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Determination of unbound fraction of imatinib and N-desmethyl imatinib, validation of an UPLC–MS/MS assay and ultrafiltration method

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ABSTRACT

Imatinib is a small-molecule tyrosine kinase inhibitor with large inter-individual but low intra-individual pharmacokinetic variability with consistent concentration–efficacy and concentration–toxicity relationships. For these reasons imatinib therapeutic drug monitoring is based on total plasma concentrations. However, since a significant impact of unbound imatinib concentrations on clinical response and/or toxicity evaluation has been suggested, the quantification of free fraction of imatinib and its active metabolite are of interest for therapeutic monitoring. Hence a reliable method for both separation and assay of the free fraction is needed. Using plasma samples spiked with imatinib (from 1000 to 7500 ng/mL) and its metabolite (from 1000 to 2500 ng/mL), an ultrafiltration procedure and an UPLC assay which give reproductive values for unbound fractions of imatinib (mean $3.0 \pm 1.0\%$) and metabolite N-desmethyl imatinib ($3.6 \pm 1.8\%$) have been developed. The validation of the analytical UPLC–MS/MS method associated to ultrafiltration for quantification of imatinib and N-desmethyl imatinib was reported. The LOQ was set at 10 ng/mL for imatinib and 20 ng/mL for N-desmethyl imatinib, intraday CV (%) ranged from 2.7 to 4.8% for imatinib and from 5.4 to 12.4% for N-desmethyl imatinib and interday CV (%) ranged from 5.6 to 6.5% for imatinib and from 5.4 to 16.1% for N-desmethyl imatinib. Methodological modifications were attempted to overcome non specific binding (NSB) on the ultrafiltration device. Two types of devices previously used for unbound determination of drugs were tested. Our results clearly showed that the methodology and the features of devices used for ultrafiltration could totally compromise the determination of unbound concentrations of a drug.

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1. Introduction

Imatinib (IM) is a small-molecule tyrosine kinase inhibitor that was first developed for the treatment of chronic myeloid leukemia (CML) to inhibit the action of the BCR-ABL fusion protein, and for gastrointestinal stromal tumors (GISTs) characterized by the exon 11 KIT mutation [1,2]. In CML, IM exerts its therapeutic effect through competitive inhibition at the adenosine triphosphate binding site, thereby inhibiting tyrosine phosphorylation of proteins involved in Bcr-Abl signal transduction [3]. At the clinical level, this inhibition induces apoptosis in Bcr-Abl-positive cells, with no effect on normal cells [1,4,5]. IM is also the first-line treatment of gastrointestinal stromal tumor (GIST) due to its inhibitory effect on C-Kit receptor [6].

Therapeutic drug monitoring (TDM) based on total plasma concentrations is currently performed for IM due to its large inter-individual but low intra-individual pharmacokinetic variability, with consistent concentration–efficacy and concentration–toxicity relationships [7]. However, some results suggested a significant correlation between unbound drug concentrations [8–10] and clinical response and/or toxicity, compared with total plasma concentrations, involving the evaluation of IM and N-desmethyl imatinib (NDI; main active metabolite) unbound concentrations. Different procedures were reported for IM and NDI monitoring in plasma [11,12], but quantification of NDI in ultrafiltrate has not been validated so far, due to difficulties in obtaining a reproducible process for the separation of free fraction and a sufficiently sensitive analytical method. We have optimized and validated an ultrafiltration process combined with an UPLC–MS method which could be applied for routine quantification of total and unbound concentrations of both IM and NDI. The originality of our approach was to add 200 μL of blank plasma into the collecting cup to overcome the poor solubility of IM and NDI in plasma ultrafiltrate. Indeed, IM is a quadrivalent base which is pH-sensitive due to the presence of an

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amine and a pyridine group with pK_a range from 1.52 to 8.07. It is soluble in an aqueous media with pH lower than 5.5 and in polar organic solvents but poorly soluble in non-polar organic solvents due to its low partition coefficient ($\log P = 1.267$ at 37 °C) [13].

To date, the two methods most commonly used to determine plasma unbound concentrations are equilibrium dialysis and ultrafiltration. In a lead optimization setting, ultrafiltration generally has the advantage over equilibrium dialysis, being a less time-consuming process, and therefore, likely to have a higher throughput. The disadvantage of both equilibrium dialysis and conventional ultrafiltration is that they can be susceptible to non-specific binding (NSB) of drugs to the polymer-constructed components of filter membranes or glass and plastic recipients. NSB could be reduced using different approaches of pre-treatment (tween 80, benzalkonium chloride [14]) of the filter.

On the other hand, Taylor and Harker [15] have suggested a modified ultrafiltration technique suitable for a corticosteroid series known to demonstrate non-specific binding and poor solubility ($\log P$ between 2.08 and 4.73). The method involved a modification of standard ultrafiltration (UF) techniques. For each experimental plasma sample, a control plasma sample is also processed by ultrafiltration. The retentates from experimental and control plasma samples are mixed into the filtrate of the partner sample. The resulting regenerated plasma samples, one representing the unbound drug and the other the bound drug, are then analyzed. Varying degrees of NSB were demonstrated with a number of corticosteroids, and this effect was eliminated using the modified method. We compared Taylor's methodology [15] with our approach to overcome low solubility and non-specific binding of IM. In fact, Taylor's strategy seems to be an interesting alternative method to evaluate the unbound fraction as a reference value, even through this system is less adapted for routine quantification of the unbound drug. For a routine application, we also tested a conventional ultrafiltration procedure where the IM assay is directly collected in the ultrafiltrate fraction after one centrifugation step. Our objective was to develop an ultrafiltration procedure allowing the separation of unbound fraction of IM and NDI followed by its assay. We reported here the validation of the ultrafiltration process coupled with an analytical method that allowed analysis of both total and unbound IM and NDI in plasma for a routine application.

2. Materials and methods

2.1. Chemicals

Imatinib mesylate and deuterated imatinib mesylate were provided by Novartis. N-desmethyl imatinib (NDI) was purchased from Alsachim. MilliQ water was prepared with a Millipore apparatus. Human blank plasma was obtained from ESF "Etablissement Français du Sang". Hexane (Chromasolv for HPLC quality) was purchased from Sigma–Aldrich, ethyl acetate, methanol and acetonitrile (HPLC grade) from Scharlau, sodium hydroxide (32%), formic acid from Merck, and ammonium formate from Fluka.

2.2. IM and NDI quantification

2.2.1. UPLC–MS/MS

The UPLC system consisted of an Acquity UPLC® separation module (Waters, Milford, USA) controlled by Empower™ CDS software. For the detection, the mass spectrometer Acquity detector (Waters, Milford, USA) with electrospray ionization (ESI) in positive ion mode was utilized. The mass spectrometer was operated in the multiple-reaction monitoring (MRM) mode. The temperature of the ESI source during the operation was 120 °C and the desolvation temperature was 250 °C. The gas flow of the cone was set at 1 L/h and the gas flow of the desolvation was set at 500 L/h. The capillary voltage was 55 V. The MS collision energy was 30 V. The mass spectrometer detected the precursor ion of IM on 494.21 m/z and the product ion on 394.5 m/z. The precursor ion of NDI was detected on 480.50 m/z and the product ion on 394.10 m/z. For the internal standard the precursor ion was detected on 502.38 and the production on 394.40 m/z.

The separations were performed on an Acquity UPLC® BEH shield RP18 2.1 mm × 50 mm, 1.7 μm (Waters, Milford, USA), using an analytical method adapted from Titier et al. [16]. LC eluent consisted in a gradient of phase A (2 mM ammonium formate in water, pH = 3.0) and phase B (acetonitrile + 0.1% formic acid). Phase B, initially set at 2% for 0.5 min, was increased linearly from 2% to 50% over 2 min and from 50% to 95% over 0.5 min, and was set at 95% for 1.5 min. Then phase B was decreased to initial conditions over 0.2 min and the system was re-equilibrated for 1.3 min before the next injection. The total run time was 5.5 min at a flow rate of 0.400 mL/min.

2.2.2. Sample preparation

Before injection, samples were pre-treated as follows: a plasma sample of 200 μL was added to 50 μL of EI, 200 μL NaOH 0.2 N and 1000 μL of ethyl acetate. The samples were checked for 10 min on a rotative device and centrifuged for 5 min (1000 × g, 4 °C). The organic phase was evaporated with air at 37 °C. Finally, the dry film was solubilized with 200 μL of a mixture methanol/water (1/1) implemented by 40 μL of formic acid before transfer into a vial and UPLC analysis (7 μL injected).

2.3. Analytical validation

The method was validated with plasma samples spiked with IM and NDI by dilution of stock solutions in methanol (1 mg/mL). The selectivity of the method was evaluated comparing chromatograms from IM and NDI samples at the LOQ and from blank samples ($n = 5$) to ascertain that no endogenous peak would interfere with IM or NDI signal.

To test for potential carry over from previous samples, the high level quality control (1600 ng/mL) and the highest calibration point (2000 ng/mL) were injected before one blank. Carry over was evaluated with 3 independent repetitions *per* concentration level and was estimated by the ratio of blank on spiked sample areas.

The effect of different biological matrices (matrix effect) was evaluated by analyzing the plasma of 4 volunteers (2 men and 2 women) spiked with analytes to obtain a final concentration of 1000 ng/mL. Analytes from these different plasmas were quantified to verify whether the matrix had an effect on bias from the theoretical value.

Calibration was performed after sample extraction (see Section 2.2.2) from 10 to 2000 ng/mL for IM and from 20 to 2000 ng/mL for NDI. Three levels of quality control (50, 600 and 1600 ng/mL for each compound) were used for the determination of intraday and interday precision and accuracy. A set of 6 calibrations were analyzed (one *per* day for 6 days) to calculate precision and accuracy parameters at the LOQ.

IM and NDI stability in plasma samples were previously evaluated at −20 °C. Streit et al. [17] showed that IM and NDI are stable for 7 days whereas Titier et al. [16] validated the IM stability for 30 days and Parise et al. [11,12] for 12 months. NDI stability in plasma was also validated for one month [18] and no significant loss of IM or NDI was observed after three freeze/thaw cycles [11,16].

2.4. Ultrafiltration

Ultrafiltration with the Centrifree® YM-30 device is one of the most commonly used processes to determine plasma unbound

concentrations whereas the Amicon® ultra-0.5 device was previously used for unbound determination of low aqueous soluble drugs. In order to decide which method was the most adapted to routine usage, we tested both these ultrafiltration devices with their respective procedures.

2.4.1. Determination of the unbound plasma fraction by conventional ultrafiltration with Centrifree® YM-30 device

2.4.1.1. Ultrafiltration procedure. Stock solutions (1 mg/mL) of IM and NDI in methanol were added to human blank plasma to reach the nominal concentrations 1000/–, 2000/–, 3000/1000, 5000/1600 and 7500/2500 ng/mL (final methanol < 1%, v/v). These concentrations were selected in order to detect at least 0.5% free fraction in plasma and to take into account the IM/NDI ratio of around 3 observed in patients during TDM. Plasma samples were placed in a rolling incubation at ambient temperature for 1 h, before storage at −20 °C for one week at most until ultrafiltration and assay. Three agitation durations were tested to ensure equilibrium and 1 h was selected giving reproducible results (data not shown). Comparison of the concentrations (3000/1000; 7500/2500 (IM/NDI)), from one freeze/thaw cycle and fresh samples ($n = 3$) gave similar results.

Two hundred microlitres of aliquot of IM and NDI solution *per* concentration was added to the sample reservoir of the Centrifree® YM-30 (Millipore) unit after thawing plasma samples at ambient temperature (6 replicates) and 200 μL of blank plasma was added in the collection tube of the ultrafiltration device. Tubes were then centrifuged for 40 min at 2000 × *g* and 20 °C. Two hundred microlitres of the solution (ultrafiltrate mixed with plasma) collected in the filtration collection cup was extracted before analysis as described in Section 2.2.2.

IM plasma unbound fraction was calculated by dividing plasma unbound imatinib concentration corresponding to the filtrate collection, by total plasma imatinib concentration (concentration of the solution added in the sample reservoir).

2.4.1.2. Non specific binding. To evaluate the NSB on Centrifree® YM-30 filters, Sorensen buffer (pH 7.4) was spiked with both IM and NDI (from stock solutions in methanol, final methanol < 1%) to give the following nominal concentrations (IM/NDI): 3000/1000, 5000/1600 and 7500/2500 ng/mL. Solutions were checked (vortex) for 2 min and added immediately in the sample reservoir, submitted to centrifugation (40 min, 2000 × *g* and 20 °C), extraction and analysis. NSB, expressed as a percentage, corresponds to the difference between IM/NDI quantity added in the sample reservoir before centrifugation and IM/NDI quantity recovered in the ultrafiltrate.

2.4.2. Ultrafiltration alternative method with Amicon® ultra-0.5 device for comparison with the conventional method

Taylor and Harker [15] suggested a modified ultrafiltration technique, using Amicon® ultra-0.5 device, suitable to reduce NSB of poorly soluble drugs. The method involved a modification of standard ultrafiltration (UF) techniques. We wanted to check if this technique could be used as a reference method for imatinib. For a complete evaluation of efficacy of this device (Amicon® ultra-0.5) in separating unbound drugs, we tested both methodologies: standard *vs* modified ultrafiltration (Amicon® ultra-0.5 inverted).

2.4.2.1. Standard ultrafiltration method. Plasma samples prepared from stock solutions at nominal concentrations 1000, 2000, 3000, 4000, 5000 and 7500 ng/mL, placed in a rolling incubation at ambient temperature for 1 h to ensure equilibrium, and stored at −20 °C for one week at most were used for standard ultrafiltration. After thawing plasma samples at ambient temperature, five replicates of 200 μL aliquot *per* concentration were added to the sample reservoir of the UF unit Amicon® ultra-0.5 (0.5 mL, 30K; Millipore). The low solubility of imatinib in ultrafiltrate was a limiting factor leading to high variability of our results when ultrafiltrate was directly injected into UPLC–MS. Hence, in these experiments, ultrafiltration and extraction were performed simultaneously. Then, in the filtration collection cup, 50 μL working solution of deutered imatinib at 100,000 ng/mL (EI), 20 μL NaOH 2 N and 500 μL of extraction solution were added. The UF unit Amicon® ultra-0.5 was centrifuged for 6 min (4000 × *g*, 4 °C). After centrifugation, 500 μL of extraction solution was added again in the filtrate collection tube. Tubes were then checked for 10 min on a rotative device and centrifuged for a second time for 5 min (1000 × *g*, 4 °C). The organic phase was removed and evaporated with air at 37 °C, and the reconstituted solution was analyzed by UPLC–MS/MS. The same experiments were repeated for samples at 1000, 4000 and 10,000 ng/mL with a centrifugal force of 2000 × *g* for 40 min at 20 °C to evaluate the impact of different centrifugal conditions.

2.4.2.2. Modified ultrafiltration method. The filtrate collection tubes were weighed before and after centrifugation. For each UF unit loaded with 200 μL IM plasma sample (concentrations ranging from 1000 to 7500 ng/mL and placed in a rolling incubation at ambient temperature for 1 h to ensure equilibrium), a partner UF unit was loaded with 200 μL control plasma (IM/NDI free plasma). All ultrafiltration units were centrifuged at 4000 × *g* for 6 min at 4 °C. The sample reservoirs containing plasma retentate were then inverted and placed on the filtrate collection tubes of the respective partner UF units. The UF units were centrifuged a second time at 4000 × *g* for 6 min at 4 °C to mix the retentate with the ultrafiltrate of the partner samples. This process led to two sorts of plasma samples, corresponding respectively to unbound and bound imatinib fractions; both were quantified by UPLC–MS/MS.

2.4.2.3. Non specific binding. To evaluate the NSB on Amicon® ultra-0.5 filters, blank plasma spiked with IM at nominal concentrations 1000, 3000, 4000, 5000 and 7500 ng/mL (placed in a rolling incubation at ambient temperature for 1 h to ensure equilibrium) was added in the sample reservoir and submitted to ultrafiltration at 4000 × *g* for 6 min at 4 °C. After ultrafiltration, the collected ultrafiltrates were kept for quantification. Each filter was reversed on a new tube and centrifuged at 1000 × *g* for 5 min at 4 °C, to discard the retentate from the sample reservoir. The filter was reversed for a second time and placed on a new tube. A volume of 200 μL MeOH was added on the filter. The filtrate collection tubes were weighed before and after centrifugation of samples at 4000 × *g* for 6 min at 4 °C. The volume of methanol collected in the filtrate collection tube (around 70 μL) was directly analyzed in LC–MS. NSB, given as a percentage, corresponds to the ratio between the quantity of IM recovered in methanol (washing fraction) and the quantity of IM added in the sample reservoir before centrifugation.

3. Results and discussion

The UPLC–MS method was validated in terms of specificity, sensitivity, linearity, recovery, accuracy and precision. The method was specific as no interference with IM and NDI was observed when analyzing a different set of blank samples. The peaks were identifiable and reproducible at tr = 1.78 min for IM, 1.73 min for NDI and 1.77 min for EI. Typical chromatograms obtained from a blank and the lowest calibration points (LOQ at 10 ng/mL for IM and 20 ng/mL for NDI) are reported in Fig. 1. When analysing blanks after the largest calibration point or the largest QC control, we observed a residual peak of imatinib in blank. However, the carry over evaluated by the peak ratio in blank *vs* concentrated samples was less than 0.06% for IM. Moreover, as shown in Fig. 1, residual blank peak area accounts for only 2.6% of the lowest calibration curve of IM (LOQ) whereas no carry over was observed for NDI.

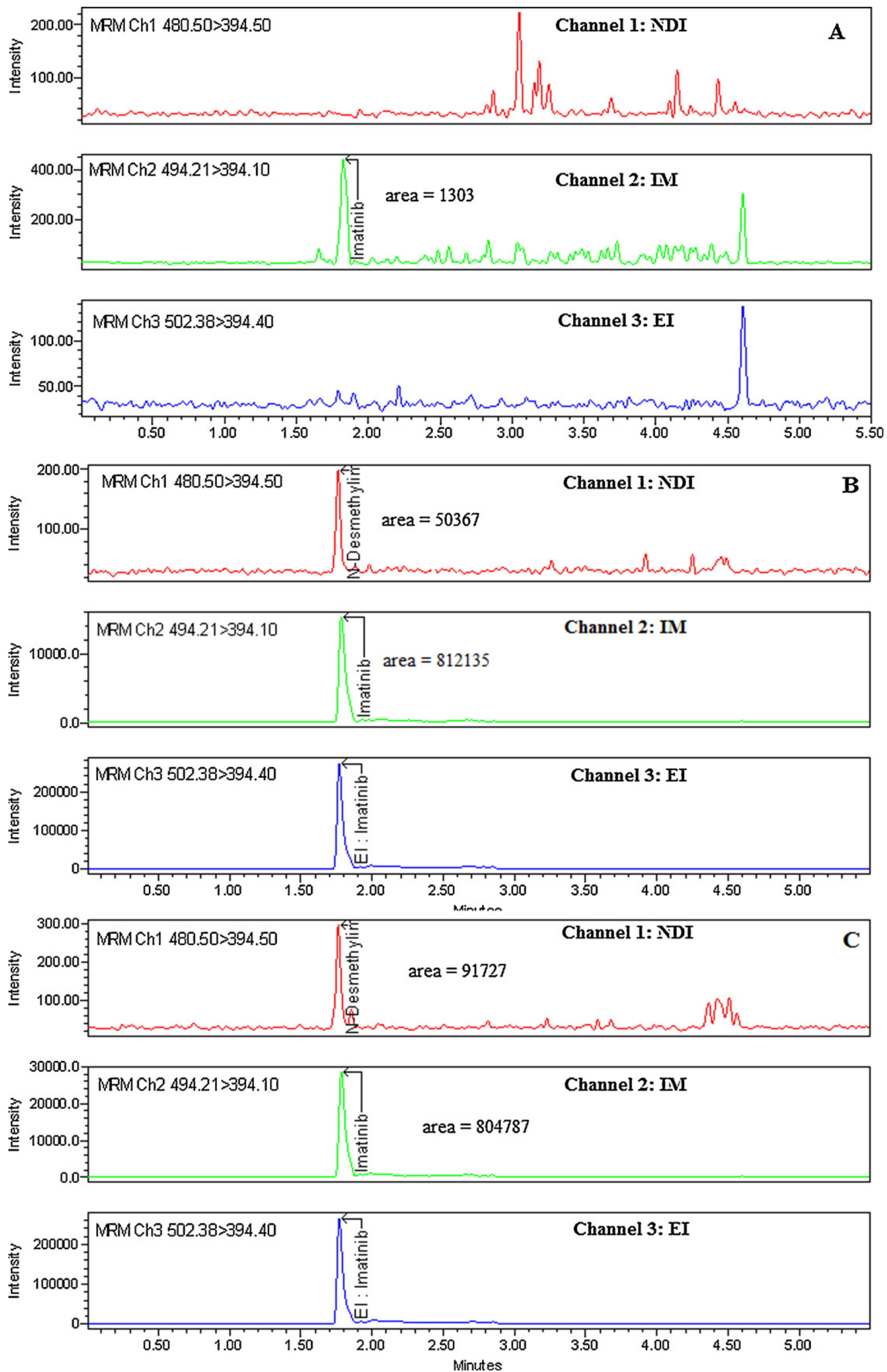

Fig. 1. Chromatogram (three MRM transition channels) for: (A) blank sample, (B) IM and NDI at 10 ng/mL and (C) IM and NDI at 20 ng/mL.

Table 1
Intra and inter-day accuracy and precision for UPLC–MS validation of imatinib and N-desmethyl imatinib quantification.

	Level (ng/mL)	Intra-day (n = 5)		Inter-day (n = 10)	
		CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
Imatinib	50	2.7	1.8	6.5	−2.6
	600	4.4	4.8	6.05	0.6
	1600	4.8	1.2	5.6	0.2
N-desmethyl imatinib	50	12.40	−8.5	10.2	5.6
	600	8.2	2.6	6.8	0.1
	1600	5.4	2.8	5.4	−5.2

We also assessed that the plasma matrix had no significant effect on the response of analytes: the measured mean concentrations (n = 4) were respectively 987.0 ± 41.7 ng/mL (RSD = 4.2%) for IM and 963.2 ± 65.0 ng/mL (RSD = 6.8%) for NDI without significant bias from the target value (1000 ng/mL). Furthermore, similar extraction yields obtained with solutions in Sorensen buffer and plasma also demonstrate that the plasma matrix had no significant effect on the response of analytes (data not shown).

Calibration curves (n = 5) were constructed by correlating peak area ratio (IM or NDI *vs* EI) as a function of the concentration of the spiked standard solutions. Regression analysis was performed with weighting 1/X. The bias (%) and the coefficient of variation (CV, %) were calculated as a measure of the intra- and inter-day accuracy and precision respectively (Table 1). Interday validation results from analysis of five replicates the first day and one replicate *per* day for 5 days for each level of QC.

The intra-day bias ranged from 1.2 to 4.8% for IM, and from −8.5 to 2.8% for NDI. The intra-day CV (%) ranged from 2.7 to 4.8% for IM, and from 5.4 to 12.4% for NDI. The inter-day bias ranged from −2.6 to 0.6% for IM, and from −5.2 to 5.6% for NDI. The inter-day CV (%) ranged from 5.6 to 6.5% for IM, and from 5.4 to 10.2% for NDI (Table 1).

The lower limit of quantification (LOQ) was defined as the lowest concentration giving a signal-to-noise ratio greater than 10 (S/N > 10) with intra-day and inter-day accuracy and precision under 20%. The precision (CV, %) and bias, assessed from six replicates at LOQ (10 ng/mL for IM and 20 ng/mL for NDI) were respectively 11.2% and −10.4% for IM and 15.2% and 15.3% for NDI. These results agreed with the required criteria and the LOQ was set to 10 ng/mL and 20 ng/mL for IM and NDI respectively.

This analytical method was designed to quantify both total and unbound concentrations of IM and NDI in patients treated with IM. The range from 10 ng/mL (20 ng/mL for NDI) to 2000 ng/mL was selected based on the kinetics previously reported for IM and NDI [11,12,16]. Finally, the applicability of this process (ultrafiltration coupled with LC–MS method) to samples of patients was assessed by the determination of concentrations of IM and NDI in the plasma of patients who received IM orally. Analyses of free fractions of IM and NDI from patients (included in TDM) are now in progress and preliminary results confirm the applicability of this method to the quantification of the free fractions of IM and DNI. Fig. 3 shows, as an example, a chromatogram obtained for the quantification of fu from a patient sample, where fu of 3.4% and 4.5% were respectively measured for IM and NDI.

TDM of total IM was initially performed in our laboratory using a method adapted from Titier [16] where 2 mM ammonium formate in water, pH = 3.0 (phase A) was replaced by 0.1% formic acid in water; liquid extraction with hexane/ethyl acetate (3/7, v/v) and solubilization of the dry residue in ammonium formate 4 mM pH = 3.25/methanol (1/1, v/v) were unchanged. To improve the NDI sensitivity, the following new conditions were selected: LC eluent (gradient) composed with 2 mM ammonium formate in water, pH = 3.0 (phase A) and acetonitrile + 0.1% formic acid (phase B); liquid extraction with ethyl acetate and solubilization of dry residue in water/methanol/formic acid (1/1/0.2). As shown in Fig. 2, LOQ for NDI was set at 20 ng/mL with these new conditions whereas NDI was undetected at 80 ng/mL with the previous ones.

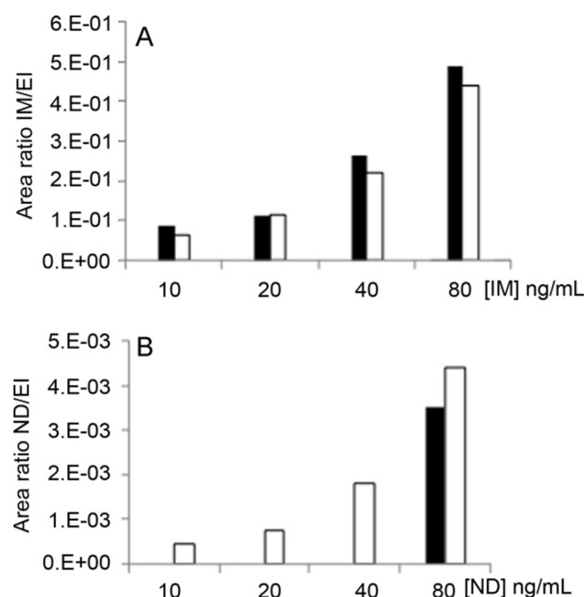

Fig. 2. Improvement of UPLC–MS sensitivity for IM (A) and NDI (B) quantification (■) LC eluent (gradient) composed with 2 mM ammonium formate in water, pH = 3.0 (phase A) and acetonitrile + 0.1% formic acid (phase B), liquid extraction with ethyl acetate and solubilization of dry residue in water/methanol/acid formic (1/1/0.2) (□) LC eluent (gradient) composed (0.1% formic acid in water (phase A), 0.1% formic acid in acetonitrile (phase B), liquid extraction with hexane/ethyl acetate (3/7, v/v) and solubilization of the dry residue in ammonium formate 4 mM pH = 3.25/methanol (1/1, v/v)).

Extraction recovery with ethyl acetate was given by the ratio of the peak areas of the analyte from a plasma solution after extraction *vs* a methanol solution without extraction. Extraction recoveries were 35.5 ± 4.1%; 28.2 ± 2.4% and 32.6 ± 4.7% for IM and 30.9 ± 1.4%; 24.3 ± 6.8% and 26.6 ± 3.0% for NDI respectively at three QC levels at 40; 1200 and 2000 ng/mL. Extraction efficiency for IM was similar with both solvents (mixture hexane/ethyl acetate *vs* ethyl acetate alone) and was improved for the metabolite using ethyl acetate alone. Extraction recovery was of a similar order of magnitude for ultrafiltrate samples (data not shown).

Previous tests without extraction were performed using methanol (results not shown) in the filtration collection cup directly injected in UPLC–MS but signals during detection were not reproducible. Indeed, the presence of salts in the ultrafiltrate did not allow direct UPLC–MS analysis due to the decrease of signal and of analytical sensitivity after few injections. Thus, an extraction step to clean samples up was necessary before analysis. An alternative to avoid manual extraction would involve an online extraction process combined with LC–MS apparatus as used by Streit et al. [17].

The challenge for the determination of unbound fraction with lipophilic compounds is to find the good compromise between a sufficiently sensitive analytical procedure applied to ultrafiltrate and a method able to isolate the free drug (*e.g.* equilibrium dialysis, ultrafiltration).

Using the Centrifree® YM-30 device, IM and NDI unbound fractions were respectively between 1.97 ± 0.68% and 4.15 ± 1.01% and 2.97 ± 0.32% and 4.00 ± 2.01% using healthy human plasma (Table 2). Although the methods used were different, our results agreed with values reported by other authors. Smith et al. [19]

Table 2
Determination of unbound fraction (fu %) of imatinib and N-desmethyl imatinib with Centrifree® YM-30 device.

Total plasma imatinib concentration (ng/mL)	% fu (n = 6) Imatinib	Total plasma N-desmethyl imatinib concentration (ng/mL)	% fu (n = 5) N-desmethyl imatinib
1000	3.53 ± 1.01	1000	4.00 ± 2.01
2000	4.15 ± 1.01	1600	2.91 ± 0.32
3000	1.97 ± 0.68	2500	3.85 ± 2.47
5000	2.98 ± 0.85		
7500	2.87 ± 0.85		

evaluated the effect of St. John's Wort on the pharmacokinetics of IM during an open-label, complete crossover, fixed-sequence, pharmacokinetic study including ten healthy adult volunteers. Single 400 mg oral doses of IM were administered before and after 2 weeks of treatment with 300 mg of St. John's Wort 3 times/day. Unbound concentrations of IM were determined for all 3- and 24-h samples by plasma ultrafiltration at 37 °C (Centrifree®, Amicon). Ultrafiltrate (0.1 mL) was directly injected and analyzed by LC–MS/MS. Unbound fraction ranged from 2.3 to 9.7% (mean 5%) for IM, and from 2.7 to 9.1% (mean 4.9%) for IM associated with St. John's Wort, with no significant difference. Kretz et al. [20] evaluated blood-binding parameters of IM and its metabolite NDI in healthy males (n = 3) and female AML patients (n = 5). Protein binding of IM and NDI were determined *in vitro* using $^{(14)}$C labeled compounds. Plasma was centrifuged in Centrifree devices (10 min, 2000 × g, 37 °C). Radioactivity was determined in plasma and in ultrafiltrate. The unbound fraction of IM is around 5.0% in healthy humans for plasma concentrations ≤ 5000 ng/mL, and is greater for concentrations over 5000 ng/mL (8.3% for 12,000 ng/mL; 11.2% for 26,000 ng/mL). Reardon et al. [21] performed a phase II study to evaluate the combination of IM (mesylate) plus hydroxyurea, a ribonucleotide reductase inhibitor, in patients with recurrent glioblastoma multiforme (GBM). Patients received IM plus hydroxyurea (500 mg twice a day) orally on a continuous daily schedule. The IM dose was 500 mg twice a day for patients on enzyme-inducing antiepileptic drugs (EIAEDs) and 400 mg once a day for those not on EIAEDs. IM protein binding was determined by equilibrium dialysis. Initial studies used control human plasma to which 5000 ng/mL of IM and 0, 25, 50 or 100 μmol/L of hydroxyurea were added. Subsequent studies evaluated IM protein binding in day 1 and 28 plasma samples from patients. IM was analyzed using a validated LC–MS [11]. In patients without EIAEDs (n = 18), the unbound fraction was 5.6% and 4.9% on days 1 and 28 respectively, with no significant difference. In patients with EIAEDs (n = 15), the unbound fraction was 4.7% and 4.8% on days 1 and 28 respectively, with no significant difference. Gibbons et al. [22] evaluated the safety, dose-limiting toxicities, maximum-tolerated dose, and pharmacokinetics of IM in sixty adult patients, with advanced solid tumors and varying renal function (normal, creatinine clearance [CrCL] ≥ 60 mL/min; mild dysfunction, CrCL 40–59 mL/min; moderate dysfunction, CrCL 20–39 mL/min; and severe dysfunction, CrCL < 20 mL/min), receiving daily IM doses of 100–800 mg. Treatment cycles were 28 days long. On day 15, IM protein binding was determined in the 24-h sample by equilibrium dialysis. Imatinib was analyzed by LC–MS [11]. The reported unbound fractions were 6.2%, 6.1% and 4.7% for normal (n = 12), mild (n = 18) and moderate (n = 15) kidney dysfunction, respectively. More recently, Streit et al. [17] monitored total and unbound IM/NDI concentrations in CML and GIST patients using LC–MS/MS. For unbound IM and NDI determinations, 200 μL of EDTA plasma was added to the sample reservoir of a Centrifree micropartition system and the tube was centrifuged for 40 min at 2000 × g and 20 °C. Then, 50 μL of the resulting ultrafiltrate was mixed with 50 μL of the IS solution (0.1 mg/L D8-imatinib in methanol) in an autosampler vial, before direct injection (25 μL) onto the LC–MS/MS system for an online extraction using an Oasis HLB column. IM unbound fraction varied widely from 3.0% to 14.6% in CML patients and from 2.6% to 9.7% in GIST patients. The mean percentage protein binding in trough-only samples in CML patients was 5.0% (range, 3.0–7.9%) and in GIST patients was 4.5% (range: 2.8–7.4%). Streit et al. [17] found NDI unbound fraction concentration-dependent and not reproducible, contrary to our results, probably due to a low sensitivity of their analytical method. Authors reported a LOQ of 8.3 ng/mL when a plasma sample volume of 50 μL was used (415 pg injected) *vs* a LOQ of 20 ng/mL (7 μL and 140 pg injected) in our method.

Non-specific binding of drugs on the filter device (polymer components of filter membrane or glass and plastic recipients) is a limiting factor for ultrafiltration. We found NSB values around 30–35% for imatinib, as reported by Streit et al. (30% for concentrations ranging from 4.2 to 125.6 ng/mL) [17], reaching 40% at the highest concentration (7500 ng/mL). However, lower NSBs around 22–25% were obtained for NDI plasma concentrations ranging from 1000 to 2500 ng/mL (Table 3). Similarly to Streit et al. [17] who attempted a pretreatment of filters with IM to no avail, we also failed to reduce NSB using pre-treatments with benzalkonium chloride [14] or sodium hydroxide as suggested by the manufacturer (data non shown). Despite this, our methodology gives reproducible unbound fractions and can be used for TDM.

Taylor's modified method [15] was presented as a way of overcoming the low solubility and the large NSB observed with conventional (standard) ultrafiltration techniques for a panel of high lipophilic corticosteroids. This strategy, which theoretically avoids NSB due to adsorption of compounds on collection tubes, seemed very attractive as a reference method for determination of unbound concentrations. However this method assumes the filter device (*e.g.* Amicon® ultra-0.5) is suitable to be returned for

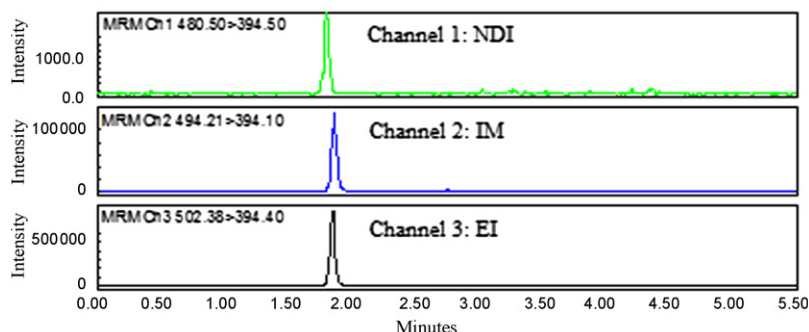

Fig. 3. Chromatogram from patient sample for the quantification of fu for IM and NDI.

Table 3
Non specific binding of imatinib and N-desmethyl imatinib on ultrafiltration devices.

	% fixation on the filter	
	Amicon® ultra-0.5 (mean, $n = 4$; *$n = 1$)	Centrifree® YM-30 (mean, $n = 3$)
Total plasma imatinib concentration (ng/mL)		
1000	14.1 ± 1.4	–
3000	13.9*	31.0 ± 1.4
4000	11.5 ± 1.8	–
5000	13.04*	35.3 ± 2.2
7500	10.0 ± 2.5	43.7 ± 0.9
Total plasma N-desmethyl imatinib concentration (ng/mL)		
1000	–	22.5 ± 2.2
1600	–	21.9 ± 2.3
2500	–	25.2 ± 0.7

successive centrifugations. With this device, the imatinib NSB on the filter was around 10–14% and was constant for IM total concentrations ranging from 1000 to 7500 ng/mL (Table 3). These results were lower than those obtained with the Centrifree® YM-30 device, probably due to the different methodology used depending on the features of the filter: in one case (Amicon®) a plasmatic solution is used, in the other (Centrifree®) a solution in Sorensen. Finally, these results seem to indicate that the value of NSB around 30% measured with Centrifree® YM filters may be overestimated compared to the true value and hence does not reflect the exact NSB present during ultrafiltration of plasma samples.

However, with Amicon® ultra-0.5, IM concentrations detected in ultrafiltrates were close to but generally under the lower limit of quantification (LOQ). Thus, accurate unbound concentrations and fractions could not be calculated. Under these conditions, with the hypothesis that the concentrations near the LOQ reflected the real unbound fraction, fu (%) could not exceed 1.5% (value estimated by dividing the LOQ by IM total concentration) independently of IM total concentration, centrifugal conditions ($4000 \times g$/6 min/4 °C *vs* $2000 \times g$/40 min/20 °C) and ultrafiltration process (standard *vs* modified). Similar results were obtained with high non therapeutic IM total concentrations (10,000, 25,000, 50,000 and 75,000 ng/mL; results not shown). This suggests that ultrafiltration with Amicon® ultra-0.5 cannot be used to separate unbound imatinib from plasma. Indeed, with Taylor's ultrafiltration methods, we failed to calculate the free fraction due to the very low IM concentrations in ultrafiltrate (≤LOQ). Although the composition of the filter device (modified cellulose) is reported to give little NSB [23], a significant IM adsorption on the filter (up to 10%) was revealed during our experiments (both with the Amicon® ultra-0.5 and with the Centrifree®YM-30 devices), whereas adsorption onto the plastic component of the collection tube during ultrafiltration was not detected (results not shown). Variability in duration and force of centrifugation may induce a molecular sieve effect, influencing the ultrafiltration rate [23,24], however similar results were obtained whatever the conditions applied for ultrafiltration with Amicon® ultra-0.5 device (40 min, $2000 \times g$, 20 °C *vs* 6 min, $4000 \times g$, 4 °C). Molecular sieving during ultrafiltration is a function of total pore area *per* unit path length, pore radius, molecular radius and filtration rate [24] and the divergence of results with each device was probably related to the shape of the filters. Actually, the liquid flow was quite different between methods with a normal flow filtration occurring in the direction of the membrane surface with Centrifree® YM-30 filters, and a tangential filtration-like flow with vertical membrane panels such as Amicon® ultra-0.5 filter [25]. In this case, particulates and macromolecules were swept along by the tangential flow and concentrations of analytes were under the LOQ.

4. Conclusion

Although we were not able to overcome NSB completely, we propose an ultrafiltration procedure which gives reproducible values for unbound fractions for IM (1.97 ± 0.68% to 4.15 ± 1.01%) and for the first time for NDI (2.91 ± 0.32% to 4.00 ± 2.01%) using spiked plasma samples ranging from 1000/1000 to 7500/2500 ng/mL (concentrations in agreement with values measured in patients). We have also validated the analytical UPLC–MS/MS method associated to ultrafiltration for quantitative determination of IM and NDI.

The difference in unbound fraction determination with the Amicon® ultra-0.5 compared to the Centrifree® filter emphasises the importance of the methodology and the device features used for the ultrafiltration step. This could totally compromise the determination of unbound drug concentrations.

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