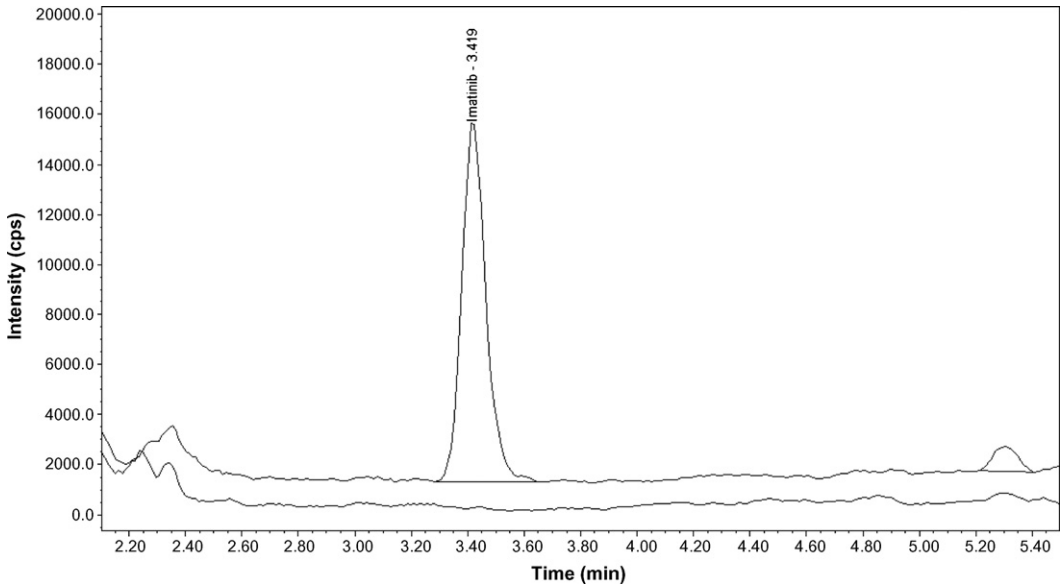

Fig. 2. Representative chromatograms SIR (Single Ion Regarding) overlapped of a blank plasma extracted and STD1 (lowest calibration standard) of imatinib.

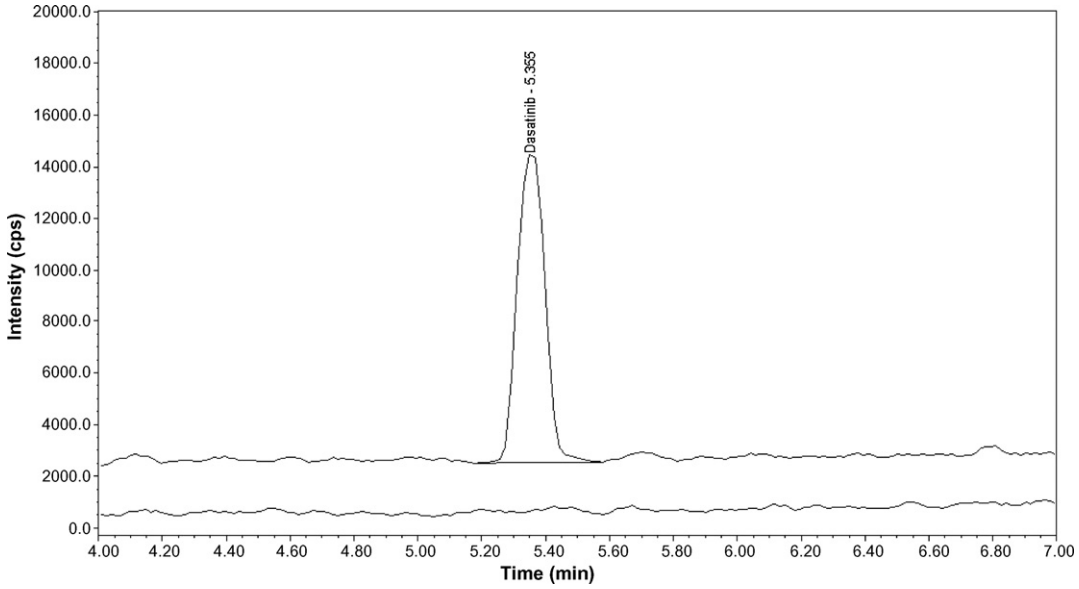

Fig. 3. Representative chromatograms SIR (Single Ion Regarding) overlapped of a blank plasma extracted and STD1 (lowest calibration standard) of dasatinib.

a degradation of the peak area more than 20% when stored at room temperature for more than 36 h.

3.6. Analysis of samples treated patients

Method developed was applied for assaying of 40 samples, corresponding to different points of time–concentration curve, obtained from 14 patients treated with imatinib, dasatinib and nilotinib (8 with imatinib, 3 with dasatinib, 3 with nilotinib). Values obtained resulted in the expected range of concentrations according to data present in literature [11,14,15,19].

4. Discussion

Targeted therapies using imatinib, dasatinib and nilotinib, based on the inhibition of protein tyrosine kinases, represent the thera-

Table 3
Accuracy (%), intra-day and inter-day precision (R.S.D.%) assayed for all drugs ($n = 12$).

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	1.8	4.9	8.9	−10.0	5.1	10.9	9.2	5.2	12.6
Dasatinib	4.3	5.8	8.7	−12.1	3.5	9.0	12.8	2.9	11.4
Nilotinib	1.9	3.1	10.3	−5.5	4.9	11.8	11.9	3.7	19.2

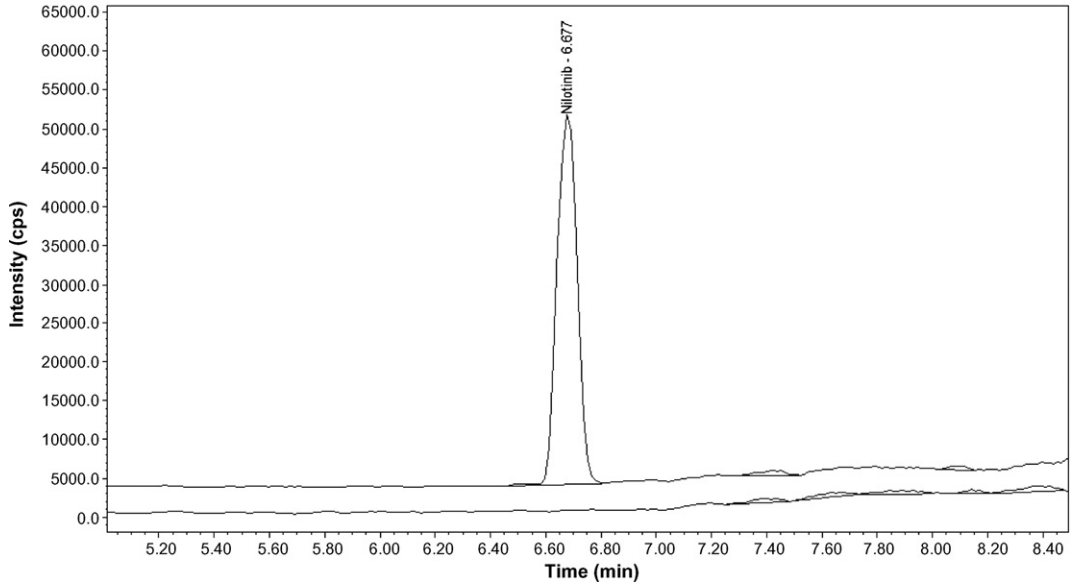

Fig. 4. Representative chromatograms SIR (Single Ion Regarding) overlapped of a blank plasma extracted and STD1 (lowest calibration standard) of nilotinib.

peutic strategies for treatment of CML; TDM, recently, has become an essential tool for the management of CML patients. In order to manage primary or acquired resistance to imatinib, clinical studies using dasatinib or nilotinib as second line therapy are currently under investigation. Clinical studies using combination of therapies with different TKIs in sequential or simultaneous administration are under evaluation. Some data regarding experiments in vitro and in vivo, in animals and in patients, have already been published [16,21], as evidence of the beneficial effects of this combination. In this context, aim of our study was to set up an HPLC–MS method for the simultaneous quantification of the three major antileukemia drugs in human plasma. Up to now, no HPLC methods for this simultaneous determination are currently available. The method we've developed and validated, based on a protein precipitation extraction procedure, can be easily adaptable in any laboratory, in contrast to an automated solid-phase extraction [3] or a semi-automated high-throughput precipitation procedure [20], which require instruments available only in a few laboratories. Our method is suitable to be used in clinical routine due to its high extraction efficiency, its good reproducibility and due to the simultaneous quantification of the three drugs using a small volumes of plasma (250 μl). In fact, although no therapeutic window has been clearly defined for the imatinib, dasatinib and nilotinib, it could be interesting to try to quantify plasma concentrations of the three drugs and assess concentration-response relationship. At present some reports showed that the imatinib trough plasma concentration should be more than 570 ng/ml to insure efficacy [22]. More recently Picard et al. established a higher trough plasma imatinib threshold (about 1000 ng/ml) for achievement of major molecular response [23]. Moreover, measurement of antileukemia plasma concentrations can be useful 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 [13]. The LOQ (78.1 ng/ml) of the developed assay makes our method convenient to perform imatinib therapeutic monitoring in patients.

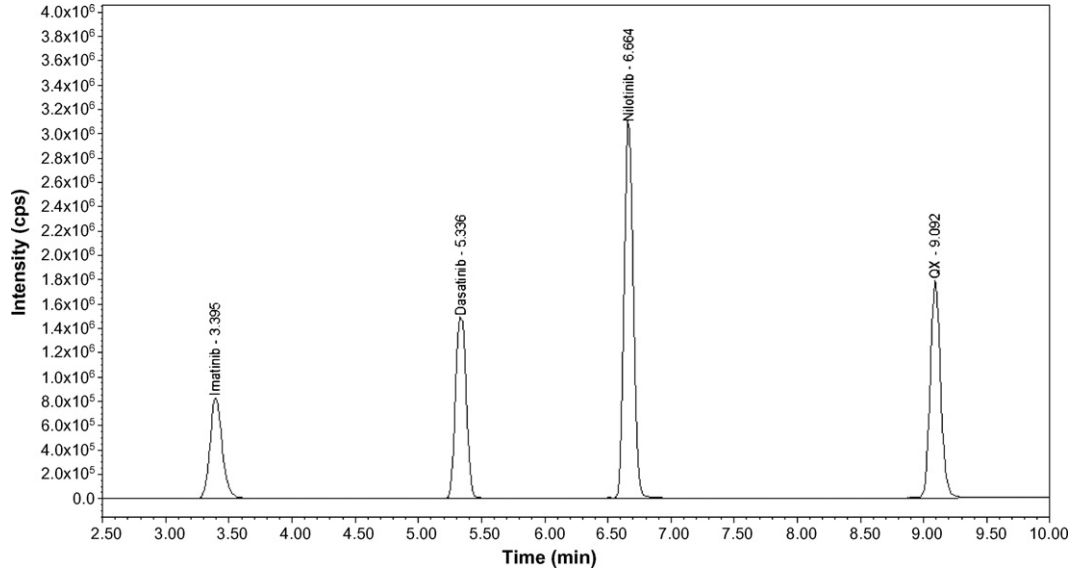

Fig. 5. Representative chromatogram SIR (Single Ion Regarding) of a imatinib, dasatinib and nilotinib STD8 (highest calibration standard) plasma extracted and quinoxaline (QX, internal standard).

Calibration curves included a wide range of imatinib, dasatinib and nilotinib concentrations, optimized according to expected ranges of drug concentrations in plasma patients. Choice of limits of these ranges was based on the values available in the clinical reports [11,22,23]. Reliability of our method has been demonstrated for all drug concentrations and 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", allowed accurate measurement of drugs plasma levels.

Very few studies are available up to now about pharmacokinetics of nilotinib, even less for dasatinib. If a threshold for efficacy of treatment with imatinib is anyway defined, for dasatinib and nilotinib this information seems to be still far away to be achieved. This observation suggests that the possibility to analyze in a single chromatographic run the three drugs can be useful for monitoring the substances in a concomitant treatment and to obtain informations about pharmacokinetics/pharmacodynamics relationship of dasatinib and nilotinib.

5. Conclusion

We report a new method using HPLC–MS for the simultaneous 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 body fluids and/or tissues during animal and clinical pharmacokinetic studies of concomitant administration. The described HPLC–MS method allows the rapid, simple, sensitive, and selective simultaneous quantification of the three major antileukemia drugs. This method could be currently used for the clinical monitoring of plasma TKIs concentrations in patients treated simultaneously, as demonstrate any initial analyses performed in plasma patients treated with imatinib, dasatinib or nilotinib.

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