Journal of Chromatography B, 937 (2013) 33–43

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

A validated assay for the simultaneous quantification of six tyrosine kinase inhibitors and two active metabolites in human serum using liquid chromatography coupled with tandem mass spectrometry

Nielka P. van Erp[a,b,*], Djoeke de Wit[a], Henk-Jan Guchelaar[a], Hans Gelderblom[c], Trees J. Hessing[a], Jan den Hartigh[a]

[a] Department of Clinical Pharmacy & Toxicology, Leiden University Medical Center, Leiden, The Netherlands
[b] Department of Clinical Pharmacy, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands
[c] Department of Clinical Oncology, Leiden University Medical Center, Leiden, The Netherlands

ARTICLE INFO

Article history:
Received 18 March 2013
Accepted 12 August 2013
Available online 17 August 2013

Keywords:
LC–MS/MS
Tyrosine kinase inhibitors
Metabolites

ABSTRACT

A sensitive, sophisticated and practical bioanalytical assay for the simultaneous determination of six tyrosine kinase inhibitors (imatinib, sunitinib, nilotinib, dasatinib, pazopanib, regorafenib) and two active metabolites (N-desmethyl imatinib and N-desethyl sunitinib) was developed and validated. For the quantitative assay, a mixture of three stable isotopes as internal standards was added to human serum, standards and controls. Thereafter, samples were pre-treated using protein precipitation with methanol. The supernatant was diluted with water and injected into an ultra pressure liquid chromatographic system with an Acquity TQ tandem mass spectrometry detector. The compounds were separated on an Acquity BEH C18 analytical column (100 mm × 2.1 mm ID, 1.7 μm particle size) and eluted with a linear gradient system. The ions were detected in the multiple reaction monitoring mode. The lower limit of quantification and the linearity of all compounds generously met with the concentrations that are to be expected in clinical practice. The developed bioanalytical assay can be used for guiding TKI therapy in daily clinical practice as well as for investigator-initiated research.

1. Introduction

In recent years, multiple tyrosine kinase inhibitors (TKIs) have been approved as mono-therapy for cancer treatment such as in renal cell cancer, gastro intestinal stromal tumors and leukemia, and numerous others are under investigation. Since these targeted anticancer compounds specifically inhibit cellular processes that are deregulated in various types of tumor cells, they were initially considered to be less toxic than conventional chemotherapy. However, it appears that similar to conventional chemotherapy, dose interruptions or reductions due to adverse effects are necessary in a large number of patients which indicates that TKIs have a narrow therapeutic window [1–4]. TKIs show large interpatient variability in pharmacokinetics, which results in highly variable plasma concentrations and consequently drug-exposure [5–9]. For several TKIs an indication for the optimal drug exposure (therapeutic window) has been derived from retrospective data-analysis [10–16]. The highly variable drug exposure will result in exposure levels outside the therapeutic window in a considerable number of patients. This might explain, at least in part, the toxicity and suboptimal response seen in some individuals.

Therapeutic drug monitoring (TDM) comprises the measurements, interpretation and adjustment of therapy in order to reach exposure levels within the target range. TKIs have most of the characteristics that are required for TDM, such as a narrow therapeutic window, large inter-patient variability compared to intra-patient variability and the chronic use until disease progression [17]. Therefore, TDM might be a very promising tool for this new class of drugs in order to improve treatment benefit by reducing toxicity and increasing efficacy.

To support clinical pharmacological studies and to address observations (toxicities and inefficacies) in daily clinical practice, it was essential to develop and validate a quantitative bioanalytical assay in which the mostly used TKIs can be quantified. We hereby present an assay in which imatinib, N-desmethyl imatinib, sunitinib, N-desethyl sunitinib, nilotinib, dasatinib, pazopanib and regorafenib can be determined simultaneously. The described

* Corresponding author at: Leiden University Medical Center, Department of Clinical Pharmacy & Toxicology, Room L0-042, Albinusdreef 2, 2333 ZA Leiden, The Netherlands. Tel.: +31 243616405.
E-mail address: P.vanErp@akf.umcn.nl (N.P. van Erp).

1570-0232/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jchromb.2013.08.013

Table 1
Target concentrations of the six TKIs and two metabolites in the calibration standards (CS) and quality control (QC) samples.

Sample type	Imatinib (mg/L)	Desmethyl imatinib (mg/L)	Nilotinib (mg/L)	Sunitinib (μg/L)	Desethyl sunitinib (μg/L)	Dasatinib (μg/L)	Pazopanib (mg/L)	Regorafenib (μg/L)
CS zero	0	0	0	0	0	0	0	0
CS 0	0	0	0	0	0	0	0	0
CS 1	0.1	0.1	0.1	2	2	5	1.0	0.1
CS 2	0.5	0.5	0.5	10	10	25	5.0	0.5
CS 3	1.0	1.0	1.0	40	40	100	10	1.0
CS 4	2.0	2.0	2.0	80	80	200	20	2.0
CS 5	3.0	3.0	3.0	160	160	300	30	3.0
CS 6	5.0	5.0	5.0	200	200	500	50	5.0
QC LLOQ	0.1	0.1	0.1	2	2	5	1	0.1
QC low	0.3	0.3	0.3	5	5	15	3	0.3
QC medium	1.5	1.5	1.5	50	50	150	15	1.5
QC high	4.0	4.0	4.0	180	180	400	40	4.0

LLOQ: lower limit of quantification

validations were performed according to the FDA guidelines for bioanalytical method validation [18].

2. Materials and methods

2.1. Chemicals and reagents

Imatinib mesylate and N-desmethyl imatinib mesylate were kindly provided by Novartis International Pharmaceuticals (Cork, Ireland). Nilotinib base and the isotope $^{13}C3^{15}N$ nilotinib hydrochloride were kindly provided by Novartis Pharma A.G. (Basel, Switzerland). Sunitinib maleate and N-desethyl sunitinib were kindly provided by Pfizer Inc. (Groton, USA). Regorafenib and pazopanib hydrochloride were obtained from Axon Medchem (Groningen, The Netherlands). Dasatinib base was obtained from LC laboratories (Woburn, MA, USA). The isotopes $^{2}H8$ dasatinib base and $^{13}C^{2}H3$ pazopanib hydrochloride were obtained from Alsachim (Strasbourg, France). DMSO (Uvasol), methanol (Lichrosolv), formic acid (Emsure) and ammonium acetate were obtained from Merck (Darmstadt, Germany). Methanol absolute (HPLC supra-gradient) was purchased from Biosolve (Valkenswaard, The Netherlands). Millipore quality water was used.

2.2. Preparation of stock solutions, calibration standards and quality controls samples

Two independent stock solutions were made for each analyte: imatinib, N-desmethyl imatinib, nilotinib, sunitinib, N-desethyl sunitinib, dasatinib, pazopanib and regorafenib. One stock solution was used for the preparation of calibration standards (CS) and the other for the quality control (QC) samples. All stock solutions were prepared in DMSO and contained 1 mg/mL free base except for pazopanib solution, which contained 2 mg/mL free base. Stock solutions of different analytes were mixed and diluted with methanol to obtain four QC and CS substock solutions. Subsequently, the substock solutions were combined and further diluted with methanol to prepare the working solution for the CS. The QC samples were prepared by adding combinations of the (sub)stock solutions to blank human serum. The (sub)stock solutions were diluted at least 15-fold. CS were prepared by adding 5 μL of the working solution to 50 μL blank human serum. The target concentrations of the CS and QC samples are listed in Table 1.

Stock solutions of the internal standards were also prepared in DMSO at a concentration of approximately 1 mg/mL. The internal standard working solution contained a mixture of the three internal standards in methanol: 1 mg/L $^{2}H8$ dasatinib, 5 mg/L $^{13}C^{2}H3$ pazopanib and 5 mg/L $^{13}C3^{15}N$ nilotinib.

All stock, substock and working solutions were stored at −20 °C. The QC samples were prepared in bulk and divided into aliquots of 75 μL in polypropylene vials which were stored at −20 °C. The CS were freshly prepared before each validation run.

2.3. Sample pre-treatment

Protein precipitation was used as sample pre-treatment for serum samples. To 50 μL of serum sample, 10 μL internal standard substock and 500 μL methanol were added. After vortex mixing for 3 min, samples were centrifuged at 13,000 × *g* for 5 min. To 200 μL supernatant, 200 μL water was added and mixed.

2.4. Liquid chromatography

The ultra pressure liquid chromatographic (UPLC) system used consisted of a coupled binary solvent manager, sample manager, column heater and mass spectrometry detector (Acquity, UPLC, Waters, Wilford, MA, USA). Chromatographic separation of the eight compounds and three internal standards in this assay was carried out using an Acquity UPLC BEH C18 chromatographic column (100 × 2.1 mm ID, particle size 1.7 μm, Waters) protected with an Acquity UPLC BEH C18 pre-column (5 × 2.1 mm ID, particle size 1.7 μm, Waters). For the analysis of pazopanib 1 μL and for the other compounds 10 μL was injected onto the column. The compounds were eluted with a linear gradient system at a flow rate of 0.4 mL/min. Mobile phase A consisted of 0.1% (v/v) formic acid and 2 mM ammonium acetate in water and mobile phase B consisted of 0.1% (v/v) formic acid and 2 mM ammonium acetate in methanol. The following linear gradient was used in this assay [time scale (min–min) mobile phase A (%)/mobile phase B (%)]: 0-1 75/25; 1-3 75/25 → 50/50; 3-5 50/50 → 10/90; 5-6 10/90; 6-6.5 10/90 → 75/25; 6.5-7 75/25. The column temperature was maintained at 50 °C and the auto-sampler temperature at 15 °C. The total run time was 7.0 min.

2.5. Mass spectrometry

The LC eluate was directed into a tandem quadruple, atmospheric pressure ionization (API) mass spectrometer (TQ detector, Acquity, Waters, Wilford, MA, USA). The detector was equipped with an electrospray ionization (ESI) source operating in the positive ion mode and configured in multiple reaction monitoring (MRM) mode. The data were acquired and processed using Masslynx™ Sofware (version 4.1, Waters). The general MS settings and analyte specific parameters for the assay are summarized in Table 2.

Table 2
General settings and analyte specific parameters for the analysis of all analytes.

General settings	
Capillary voltage (V)	1000
Extractor voltage (V)	1
Source temperature (°C)	150
Desolvation temperature (°C)	400
Cone gas flow (L/h)	50
Desolvation gas flow (L/h)	900
Collision gas flow (mL/min)	0.25

Analyte specific parameters	Imatinib	Desmethyl imatinib	Nilotinib	Sunitinib[a]	Desethyl sunitinib[a]	Dasatinib	Pazopanib	Regorafenib	IS-1	IS-2	IS-3
Parent mass (*m/z*)	494.1	480.1	530.1	399.2	371.1	488.1	438.1	483.0	496.1	442.1	534.1
Product mass (*m/z*)	393.9	393.9	288.9	282.9	282.9	400.9	357.0	269.9	405.5	361.0	292.9
Cone voltage (V)	45	50	55	40	35	60	50	45	60	40	55
Collision energy (V)	30	30	32	25	25	32	30	35	32	30	30
Dwell time (ms)	31	31	31	31	31	31	31	31	31	31	31
Typical retention time (min)	3.8	3.8	5.0	3.2–4.3	2.9–4.1	4.1	3.6	5.7	4.0	3.6	5.0

IS-1 = ^{2}H8 dasatinib; IS-2 = ^{13}C^{2}H3 pazopanib; IS-3 = ^{13}C3^{15}N nilotinib
[a] Diastereomeric cis/trans isomers: sunitinib → RT 3.2 and 4.3 and desethyl sunitinib → RT 2.9 and 4.1 are the trans- and cis-isomers respectively.

2.6. Quantification

The six TKIs and two metabolites were quantified in serum by describing the relationship between the peak area ratio with the internal standard *versus* the nominal concentration. ^{2}H8 dasatinib, ^{13}C^{2}H3 pazopanib and ^{13}C3^{15}N nilotinib were used as internal standards for their target analytes. ^{2}H8 dasatinib was also found to be a suitable internal standard for the quantification of sunitinib and N-desethyl sunitinib. ^{13}C3^{15}N nilotinib was used as the internal standard for imatinib, N-desmethyl imatinib and regorafenib. The calibration lines were chosen to cover the clinically relevant range of concentrations that are expected in patients treated with the registered dose.

2.7. Validation procedures

The validation of the assay was performed according to the FDA guidelines for validation of bioanalytical assays including linearity, accuracy, precision, selectivity, recovery and stability [18].

The linearity of the assay was assessed by preparing and analyzing 6 non-zero calibration standards (CS) in six independent analytical runs. Least squares linear regression analysis was applied to describe the relationship between the peak area ratio with the internal standard *versus* the nominal concentration. The lowest total bias and the most constant bias across the range were obtained using a weighting factor of $1/\chi$.

The within-run and between-run precision and accuracy were determined. To test the within-run precision, 6 replicates of the QC samples; QC low, QC medium and QC high were analyzed in one analytical run. To test the between-run precision, the QC samples QC LLOQ, QC low, QC medium and QC high were analyzed in six analytical runs on six different days. Precision was expressed as CV values and accuracy as deviations from the nominal concentrations.

Initially the cross-analyte interference was investigated by injecting dilutions of each analyte (TKIs, metabolites and IS) separately.

The selectivity of the assay was tested in six different batches of blank control serum and plasma. The selectivity was analyzed for blank serum, blank plasma, low (∼ the LLOQ concentration) and high (∼ highest standard) controls of each analyte prepared in duplo in 6 individual batches of blank control serum and plasma.

The total recovery, covering sample preparation and matrix effect, was determined six times at two concentration levels for each analyte (∼ LLOQ and highest standard) and the internal standards.

Carry-over was tested by injecting a blank sample after the highest calibration standard.

The stability of imatinib, desmethyl imatinib, nilotinib, sunitinib, desethyl sunitinib, dasatinib, pazopanib, and regorafenib in serum was tested at ambient temperature and at 2–8 °C after 0, 2, 3 days; 1, 2 weeks; and 1, 2 and 3 months. The long term stability at −20 °C was additionally tested at 9 months. Deviations were calculated against the initial concentrations. Analytes are considered stable in the matrix if the deviation is within ±15%. The stability of the individual stock solutions at −20 °C was determined with $n = 6$. Analytes were considered to be stable in stock solution if 90–110% of the initial concentration was found. The stability of the individual stock solutions was tested at 12 months.

3. Results and discussion

3.1. Method development

3.1.1. Sample pre-treatment

The most simple form of sample pre-treatment was initially tested; protein precipitation. Acetonitrile (ACN) and methanol were tested for protein precipitation. The in vial stability appeared to be less favorable for ACN sample pre-treatment. Additionally, peak symmetry appeared to be less favorably after ACN precipitation. Methanol was therefore selected as the solvent to precipitate proteins and showed high extraction recoveries for all analytes. To improve chromatographic separation water was added to the extract.

3.1.2. Chromatography

Two different combinations of mobile phases were tested: Mobile phase 1. A: 0.05% formic acid + 5 mM ammonium acetate in water and B: 100% acetonitrile and mobile phase 2. A: 0.1% formic acid + 2 mM ammonium acetate in water and B: 0.1% formic acid + 2 mM ammonium acetate in methanol. The gradient applied was the same for both combinations. Peak separation, shape and sensitivity of the assay was poorer for the mobile phase composed of ACN (mobile phase 1) than for the mobile phase containing methanol. After selection of the mobile phase the gradient was further optimized to reduce the run time from 10 to 7 min (Fig. 1).

3.1.3. Linearity

The concentration range that needs to be covered for pazopanib is much higher than for the other TKIs. Initially 10 μL was injected

Fig. 1. Typical MRM chromatograms of calibration standard 6 (CS 6) of dasatinib, nilotinib, imatinib, desmethylimatinib, pazopanib, regorafenib, sunitinib isomers (*trans*-isomer = snn1 and *cis*-isomer = snn2), desethylsunitinib isomers (*trans*-isomer = desnn1 and *cis*-isomer = desnn2), internal standards (IS) ^{2}H8 dasatinib, ^{13}C^{15}N nilotinib and ^{13}C^{2}H3 pazopanib, additionally chromatograms of five patients treated with these tyrosine kinase inhibitors are presented.

Table 3
Analytes with their selected mass transitions and proposed fragmentation pathways.

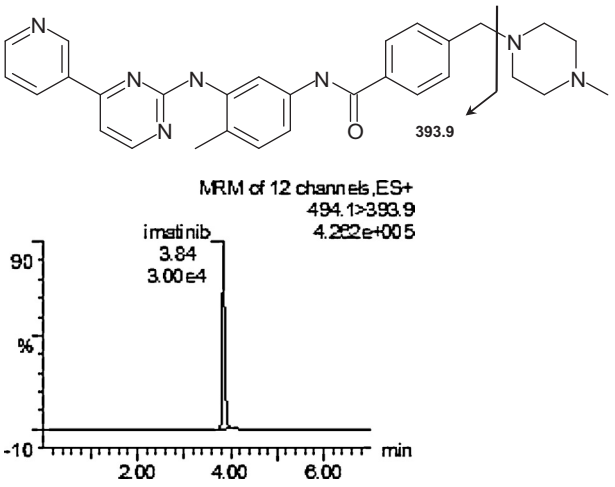
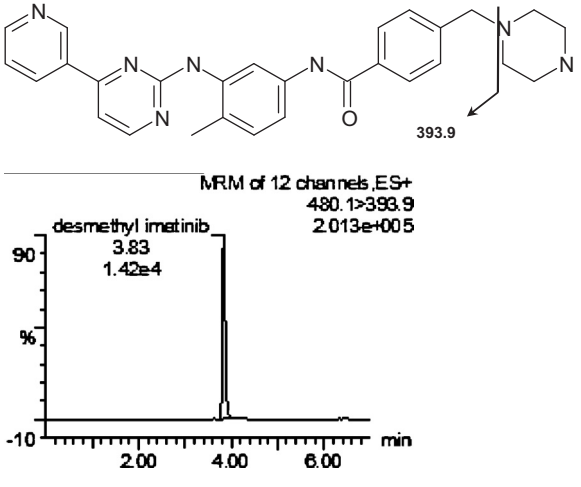
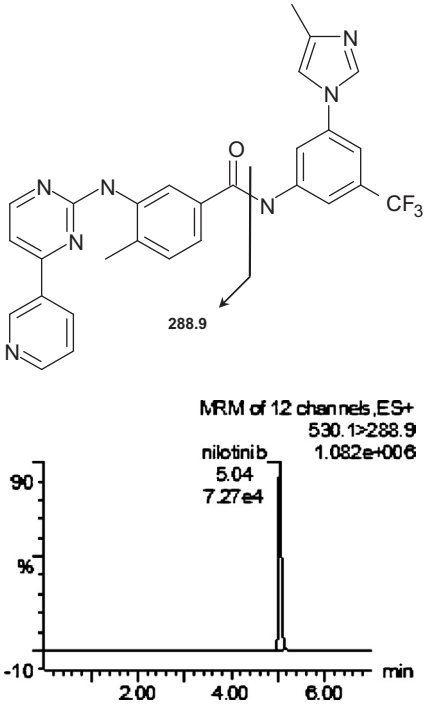
Compound	Transition	Proposed fragmentation and MRM chromatograms of calibration standard 6
Imatinib	494.1 → 393.9	
Desmethyl imatinib	480.1 → 393.9	
Nilotinib	530.1 → 288.9	

Table 3 (*Continued*)

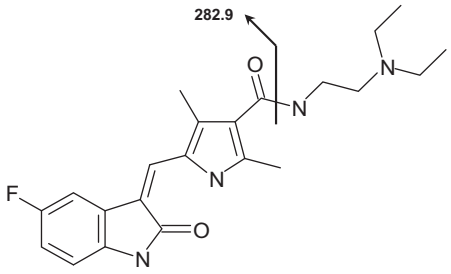
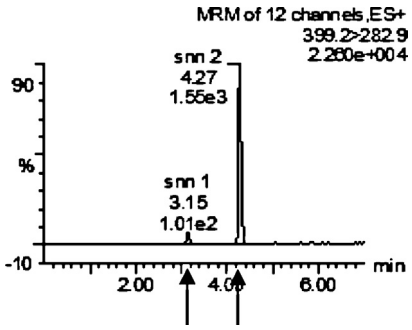
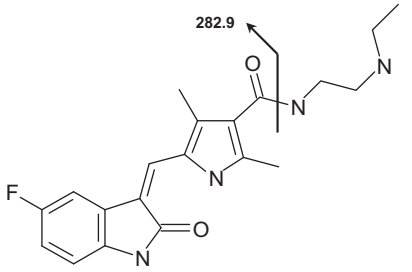
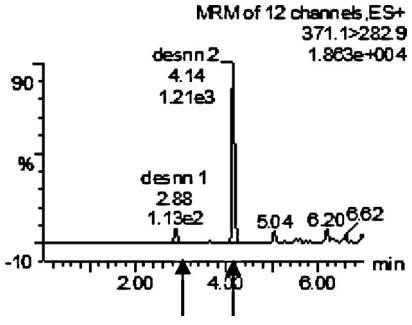
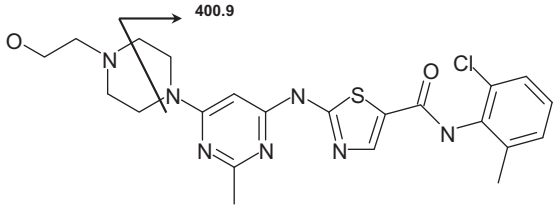
Compound	Transition	Proposed fragmentation and MRM chromatograms of calibration standard 6
Sunitinib	399.2 → 282.9	 
Desethyl sunitinib	371.1 → 282.9	 
Dasatinib	488.1 → 400.9	

Table 3 (*Continued*)

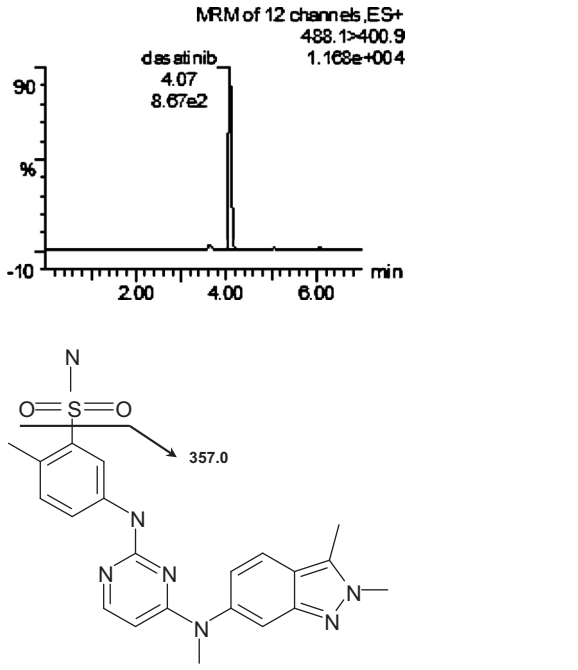
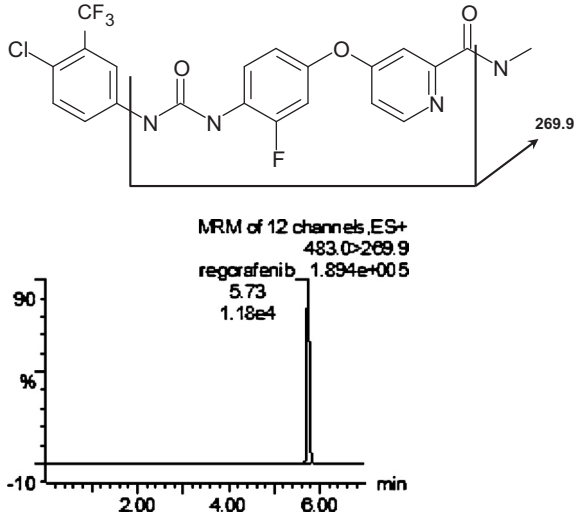
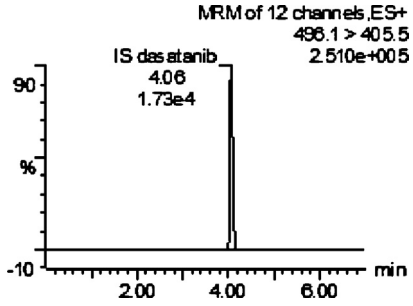
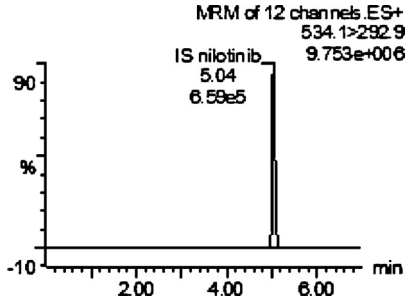
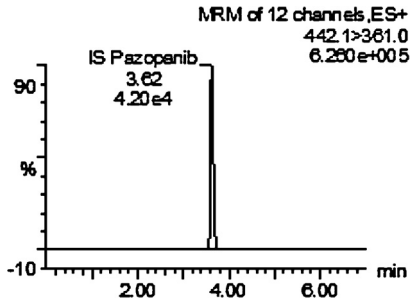
Compound	Transition	Proposed fragmentation and MRM chromatograms of calibration standard 6
Pazopanib	438.1 → 357.0	
Regorafenib	483.0 → 269.9	

Table 3 (*Continued*)

Compound	Transition	Proposed fragmentation and MRM chromatograms of calibration standard 6
IS dasatinib		
IS nilotinib		
IS pazopanib		

onto the chromatographic column for all the analytes. However, for pazopanib nonlinearity of the calibration line was seen during method development. This nonlinearity problem was solved by reducing the injection volume to 1 μL for pazopanib quantification, while keeping the injection volume for the other TKIs at 10 μL. In each analytical run the CS, QC and patient samples are injected two times. The first injection of 10 μL onto the system is for quantification all TKIs except for pazopanib. The second injection of 1 μL is for quantification of pazopanib.

3.1.4. Mass spectrometry

Mass spectrometric parameters were optimized by performing direct infusion and flow injection analysis of each analyte. In order to achieve high specificity and sensitivity, the multiple reaction monitoring (MRM) scan mode was applied to monitor the mass transition to the product ion with the highest abundance in the product ion scan for each analyte. Table 3 shows the selected transitions and the proposed corresponding fragmentation pathways.

3.1.5. Isomerization

As described in the literature, sunitinib and N-desethyl sunitinib showed light induced *trans* to *cis* transformation with an equal MS response [19]. Therefore the sum areas of both isomers of sunitinib and desethyl sunitinib were used for quantification.

3.2. Validation

3.2.1. Calibration

The linearity of the assay for all analytes expressed as correlation coefficients (R^2) were at least 0.995. The linear range varied per analyte but was in all cases between the corresponding concentration of the LLOQ and the highest CS as listed in Table 1. At all concentration levels the deviations of the back-calculated concentrations were within ±15% of the nominal concentrations and this is in accordance to FDA guidelines.

3.2.2. Precision and accuracy

Precision, expressed as CV values and accuracy, expressed as deviations from the nominal concentrations were below 7% and within ±11%, respectively. Therefore, the precision and accuracy were within the acceptance criteria of the FDA guidelines.

Additionally, the signal to noise ratio of the six TKIs and two metabolites at the LLOQ level was >5. In conclusion, the acceptance criteria for accuracy and precision were met. The assay performance data are summarized in Table 4.

3.2.3. Selectivity

No interference of the different compounds was observed in the test where dilutions of each analyte were injected separately. Only for the stable isotopes a small amount of the corresponding TKI was

Table 4
Assay performance data of six TKIs and two metabolites from 4 QC samples in six analytical runs.

Nominal conc.	Mean within run calculated conc.	Within-run precision (%CV)	Within-run accuracy (% dev.)	Mean between run calculated conc.	Between-run precision (%CV)	Between-run Accuracy (% dev.)
Imatinib (mg/L)						
0.1	–	–	–	0.10	4.0	97
0.3	0.27	3.0	91	0.28	4.2	93
1.5	1.43	3.4	96	1.44	5.7	96
4.0	4.09	1.1	102	3.93	3.8	98
Desmethyl imatinib (mg/L)						
0.1	–	–	–	0.10	3.8	102
0.3	0.31	2.7	102	0.31	3.8	104
1.5	1.57	2.6	105	1.56	6.2	104
4.0	4.20	1.4	105	4.03	4.2	101
Nilotinib (mg/L)						
0.1	–	–	–	0.10	5.4	97
0.3	0.27	1.5	91	0.28	3.3	94
1.5	1.38	1.0	92	1.43	2.2	95
4.0	3.76	0.6	94	3.75	1.7	94
Suntinib (μg/L)						
2	–	–	–	1.85	11.8	93
5	4.74	4.9	95	5.06	6.6	101
50	47.8	3.5	96	50.4	3.5	101
180	180.1	1.1	100	180.7	1.1	100
Desethyl sunitinib (μg/L)						
2	–	–	–	1.88	17.6	94
5	4.7	6.6	94	4.95	7.2	99
50	47.5	4.0	95	51.9	7.5	104
180	177.4	1.1	99	186.0	7.7	103
Dasatinib (μg/L)						
5	–	–	–	4.37	17.7	87
15	14.5	5.6	97	14.8	2.8	99
150	146.2	1.9	97	152.6	3.4	102
400	402.06	1.6	101	400.0	2.1	100
Pazopanib (mg/L)						
1	–	–	–	1.00	4.1	100
3	3.15	2.3	105	3.18	1.5	106
15	15.9	2.4	106	16.5	2.4	110
40	44.3	0.7	111	43.7	1.8	109
Regorafenib (mg/L)						
0.1	–	–	–	0.10	14.6	101
0.3	0.30	5.4	101	0.32	5.1	105
1.5	1.34	2.2	89	1.47	6.8	98
4.0	3.68	1.0	92	3.97	6.3	99

Conc., concentration; dev., deviation; CV, coefficient of variation.

measured, the measured signal was <5% of the LLOQ and therefore considered acceptable.

The blank controls (plasma and serum) showed no peaks co-eluting with one of the compounds. The variation (CV%) in the relative area of the low and high controls prepared in the 6 different batches of blank serum were <10% for all compounds tested. Only the variation of the low control of regorafenib was 15.8% which still meets the <20% variability allowed for the LLOQ. The low and high controls prepared in six different batches of blank plasma were all within 10% variation except for imatinib with 11.2 and 10.8% variation for the low and high concentration respectively. The deviation of plasma measurements from serum measurements for all analytes ranged from −15.8% to 4.9%. Based on these results we concluded that the selectivity was sufficient and that the assay was suitable for the detection of the analytes in both serum and plasma.

3.2.4. Recovery

In serum the mean total recovery of imatinib, N-desmethyl imatinib, nilotinib, sunitinib, N-desethyl sunitinib, dasatinib, pazopanib, regorafenib, ^{2}H8 dasatinib, ^{13}C^{2}H3 pazopanib and ^{13}C3^{15}N nilotinib ranged from 78% to 93% with CV% of 3.4–11.9% (except for regorafenib, LLOQ 23.5%). The variability of the detected regorafenib concentrations might be improved by introducing a structure analog as internal standard. At this point in the method validation we accepted the relative high variability in the recovery of regorafenib.

3.2.5. Carry-over

The response of the blank sample at the retention time of the analytes was <10% of the corresponding peak area of the LLOQ sample and the response of the internal standard was <1% of the normal response. Carry-over was therefore considered acceptable.

3.2.6. Stability

Except for regorafenib, all analytes were stable for at least 9 months at −20 °C. Regorafenib was stable for 3 months at −20 °C. At ambient temperature and at 2–8 °C all analytes were stable for 3 months, with the exception of imatinib QC low which was just outside the range of ±15% (18.1%).

The effect of six freeze/thaw cycles was tested for the LLOQ of all analytes in serum. The CV's were <17.7% and the observed concentrations were within 13% of the nominal concentrations. All analytes met the criteria that apply for LLOQ samples and were considered stable during six freeze/thaw cycles.

In-(autosampler)vial stability was tested by reinjecting the CS of the calibration line, and the QC low, medium and high samples

Table 5
Stability data of six TKIs and two metabolites in serum expressed as percentages (%) of the initial concentration.

Nominal conc.	Ambient 3 months	2–8 °C 3 months	−20 °C 9 months	Ambient In vial stability, 168 h
Imatinib (mg/L)				
0.3	81.9	95.2	103.7	100.5
1.5	87.9	93.0	102.5	102.2
4.0	91.0	92.8	98.9	99.7
Desmethyl imatinib (mg/L)				
0.3	87.4	94.8	107.7	100.1
1.5	92.9	91.4	102.7	100.9
4.0	89.0	89.8	100.2	100.6
Nilotinib (mg/L)				
0.3	107.4	105.2	105.9	99.8
1.5	107.3	99.9	102.8	100.0
4.0	105.4	98.5	102.5	100.0
Suntinib (μg/L)				
5	105.6	104.2	98.7	97.9
50	115.2	105.1	99.9	103.7
180	102.1	99.2	102.0	97.1
Desethyl sunitinib (μg/L)				
5	96.3	102.9	98.2	93.0
50	102.1	111.5	104.9	100.5
180	92.2	107.9	103.4	99.0
Dasatinib (μg/L)				
15	96.9	94.3	108.7	105.6
150	102.5	98.9	106.6	104.4
400	97.5	96.4	102.9	104.6
Pazopanib (mg/L)				
3	105.8	105.4	99.7	100.3
15	107.3	100.0	100.6	102.6
40	106.6	98.2	97.8	102.1
Regorafenib (mg/L)				
0.3	99.3	98.7	99.3[a]	99.2
1.5	107.8	101.4	113.3[a]	104.2
4.0	101.4	97.0	107.6[a]	105.1

[a] Regorafenib is stable for 3 months in the freezer.

24, 48 and 168 h after the original analysis. The in-vial stability at ambient temperature for all analytes was demonstrated to be at least 168 h.

The stability of the individual stock solutions was tested at 12 months and met the criteria. So, stock solutions of all analytes were at least stable for 12 months at −20 °C. The data on the stability of the analytes under different conditions are summarized in Table 5.

3.2.7. *Matrix effect*

In this study, the potential matrix effect was evaluated by spiking 6 lots of heparin plasma, EDTA plasma and serum at a low (∼ the LLOQ concentration) and a high level (∼ the highest standard) concentration of each analyte. The variability of the responses were <15%. Except for sunitinib, SU12662 low level in serum which were slightly higher 15.5% and 16.0%, respectively and desmethylimatinib low level in EDTA plasma with a variability of 15.6%. We concluded based on these data that the matrix did not appear to interfere significantly with the integrity of our analytical method.

4. Clinical application

The described validated assay aimed to support investigator initiated pharmacokinetic studies with imatinib, nilotinib, sunitinib, dasatinib, pazopanib and regorafenib. Additionally, the assay was developed to address unexplained cases of inefficacy or toxicity of TKI therapy in clinical practice. Moreover, the assay can be applied to explore the value of therapeutic drug monitoring for this class of drugs. To test the assay performance in patient material, we analyzed samples of patients that were treated with imatinib, pazopanib or sunitinib in study protocols and of patients treated with regorafenib in our clinic (Fig. 1A–D). Additionally external commercially controls from Chromsystems® to check the performance of the imatinib, N-desmethyl imatinib, nilotinib and dasatinib quantification. These QCs were all within 10% of the declared level (level 1–4).

5. Conclusion

A sensitive, sophisticated and practical bioanalytical assay for the simultaneous determination of six tyrosine kinase inhibitors (imatinib, sunitinib, nilotinib, dasatinib, regorafenib and pazopanib) and two active metabolites (N-desmethyl imatinib and N-desethyl sunitinib) was developed and validated according to FDA guidelines.

This assay has been applied to support an investigator initiated pharmacokinetic study with pazopanib and sunitinib. Moreover, the assay is being used to explore the possibilities of therapeutic drug monitoring and further understand the pharmacology of this class of drugs [20]. In the literature, multiple bioanalytical assays that quantify TKIs in human matrices have been described. Most of them are developed to quantify a single TKI [19,21,22]. For the clinical practice, it is more efficient to have a bioanalytical method available that can simultaneously determine the most abundantly used TKIs in the clinic. Thus far, nine methods have been published that are developed to simultaneously measure multiple TKIs. Six assays that are developed can simultaneously measure at most six TKIs [23–27], two methods can simultaneously determine eight

TKIs [28,29] and one method can simultaneously determine nine TKIs [30].

This present method deviates from previously published assays with regard to the TKIs that are simultaneously measured. Thus far, no multi TKI bioanalytical method has been published that incorporates pazopanib and regorafenib. Pazopanib is possibly not incorporated in other published methods due to the relatively high concentrations that need to be quantified. For pazopanib the serum concentrations are much higher as for the other TKIs. We have overcome the problem of nonlinearity at the highest concentrations by injecting less volume for pazopanib quantification on the analytical column. Regorafenib is potentially not incorporated yet since it was only very recently registered by the FDA for the treatment of metastatic colorectal cancer and is pending registration as third line treatment for GIST. In line with the method of Lankheet et al. [28] we have used protein precipitation, which is for routine measurements in the clinical setting a fast and simple sample pretreatment procedure manageable for any laboratory. The presented method robust, easy to perform is currently used for routine patient care in cases where unexpected toxicity, inefficacy and drug interactions are suspected. Additionally the assay is used to explore the benefit of routine therapeutic drug monitoring for TKIs as well as for investigator initiated studies.

Acknowledgement

The development of the assay was supported by “Stichting een gift voor GIST”. We thank “Stichting een gift voor GIST” for making this possible.

References

[1] T.E. Hutson, I.D. Davis, J.P. Machiels, P.L. De Souza, S. Rottey, B.F. Hong, R.J. Epstein, K.L. Baker, L. McCann, T. Crofts, L. Pandite, R.A. Figlin, J. Clin. Oncol. 28 (2010) 475.
[2] G.D. Demetri, A.T. van Oosterom, C.R. Garrett, M.E. Blackstein, M.H. Shah, J. Verweij, G. McArthur, I.R. Judson, M.C. Heinrich, J.A. Morgan, J. Desai, C.D. Fletcher, S. George, C.L. Bello, X. Huang, C.M. Baum, P.G. Casali, Lancet 368 (2006) 1329.
[3] B.J. Druker, M. Talpaz, D.J. Resta, B. Peng, E. Buchdunger, J.M. Ford, N.B. Lydon, H. Kantarjian, R. Capdeville, S. Ohno-Jones, C.L. Sawyers, N. Engl. J. Med. 344 (2001) 1031.
[4] T.E. Hutson, J. Bellmunt, C. Porta, C. Szczylik, M. Staehler, A. Nadel, S. Anderson, R. Bukowski, T. Eisen, B. Escudier, Eur. J. Cancer 46 (2010) 2432.
[5] N.P. van Erp, S.D. Baker, A.S. Zandvliet, B.A. Ploeger, M. den Hollander, Z. Chen, J. den Hartigh, J.M. Konig-Quartel, H.J. Guchelaar, H. Gelderblom, Cancer Chemother. Pharmacol. 67 (2011) 695.
[6] A. Broniscer, S.J. Baker, C.F. Stewart, T.E. Merchant, F.H. Laningham, P. Schaiquevich, M. Kocak, E.B. Morris, R. Endersby, D.W. Ellison, A. Gajjar, Clin. Cancer Res. 15 (2009) 701.
[7] R.S. Herbst, A.M. Maddox, M.L. Rothenberg, E.J. Small, E.H. Rubin, J. Baselga, F. Rojo, W.K. Hong, H. Swaisland, S.D. Averbuch, J. Ochs, P.M. LoRusso, J. Clin. Oncol. 20 (2002) 3815.
[8] G.D. Demetri, P.G. Casali, J.Y. Blay, M.M. von, J.A. Morgan, R. Bertulli, I. Ray-Coquard, P. Cassier, M. Davey, H. Borghaei, D. Pink, M. biec-Rychter, W. Cheung, S.M. Bailey, M.L. Veronese, A. Reichardt, E. Fumagalli, P. Reichardt, Clin. Cancer Res. 15 (2009) 5910.
[9] H.I. Hurwitz, A. Dowlati, S. Saini, S. Savage, A.B. Suttle, D.M. Gibson, J.P. Hodge, E.M. Merkle, L. Pandite, Clin. Cancer Res. 15 (2009) 4220.
[10] R.A. Larson, B.J. Druker, F. Guilhot, S.G. O'Brien, G.J. Riviere, T. Krahnke, I. Gathmann, Y. Wang, Blood 111 (2008) 4022.
[11] B.E. Houk, C.L. Bello, B. Poland, L.S. Rosen, G.D. Demetri, R.J. Motzer, Cancer Chemother. Pharmacol. 66 (2010) 357.
[12] G.D. Demetri, Y. Wang, E. Wehrle, A. Racine, Z. Nikolova, C.D. Blanke, H. Joensuu, M.M. von, J. Clin. Oncol. 27 (2009) 3141.
[13] N. Widmer, L.A. Decosterd, S. Leyvraz, M.A. Duchosal, A. Rosselet, M. Biec-Rychter, C. Csajka, J. Biollaz, T. Buclin, Br. J. Cancer 98 (2008) 1633.
[14] B. Suttle, H.A. Ball, M. Molimard, D. Rajagopalan, R.S. Swann, R.G. Amado, L. Pandite, ASCO Annual Meeting 2010, 2010.
[15] Y. Lin, H.A. Ball, B. Suttle, F. Mehmud, R.G. Amado, T.E. Hutson, L.N. Pandite, Genitourinary Cancer Symposium 2011, 2011.
[16] R.A. Larson, O.Q. Yin, A. Hochhaus, G. Saglio, R.E. Clark, H. Nakamae, N.J. Gallagher, E. Demirhan, T.P. Hughes, H.M. Kantarjian, P.D. le Coutre, Eur. J. Clin. Pharmacol. 68 (5) (2012) 723–733.
[17] M.E. de Jonge, A.D. Huitema, J.H. Schellens, S. Rodenhuis, J.H. Beijnen, Clin. Pharmacokinet. 44 (2005) 147.
[18] FDA, 2001, Guidance for Industry: Bioanalytical Method Validation. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf
[19] P. de Bruijn, S. Sleijfer, M.H. Lam, R.H. Mathijssen, E.A. Wiemer, W.J. Loos, J. Pharm. Biomed. Anal. 51 (2010) 934.
[20] N.P. van Erp, H. Gelderblom, H.J. Guchelaar, Cancer Treat. Rev. 35 (2009) 692.
[21] R.W. Sparidans, T.T. Ahmed, E.W. Muilwijk, M.E. Welzen, J.H. Schellens, J.H. Beijnen, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 905 (2012) 137.
[22] N.A. Lankheet, E.E. Schaake, H. Rosing, J.A. Burgers, J.H. Schellens, J.H. Beijnen, A.D. Huitema, Bioanalysis 4 (2012) 2563.
[23] A. Chahbouni, J.C. den Burger, R.M. Vos, A. Sinjewel, A.J. Wilhelm, Ther. Drug Monit. 31 (2009) 683.
[24] S. De Francia, A. D'Avolio, F. De Martino, E. Pirro, L. Baietto, M. Siccardi, M. Simiele, S. Racca, G. Saglio, F. Di Carlo, G. Di Perri, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 877 (2009) 1721.
[25] L. Gotze, A. Hegele, S.K. Metzelder, H. Renz, W.A. Nockher, Clin. Chim. Acta 413 (2012) 143.
[26] 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: Analyt. Technol. Biomed. Life Sci. 877 (2009) 1982.
[27] Y. Hsieh, G. Galviz, Q. Zhou, C. Duncan, Rapid Commun. Mass Spectrom.: RCM 23 (2009) 1364.
[28] N.A. Lankheet, M.J. Hillebrand, H. Rosing, J.H. Schellens, J.H. Beijnen, A.D. Huitema, Biomed. Chromatogr.: BMC 27 (2013) 466.
[29] L. Couchman, M. Birch, R. Ireland, A. Corrigan, S. Wickramasinghe, D. Josephs, J. Spicer, R.J. Flanagan, Anal. Bioanal. Chem. 403 (2012) 1685.
[30] S. Bouchet, E. Chauzit, D. Ducint, N. Castaing, M. Canal-Raffin, N. Moore, K. Titier, M. Molimard, Clin. Chim. Acta 412 (2011) 1060.