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ORIGINAL PAPER

An automated method for the measurement of a range of tyrosine kinase inhibitors in human plasma or serum using turbulent flow liquid chromatography–tandem mass spectrometry

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Abstract Tyrosine kinase inhibitors (TKIs) are used to treat a number of cancers, including chronic myeloid leukaemia and hepatocellular carcinoma. Therapeutic drug monitoring (TDM) may be indicated to (1) monitor adherence, (2) guide dosage, and (3) minimise the risk of drug–drug interactions and dose-related toxicity. On-line, automated sample preparation provided by TurboFlow technology (ThermoFisher Scientific) in conjunction with the sensitivity and selectivity of tandem mass spectrometry (MS/MS) detection may be applied to the analysis of single drugs and metabolites. We report the use of TurboFlow LC–MS/MS for the analysis of nine TKIs and metabolites (imatinib, *N*-desmethylimatinib, dasatinib, nilotinib, erlotinib, gefitinib, lapatinib, sorafenib, sunitinib) in human plasma or serum for TDM purposes. An Aria Transcend TLX-II system coupled with a TSQ Vantage was used. Samples (50 μL) were vortex mixed with internal standard solution (150 μL imatinib-D_8, gefitinib-D_8, sunitinib-D_{10}, and nilotinib-$^{13}C_2{}^{15}N_2$ in acetonitrile) and, after centrifugation 100 μL supernatant were injected directly onto a 50×0.5-mm Cyclone TurboFlow column. Analytes were focussed onto a 50×2.1-mm (3 μm) Hypersil GOLD analytical column and eluted with an acetonitrile/water gradient. Analytes were monitored in selected reaction monitoring mode (positive APCI). Total analysis time was 7 min without multiplexing. Calibration was linear (R^2>0.99) for all analytes. Inter- and intra-assay precision (in percent relative standard deviation, RSD) was <11 % and accuracy 89–117 % for all analytes. No matrix effects were observed. This method is suitable for high-throughput TDM in patients undergoing chronic therapy with TKIs and has been utilised in the analysis of clinical samples.

Keywords TurboFlow MS/MS · TKIs · Imatinib · Dasatinib · Nilotinib · TDM · Automated sample preparation

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Introduction

Many genes and proteins that drive tumour growth have now been identified. By identifying these genes and proteins as clinical targets, small-molecule signal-transduction inhibitors have been developed for the treatment of cancer. A group of such targets are the tyrosine kinases, enzymes that phosphorylate proteins

leading to the activation of signal-transduction pathways that in turn play a critical role in a variety of biological processes, including cell growth, differentiation, and death [1]. These proteins all contain a highly conserved kinase domain, which includes binding sites for small-molecule inhibitors (tyrosine kinase inhibitors, TKIs).

Imatinib was the first clinically useful TKI and revolutionised the treatment and prognosis of chronic myeloid leukaemia (CML) and gastrointestinal stromal tumours (GIST) [2]. Since the introduction of imatinib, a number of TKIs have been developed, all of which to date are designed to compete with ATP at the ATP-binding pocket of the enzymes that are mutated and/or overexpressed in specific tumours. To date, eight TKIs have been approved for use in the UK [3] in cancer therapy, and many more are under investigation (Table 1).

Unlike traditional anti-cancer therapies that are given by intravenous infusion, TKIs are administered orally, and their bioavailability is thus dependent on gastrointestinal absorption and first-pass metabolism. In addition, they are metabolised by cytochrome P450 enzymes, the activity of which may be subject to large inter-individual variability, and which may be influenced by a number of factors such as drug–drug interactions, food intake, and smoking habit. Moreover, some TKIs are substrates of drug transporters, i.e. efflux pumps and uptake pumps, and are extensively (>90 %) bound to plasma protein [4]. Finally, some TKIs are capable of inhibiting their own transporters and metabolising enzymes, making their disposition and metabolism at steady state difficult to predict [4–6]. A given dose can therefore yield significantly different plasma concentrations in different patients, favouring the selection of resistant clones in the case of sub-therapeutic drug exposure and increasing the risk of toxicity if dosage is excessive. Hence, the value of therapeutic drug monitoring (TDM), i.e. the measurement of plasma concentrations of drugs and sometimes metabolites, is being explored with the aim of assessing adherence and optimising dosage [7–13].

In order to maximise the clinical benefit of TDM, accurate quantitative results are required using a minimal sample size and with minimal turnaround times. Off-line sample extraction techniques (liquid–liquid or solid-phase extraction) may be lengthy and labour intensive and may be impractical with water-soluble analytes. TurboFlow technology (ThermoFisher Scientific) offers on-line, automated methodology, based on two-dimensional liquid chromatography, with the first dimension using specialised high-turbulence liquid chromatography columns and a customised valve-switching arrangement. Retained analytes are subsequently transferred to a traditional HPLC column (second dimension). Multiplexing technology, in which multiple TurboFlow-HPLC channels flow to a single detector, further increases capacity for high-throughput analyses [14, 15]. When combined with the sensitivity/selectivity of tandem mass spectrometry (MS/MS), this technology may be applied to the analysis either of single drugs (and possibly metabolites) or to groups of similar compounds.

Table 1 Some tyrosine kinase inhibitors

Generic name (*trade name*; sponsoring company)	Tyrosine kinase target	Cancer indication
1. Bcr-abl tyrosine kinase inhibitors		
Imatinib (*Glivec*; Novartis)	Bcr-abl, PDGFRα, -β, c-kit	Philadelphia-chromosome positive CML, c-kit positive GIST
Dasatinib (*Sprycel*; Bristol Myers-Squibb)	Bcr-abl, Src-family kinases, PDGFRβ, c-kit, ephrin (Eph) receptor kinases	Philadelphia-chromosome positive CML and ALL resistant or intolerant to imatinib
Nilotinib (*Tasigna*; Novartis)	Bcr-abl, PDGFRα, -β, c-kit, DDR-1, -2	Chronic/accelerated phase CML resistant or intolerant to imatinib
2. ErbB tyrosine kinase inhibitors		
Gefitinib (*Iressa*; AstraZeneca)	EGFR	EGFR mutation positive locally advanced or metastatic NSCLC after failure of chemotherapy
Erlotinib (*Tarceva*; Genentech, OSI Pharmaceuticals, Roche)	EGFR	Locally advanced or metastatic NSCLC after failure of chemotherapy or for maintenance, locally advanced or metastatic pancreatic cancer
Lapatinib (*Tykerb*; GlaxoSmithKline)	EGFR (HER-1), HER-2	HER-2 positive breast cancer
3. VEGFR tyrosine kinase inhibitors		
Sunitinib (*Sutent*; Pfizer)	PDGFRα, -β, VEGFR1, -2, -3, c-kit, RET, CSF-1R, FLT3	Advanced RCC, GIST after progression on or intolerance to imatinib
Sorafenib (*Nexavar*; Bayer)	c-Raf, B-Raf, c-kit, FLT3, VEGFR1, -2, -3, PDGFR-β	Advanced RCC and HCC

Early methods for the analysis of certain TKIs were carried out using HPLC with UV detection [16], but not all TKIs possess good UV absorbance. Assays for the measurement of TKIs have therefore been developed using LC–MS/MS and include some where several compounds can be measured in a single analysis [9, 17–19].

The aim of this work was to develop a simple, robust TurboFlow LC–MS/MS method for the analysis of imatinib and *N*-desmethylimatinib (norimatinib), dasatinib, nilotinib, gefitinib, erlotinib, lapatinib, sunitinib, and sorafenib (Fig. 1) suitable for measuring these compounds across a concentration range typically attained during therapy.

Materials and methods

Materials and reagents

Imatinib mesylate, norimatinib, imatinib-D_8, nilotinib hydrochloride monohydrate, and nilotinib-$^{13}C_2{}^{15}N_2$ were obtained from Novartis (Basel, Switzerland). Dasatinib hydrate was obtained from Sequoia Research Products (Berkshire, UK), and sorafenib tosylate was obtained from Bayer Schering Pharma (Berlin, Germany). Erlotinib hydrochloride, gefitinib, lapatinib ditosylate, and sunitinib malate were obtained from Selleck (Houston, USA). Sunitinib-D_{10} and gefitinib-D_8 were purchased from Alsachim (Illkirch-Graffenstaden, France).

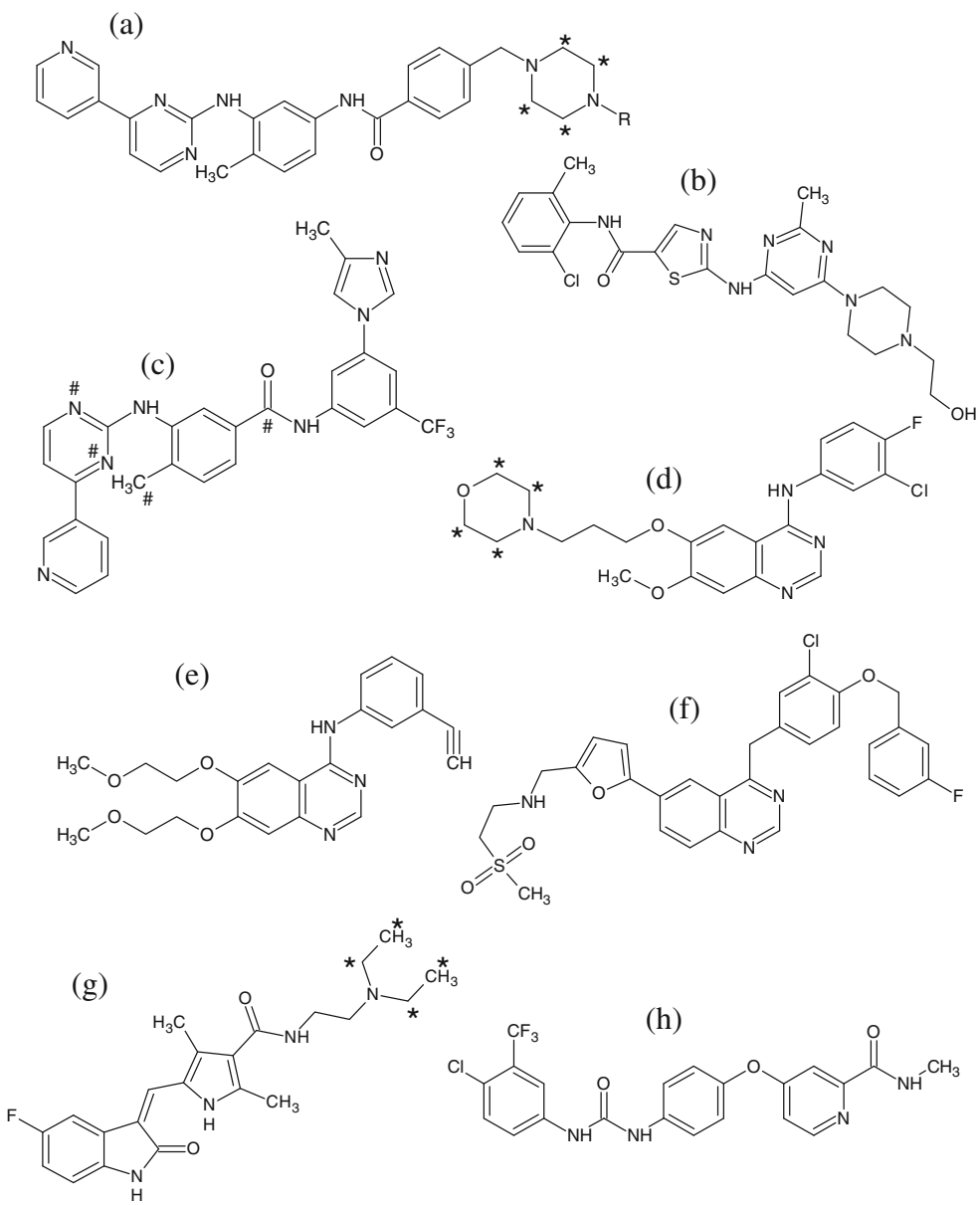

Fig. 1 Structural formulae of **a** imatinib (R=CH_3), norimatinib (R=H), and imatinib-D_8, **b** dasatinib, **c** nilotinib and nilotinib-$^{13}C_2{}^{15}N_2$, **d** gefitinib and gefitinib-D_8, **e** erlotinib, **f** lapatinib, **g** sunitinib and sunitnib-D_{10}, and **h** sorafenib. For all IS structures, the position of isotopically labelled atoms are indicated (CD_2 and CD_3 groups are indicated with a single asterisk (*), ^{13}C and ^{15}N atoms are indicated by #)

Table 2 TurboFlow LC–MS/MS of TKIs: gradient elution and valve-switching profile. Step 1: sample loading, step 2: transfer of retained analytes to the analytical column, steps 3–4: gradient elution (analytical column) and column washing (TurboFlow column), step 5: elution loop filling (loading pump), steps 6–7: system re-equilibration

			Loading (TurboFlow) pump							Eluting (analytical) pump			
Step	Start time (min:s)	Time (s)	Flow (mL/min)	Gradient	% A	% B	% C	Tee	Loop	Flow (mL/min)	Gradient	% A	% B
1	0:00	30	2.00	Step	100	–	–	Out	Out	0.80	Step	95	5
2	0:30	40	0.10	Step	100	–	–	In	In	0.70	Step	95	5
3	1:10	240	1.00	Step	–	100	–	Out	In	0.80	Ramp	–	100
4	5:10	30	1.00	Step	–	–	100	Out	Out	0.80	Ramp	–	100
5	5:40	10	1.00	Step	–	100	–	Out	In	0.80	Step	95	5
6	5:50	10	1.00	Step	100	–	–	Out	Out	0.80	Step	95	5
7	6:00	20	2.00	Step	100	–		Out	Out	0.80	Step	95	5

HPLC grade methanol, acetonitrile, acetone, and 2-propanol were all purchased from Rathburn (Walkerburn, UK). Water was deionised (18 mΩ, Elga, Marlow, UK). Ammonium acetate was purchased from Sigma-Aldrich (Poole, UK), formic acid was purchased from Fluka (Poole, UK), and coarsely filtered, pooled human serum was purchased from Sera Labs (Haywards Heath, UK). External quality control (EQC) samples containing imatinib, norimatinib, dasatinib, and nilotinib (two concentrations each analyte) were purchased from Chromsystems (Munich, Germany), lot no. 0510.

Table 3 Selected reaction monitoring parameters

Analyte	Precursor ion (*m/z*)	Fragment ions (*m/z*)	Collision energy (V)	S-lens voltage (V)
Imatinib	494.2	247.0 394.1	40 25	170
Imatinib-D_8	502.4	222.2 394.1	39 23	146
Norimatinib	480.2	247.0 394.1	47 23	139
Dasatinib	488.2	232.0 401.0	38 27	172
Nilotinib	530.2	261.0 289.0	43 31	213
Nilotinib-$^{13}C_2{}^{15}N_2$	534.3	262.0 293.0	55 28	190
Gefitinib	447.1	100.1 128.1	36 24	129
Gefitinib-D_8	455.3	108.2 136.2	39 26	145
Erlotinib	394.1	278.1 336.1	30 22	139
Lapatinib	581.1	350.0 365.1	40 35	214
Sorafenib	465.1	202.0 252.1	36 29	172
Sunitinib	399.1	283.1 326.1	26 20	112
Sunitinib-D_{10}	409.4	283.2 326.2	30 22	108
Sorafenib	465.1	202.0 252.1	36 29	172

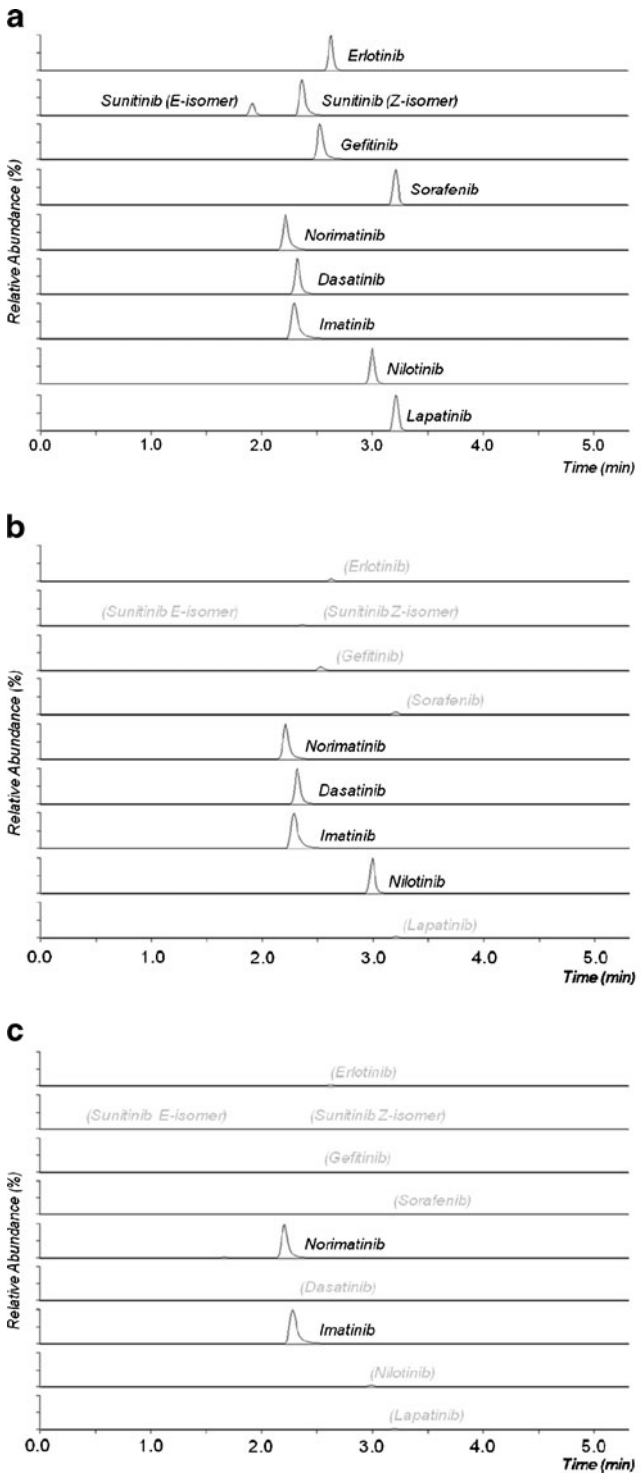

Fig. 2 Representative extracted ion chromatograms from **a** serum calibration standard 5 (concentrations: imatinib and erlotinib, 1.00 mg/L; norimatinib and gefitinib, 0.20 mg/L; nilotinib, lapatinib, and sorafenib, 2.00 mg/L; and dasatinib and sunitinib, 25 μg/L), **b** Chromsystems EQC Level II (measured [nominal] concentrations: imatinib, 1.70 mg/L [1.53–2.29 mg/L]; norimatinib, 0.29 mg/L [0.27–0.41 mg/L]; dasatinib, 204 μg/L [202–302 μg/L]; and nilotinib 1.06 mg/L [0.95–1.42 mg/L]), and **c** sample 14-h post dose from a patient prescribed 800 mg/day imatinib, imatinib and norimatinib concentrations (in milligrams per litre): 2.39 and 0.52, respectively. Chromatograms shown in order of increasing Q1 *m/z* (top to bottom): erlotinib, sunitinib (*E*- and *Z*-isomers), gefitinib, sorafenib, norimatinib, dasatinib, imatinib, nilotinib, and lapatinib. Internal standard chromatograms not shown

Preparation of calibration and internal quality control solutions

Individual stock solutions containing imatinib, norimatinib, nilotinib, gefitinib, erlotinib, lapatinib, and sorafenib (each 250 mg/L), and dasatinib and sunitinib (each 50 mg/L) were prepared in methanol. Equal volumes (1.00 mL) of each stock solution were added separately to 10- and 50-mL volumetric flasks and made up to volume with methanol to give working solutions A and B, respectively. Separate stock and working solutions were used to prepare calibration and internal quality control (IQC) solutions. Appropriate volumes of calibration working solutions were evaporated to dryness in 20-mL volumetric flasks under a gentle stream of nitrogen and reconstituted with analyte-free pooled human serum to give calibration solutions (N=7) over the following concentration ranges: imatinib and erlotinib, 0.05–5.0 mg/L; norimatinib and gefitinib, 0.01–1.0 mg/L; nilotinib, sorafenib, and lapatinib, 0.10–5.0 mg/L; and dasatinib and sunitinib, 1–150 μg/L. IQC solutions (N=3) were similarly prepared at 0.40, 1.20, and 3.00 mg/L for imatinib and erlotinib; 0.10, 0.30, and 0.80 mg/L for norimatinib and gefitinib; 0.40, 2.00, and 4.00 mg/L for nilotinib, sorafenib, and lapatinib; and 5, 50, and 120 μg/L for dasatinib and sunitinib. After thorough mixing and equilibration (24 h, 2–8 °C), calibration and IQC solutions were stored in approximately 150-μL portions in 2-mL polypropylene screw-top tubes at −20 °C until required.

Internal standard solution

A working internal standard (IS) solution containing imatinib-D_8 (200 μg/L), gefitinib-D_8 (200 μg/L), sunitinib-D_{10} (1.0 mg/L), and nilotinib-$^{13}C_2{}^{15}N_2$ (2.0 mg/L) was prepared by appropriate dilution of stock solutions (250 mg/L each compound in methanol) with acetonitrile. The solution was stored and used at 2–8 °C and returned promptly to the refrigerator after use.

Sample preparation

Portions of calibration standards and IQC solutions were thawed and mixed by inversion at room temperature prior to use. Centrifuged plasma/serum samples, calibration standards, and IQC solutions (50 μL) were vortex mixed (5 min) with 150 μL chilled working IS solution in 1.5-mL microcentrifuge tubes (Eppendorf, Cambridge, UK). After centrifugation (13,000×*g*, 5 min), the supernatant was transferred with fine-tipped pasteur pipettes (Alpha Laboratories, Eastleigh, UK) to reduced-volume glass

Table 4 (a) Intra- and (b) inter-assay accuracy and precision data

Analyte		IQC A	IQC B	IQC C
(a) Intra-assay (N=5 at each concentration)				
Imatinib	Nominal (mg/L)	0.40	1.20	3.00
	Mean measured (mg/L)	0.45	1.16	3.13
	RSD (%)	3.29	7.14	5.37
	Accuracy (% nominal)	112	97	104
Norimatinib	Nominal (mg/L)	0.10	0.30	0.80
	Mean measured (mg/L)	0.10	0.28	0.76
	RSD (%)	6.81	5.39	1.80
	Accuracy (% nominal)	95	94	94
Dasatinib	Nominal (mg/L)	5.0	50	120
	Mean measured (mg/L)	5.0	51	122
	RSD (%)	8.44	8.86	8.37
	Accuracy (% nominal)	100	103	102
Nilotinib	Nominal (mg/L)	0.40	2.00	4.00
	Mean measured (mg/L)	0.43	2.01	4.12
	RSD (%)	6.92	8.41	8.72
	Accuracy (% nominal)	108	101	103
Gefitinib	Nominal (mg/L)	0.10	0.30	0.80
	Mean measured (mg/L)	0.10	0.30	0.81
	RSD (%)	2.37	8.80	5.26
	Accuracy (% nominal)	98	100	101
Erlotinib	Nominal (mg/L)	0.40	1.20	3.00
	Mean measured (mg/L)	0.39	1.14	2.68
	RSD (%)	2.93	8.27	6.92
	Accuracy (% nominal)	97	95	89
Lapatinib	Nominal (mg/L)	0.40	2.00	4.00
	Mean measured (mg/L)	0.41	2.02	3.98
	RSD (%)	4.62	7.45	3.98
	Accuracy (% nominal)	102	101	99
Sunitinib	Nominal (mg/L)	5.0	50	120
	Mean measured (mg/L)	4.9	54	133
	RSD (%)	9.29	7.52	3.49
	Accuracy (% nominal)	98	109	111
Sorafenib	Nominal (mg/L)	0.40	2.00	4.00
	Mean measured (mg/L)	0.47	1.93	4.24
	RSD (%)	4.07	6.66	5.90
	Accuracy (% nominal)	117	96	106
(b) Inter-assay (N=5 at each concentration)				
Imatinib	Nominal (mg/L)	0.40	1.20	3.00
	Mean measured (mg/L)	0.43	1.18	3.04
	RSD (%)	8.06	6.30	8.22
	Accuracy (% nominal)	106	99	101
Norimatinib	Nominal (mg/L)	0.10	0.30	0.80
	Mean measured (mg/L)	0.10	0.29	0.76
	RSD (%)	9.12	9.15	8.89
	Accuracy (% nominal)	95	98	96
Dasatinib	Nominal (mg/L)	5.0	50	120
	Mean measured (mg/L)	4.9	51	120
	RSD (%)	11.72	5.48	4.94
	Accuracy (% nominal)	99	102	100
Nilotinib	Nominal (mg/L)	0.40	2.00	4.00
	Mean measured (mg/L)	0.44	1.99	4.14
	RSD (%)	4.24	7.47	3.31
	Accuracy (% nominal)	110	100	104
Gefitinib	Nominal (mg/L)	0.10	0.30	0.80
	Mean measured (mg/L)	0.10	0.30	0.80
	RSD (%)	4.23	4.39	2.48
	Accuracy (% nominal)	97	99	100

Table 4 (continued)

Analyte		IQC A	IQC B	IQC C
Erlotinib	Nominal (mg/L)	0.40	1.20	3.00
	Mean measured (mg/L)	0.38	1.11	2.83
	RSD (%)	5.77	8.05	4.22
	Accuracy (% nominal)	96	93	94
Lapatinib	Nominal (mg/L)	0.40	2.00	4.00
	Mean measured (mg/L)	0.40	1.92	3.93
	RSD (%)	6.33	7.56	3.66
	Accuracy (% nominal)	100	96	98
Sunitinib	Nominal (mg/L)	5.0	50	120
	Mean measured (mg/L)	5.1	52	127
	RSD (%)	8.35	5.16	3.52
	Accuracy (% nominal)	102	104	106
Sorafenib	Nominal (mg/L)	0.40	2.00	4.00
	Mean measured (mg/L)	0.46	1.97	4.15
	RSD (%)	6.39	5.55	3.68
	Accuracy (% nominal)	115	99	104

autosampler vials (Kinesis, St. Neots, UK). The vials were capped and transferred to a pre-cooled (10 °C) autosampler tray.

TurboFlow LC–MS/MS

An Aria Transcend TLX-II system (ThermoFisher Scientific, San Jose, USA) consisting of four Accela 600 high-pressure quaternary pumps (two loading pumps for the TurboFlow systems and two eluting pumps for the analytical column systems), valve interface module, and CTC PAL autosampler was used. TurboFlow and LC instrument control (including multiplexing) was performed using Aria OS (version 1.6.5, ThermoFisher Scientific). System eluents were (1) loading and eluting pumps A, 10 mmol/L ammonium acetate in deionised water/acetonitrile (99.5+5 *v*/*v*); (2) loading pump B, 0.1 % (*v*/*v*) formic acid in methanol; (3) eluting pump B, 10 mmol/L ammonium acetate in acetonitrile/deionised water (99+5 *v*/*v*); and (4) loading pump C, acetone/2-propanol/acetonitrile (1+2+2 *v*/*v*/*v*).

Sample supernatants (100 μL) were injected onto a Cyclone TurboFlow column (50×0.5 mm i.d.) under turbulent flow (100 % loading pump eluent A, 2.0 mL/min, 30 s). An on-line solvent mixing column (Agilent, CA) was used between the injection port and the TurboFlow column. Retained analytes were back-flushed from the TurboFlow column using elution solvent (100 % loading pump eluent B, 200 μL) stored in a holding loop and focussed through a T-piece onto a Hypersil GOLD C18 analytical column (3 μm aps, 50×2.1 mm i.d.; ThermoFisher Scientific, Runcorn, UK) column fitted with a 0.5-μm pre-column filter (Fisher Scientific, Loughborough, UK) and maintained at 40 °C (HotPocket, ThermoFisher Scientific). During gradient elution (Table 2; total flow rate, 1.0 mL/min) from the analytical column, the TurboFlow column was back-flushed with eluent C and the elution solvent loop re-filled with elution solvent. The whole system was then re-equilibrated prior to the next injection. The total analysis time was 7 min, including column re-equilibration. Eluent flow was diverted to waste for the first 2 min following each injection onto the TurboFlow columns, and MS/MS data were acquired for 2.5 min per analysis.

MS/MS (TSQ Vantage, ThermoFisher Scientific, Hemel Hempstead, UK) was carried out in positive ionisation mode using APCI (needle discharge current, 4 μA; temperatures: vaporiser, 420 °C; capillary, 275 °C; auxiliary, sheath, and sweep gas settings, 10, 40, and 0 arbitrary units, respectively). Collision pressure was 1.4 mTorr (0.19 Pa). Data were collected in high-resolution (0.40 *m/z* full width at half maximum peak height), selected reaction monitoring (SRM) mode, with two *m/z* transitions per analyte. MS instrument control and data acquisition were performed using Xcalibur (version 2.1.0, ThermoFisher Scientific). Analyte-specific MS parameters are detailed in Table 3. Post-analysis processing was carried out using LC Quan (version 2.6, ThermoFisher Scientific).

Assay calibration

Calibration standards (N=7) and 'matrix blanks' (analyte-free serum) were included at the beginning and end of each batch analysis, with all three IQCs included (1) after the first set of calibration standards and immediately before the last set, and (2) after every ten sample injections throughout the sequence [20, 21]. Patient samples were analysed in duplicate. Samples with analyte concentrations exceeding the calibration range were diluted as appropriate with analyte-free human serum and re-assayed. EQC samples were analysed with each sample batch. Assay acceptance criteria were (1) linear (R^2>0.98) calibration curves for each

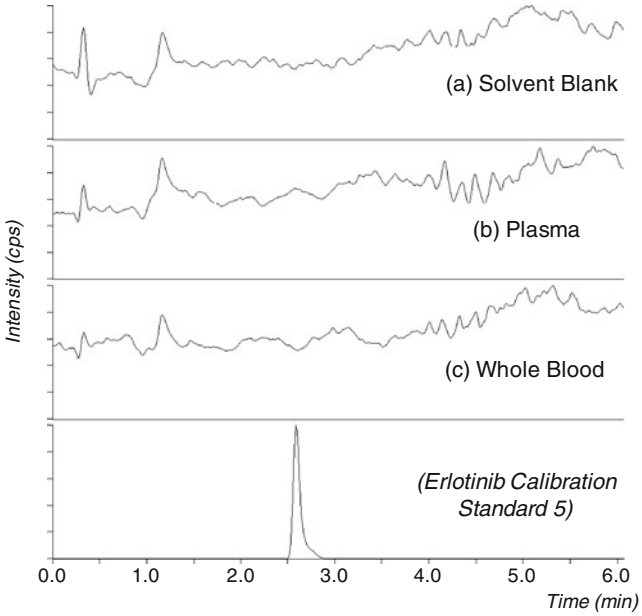

Fig. 3 Representative ion suppression chromatograms for erlotinib. Data were collected during post-column infusion of all analytes (erlotinib 25 mg/L, 5 μL/min). Extracted ion chromatograms (sum of two SRM transitions for erlotinib), showing **a** solvent blank, **b** prepared analyte-free serum, **c** prepared whole blood, and **d** erlotinib response from calibration standard 5 (see legend to Fig. 2 for concentration). Absolute intensity (in counts per second) rather than relative intensity (in percent) is shown on the *y*-axis

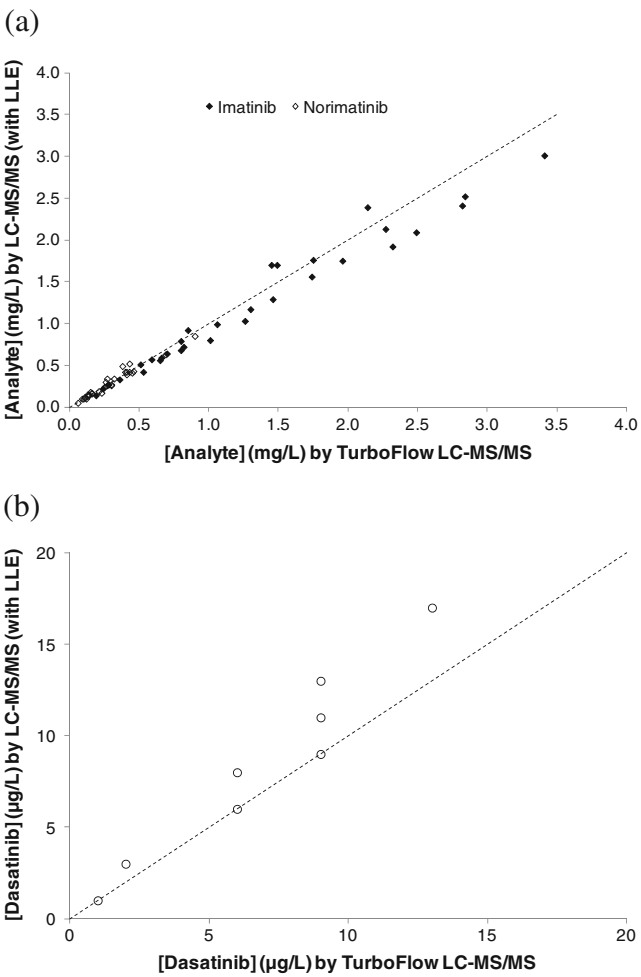

Fig. 4 Comparison of results obtained using TurboFlow LC–MS/MS versus LC–MS/MS with off-line manual liquid–liquid extraction (Birch et al., unpublished). **a** Imatinib, R^2=0.97 (y=0.89x+0.04); norimatinib, R^2=0.96 (y=0.95x+0.01), and **b** dasatinib, R^2=0.97 (y=1.45x−1.22). The *dashed lines* indicate the lines of identity

analyte, (2) IQC values within ±15 % nominal concentrations for all analytes, and (3) EQC values for imatinib, norimatinib, dasatinib, and nilotinib, all within the stated concentration ranges (Chromsystems).

Peak area ratios (analyte to IS, sum of two SRM peak areas for each analyte and IS with ion ratio confirmation) obtained on analysis of the calibration standards were plotted against analyte concentration to construct calibration graphs. Linear regression intercepts were not forced through zero, and line weighting was applied (1/concentration). IS assignment was as follows: imatinib-D_8, imatinib and norimatinib; gefitinib-D_8, gefitinib and erlotinib; sunitinib-D_{10}, dasatinib; and nilotinib-$^{13}C_2{}^{15}N_2$, nilotinib, lapatinib, and sorafenib. For sunitinib, *E*- and *Z*-isomers were separately quantified using the respective *E*- and *Z*-isomers of sunitinib-D_{10}, and the results reported as 'total sunitinib'.

Method validation

The method was validated using established guidance for bioanalytical method validation [20, 21]. Intra- and inter-assay precision (in percent RSD) and accuracy were measured by replicate analysis (N=5) of the IQC solutions on the same day and duplicate analyses (mean of duplicates) of these solutions on different days (N=5), respectively. In order to investigate recovery from the TurboFlow columns, portions (100 μL, N=6 on each TurboFlow column) of an aqueous solution (2.50 mg/L imatinib, norimatinib, nilotinib, gefitinib, erlotinib, sorafenib, lapatinib, and 0.50 mg/L dasatinib and sunitinib) were analysed (1) using the complete procedure and (2) with the TurboFlow systems bypassed (i.e. injection directly onto the analytical columns, assumed to represent 100 % recovery) and the mean peak areas compared. To assess recovery from plasma/serum, solutions containing all analytes (0.25 mg/L imatinib, norimatinib, nilotinib, gefitinib, erlotinib, sorafenib, lapatinib, and 0.05 mg/L dasatinib and sunitinib) were prepared in (1) analyte-free serum and (2) methanol. Portions of these solutions (each N=6) were individually prepared according to the protein precipitation method ("Sample preparation" section) and analysed using the TurboFlow procedure. Mean peak areas for each analyte from the methanolic and plasma solutions were then compared.

Table 5 Summary patient sample analysis data for gefitinib, nilotinib, and sorafenib

Analyte	Dose range (mg/day)	No. of samples (no. of patients)	Plasma [analyte] (mg/L)	
			Median	Range
Nilotinib	400–800	7 (6)	1.58	0.42–2.21
Gefitinib	125[a]–250	17 (6)	0.22	0.11–0.51
Sorafenib	400	6 (4)	2.96	0.91–5.66

[a]Six samples from two patients prescribed 250 mg on alternate days

To investigate ion suppression/enhancement, analyte-free human serum and whole blood (each from six separate sources) were analysed following addition of acetonitrile instead of IS solution. The detector response for each analyte transition was monitored whilst a methanolic solution containing all analytes (25 mg/L each imatinib, imatinib-D_8, norimatinib, nilotinib, nilotinib-$^{13}C_2{}^{15}N_2$, gefitinib, gefitinib-D_8, erlotinib, sorafenib, and lapatinib, 5 mg/L each dasatinib, sunitinib, and sunitinib-D_{10}) was infused (5 μL/min) by syringe post-column [22]. Analyte stability was evaluated by analysis of IQC samples (1) through three freeze–thaw cycles (N=3 each concentration), (2) before and after standing for 1 day at room temperature (N=3 each concentration), and (3) before and after standing for 1 week at 4 °C (N=3 each concentration).

Clinical samples

Samples previously analysed for imatinib and norimatinib (N=32) and dasatinib (N=9) by an in-house LC–MS/MS method, with off-line liquid–liquid extraction at pH 10.6 into butanol/butyl acetate (Birch et al., unpublished), were analysed by the TurboFlow LC–MS/MS method and the results compared. Plasma samples from patients undergoing therapy with other TKIs (nilotinib, gefitinib, sorafenib) were also analysed. For erlotinib, previously frozen paired plasma and whole blood samples (N=51) were analysed. Haemolysed whole blood was mixed thoroughly and assayed in the same way as plasma. Ethics: Guy's and St. Thomas' Ethics Committee Approval Number 10/H1109/47.

Results and discussion

Typical chromatograms are shown in Fig. 2. Calibration graphs were linear ($R^2 \geq 0.99$ for all analytes) over the calibration ranges. Intra- and inter-assay imprecision and accuracy data are summarised in Table 4. The limit of accurate measurement (signal at least five times the SD of the background noise) was less than 10 μg/L for imatinib, norimatinib, nilotinib, gefitinib, erlotinib, sorafenib, and lapatinib, and less than 1 μg/L for dasatinib and total sunitinib (50 μL samples). No significant matrix effects were observed, from either prepared plasma or whole blood samples (Fig. 3).

Mean (N=6) TurboFlow recoveries from aqueous solutions were 85–90 % for all analytes, except for gefitinib (61 %) and sorafenib (38 %). Mean recovery of all analytes from plasma versus methanolic solutions was 80–109 % with the TurboFlow columns used for validation except for lapatinib (mean 65 %). Although low for some analytes, the recovery was reproducible, as demonstrated by the precision and accuracy data (Table 4). The differences observed in peak area may be due to differences in solubility of the analytes between plasma and methanol when the test solutions were prepared, i.e. the presence of protein/lipid in the plasma promoted dissolution of the analytes as compared to methanol. Indeed, a solution of all analytes prepared in deionised water showed significantly lower peak areas for all analytes, especially for the relatively hydrophobic analytes lapatinib and sorafenib (data not shown).

Lapatinib IQC concentrations showed a mean decrease of 11 % across all nominal IQC concentrations after standing at room temperature and exposure to ambient light (24 h), and through three freeze–thaw cycles (thawing was carried out in ambient light). No significant differences were observed in calculated IQC concentrations over three freeze–thaw cycles and after standing at room temperature for 24 h for all other analytes. The difference in results after standing at 4 °C (in the dark) for 1 week was less than 15 % that of the nominal IQC values for all analytes. Prepared samples in the autosampler rack were stable for at least 12 h. These observations are in agreement with published data [11].

Table 6 Summary patient sample analysis data for erlotinib

Dose (mg/day)	No. of samples (no. of patients)	Plasma [erlotinib] (mg/L)		Whole blood [erlotinib] (mg/L)	
		Median	Range	Median	Range
100	24 (11)	1.22	0.14–2.04	1.15	0.16–2.20
150	23 (13)	1.27	0.53–4.35	1.21	0.56–3.25
Unknown	4 (4)	0.88	0.17–2.74	1.02	0.14–2.28

Table 7 Some pharmacologically active metabolites of TKIs

Parent drug (observed nominal $[M+H]^+$ *m/z*)	Active metabolite	Expected metabolite Q1 nominal *m/z* ($[M + H]^+$)	References
Dasatinib (488)	*N*-Desalkyldasatinib	444	[4]
	Dasatinib *N*-oxide	504	
	Dasatinib acid	502	
	Hydroxydasatinib[a]	504	
Gefitinib (447)	*O*-Desmethylgefitinib	433	[24]
Erlotinib (394)	*O*-Desmethylerlotinib	380	[4]
Lapatinib (581)	*O*-Debenzylapatinib	483	[25]
Sorafenib (465)	Sorafenib *N*-oxide	481	[4]
Sunitinib (399)	*N*-Desethylsunitinib	371	[4, 26]

[a]At least two hydroxylated metabolites have shown pharmacological activity

Method comparison and clinical samples

Reanalysis of the imatinib and dasatinib samples by the TurboFlow method showed good agreement with previously reported LC–MS/MS values (Fig. 4). Results from patients prescribed TKIs other than dasatinib/imatinib/nilotinib are summarised in Table 5. Samples from patients prescribed sunitinib and lapatinib were not available for this evaluation. Results from 51 paired plasma and whole blood samples from patients prescribed erlotinib are summarised in Table 6. There was a good correlation between plasma and whole blood erlotinib concentrations (R^2=0.93; y=0.78x+0.22). The plasma erlotinib concentrations were typically higher than whole blood concentrations [median (range) plasma/whole blood ratio, 1.02 (0.76–1.46)]. The median (range) haematocrit was 38.2 % (28.8–49.2 %) in these samples.

Although interference from metabolites (especially labile metabolites such as *N*-oxides—Table 7) and other compounds is possible, no additional peaks were observed in the chromatograms of the patient samples analysed in this work, and assay selectivity was ensured as far as possible by (1) TurboFlow retention/elution, (2) LC retention time, (3) MS/MS ionisation conditions, and (4) SRM data acquisition with ion ratio confirmation.

Practical considerations

Plasma protein precipitation followed by TurboFlow sample preparation together with chromatographic separation and MS/MS detection gives a selective, sensitive, and highly automated analytical system. Less sample and extract handling are required than if liquid–liquid or solid-phase extraction were to be employed, and the multiplexing capability of the Aria Transcend TLX-II system doubles assay throughput, thus significantly reducing costs. A major feature is that TurboFlow processing helps minimise matrix effects and hence helps maximise sensitivity/selectivity as compared to simply analysing plasma or protein-precipitated plasma directly. In addition, the method could be easily and quickly adapted to either incorporate active metabolites (Table 7) as reference materials become available in the future, or additional TKIs such as pazopanib (GlaxoSmithKline), vandetanib (AstraZeneca), afatinib (Boehringer Ingelheim), and axitinib and neratinib (both Pfizer).

Method validation, including duplicate analysis of all clinical samples, was carried out using just two TurboFlow columns (one for each system in multiplex mode), suggesting that TurboFlow column lifetime for this assay exceeds 500 injections. Though direct injection of serum/plasma is possible using TurboFlow technology [23], prior off-line protein precipitation serves to improve assay reliability and maximise TurboFlow column life (He and Kozak, unpublished data), especially for highly protein-bound analytes. Off-line protein precipitation also allows for the analysis of whole blood, useful for the investigation of plasma/whole blood distribution or in post-mortem work. Modern liquid-handling equipment allows protein precipitation to be largely automated using 96- or 384-well microplates.

Conclusions

A rapid, automated method has been developed and validated for the measurement of eight TKIs and one metabolite (norimatinib) in human plasma/serum, and can be used for haemolysed whole blood if necessary. Only 100 μL of sample is required for a duplicate analysis, and the method is suitable for the measurement of the analytes studied over the range of concentrations encountered in therapy. TDM of TKIs may in time become a valuable adjunct to the clinical use of these agents.

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