Journal of Chromatography B, 878 (2010) 1059–1068

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Simple and selective method for the determination of various tyrosine kinase inhibitors used in the clinical setting by liquid chromatography tandem mass spectrometry

R. Honeywell[a], K. Yarzadah[a], E. Giovannetti[a], N. Losekoot[a], E.F. Smit[b], M. Walraven[a], J.S.W. Lind[b], C. Tibaldi[c], H.M. Verheul[a], G.J. Peters[a,*]

[a] Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands
[b] Department of Pulmonary Diseases, VU University Medical Center, Amsterdam, The Netherlands
[c] Department of Medical Oncology, Livorno Civil Hospital, Livorno, Italy

ARTICLE INFO

Article history:
Received 5 July 2009
Accepted 8 March 2010
Available online 15 March 2010

Keywords:
Gefitinib
Erlotinib
Sunitinib
Sorafenib
LC–MS/MS
Analysis

ABSTRACT

A fast, sensitive, universal and accurate method for the determination of four different tyrosine kinase inhibitors from biological material was developed using LC–MS/MS techniques. Utilizing a simple protein precipitation with acetonitrile a 20 μl sample volume of biological matrixes can be extracted at 4 °C with minimal effort. After centrifugation the sample extract is introduced directly onto the LC–MS/MS system without further clean-up and assayed across a linear range of 1–4000 ng/ml. Chromatography was performed using a Dionex Ultimate 3000 with a Phenomenex prodigy ODS3 (2.0 mm × 100 mm, 3 μm) column and eluted at 200 μl/min with a tertiary mobile phase consisting of 20 mM ammonium acetate:acetonitrile:methanol (2.5:6.7:8.3%). Injection volume varied from 0.1 μl to 1 μl depending on the concentration of the drug observed. Samples were observed to be stable for a maximum of 48 h after extraction when kept at 4 °C. Detection was performed using a turbo-spray ionization source and mass spectrometric positive multi-reaction-monitoring-mode (+MRM) for Gefitinib (447.1 *m/z*; 127.9 *m/z*), Erlotinib (393.9 *m/z*; 278.2 *m/z*), Sunitinib (399.1 *m/z*; 283.1 *m/z*) and Sorafenib (465.0 *m/z*; 251.9 *m/z*) at an ion voltage of +3500 V. The accuracy, precision and limit-of-quantification (LOQ) from cell culture medium were as follows: Gefitinib: 100.2 ± 3.8%, 11.2 nM; Erlotinib: 101.6 ± 3.7%, 12.7 nM; Sunitinib: 100.8 ± 4.3%, 12.6 nM; Sorafenib: 93.9 ± 3.0%, 10.8 nM, respectively. This was reproducible for plasma, whole blood, and serum. The method was observed to be linear between the LOQ and 4000 ng/ml for each analyte. Effectiveness of the method is illustrated with the analysis of samples from a cellular accumulation investigation and from determination of steady state concentrations in clinically treated patients.

1. Introduction

Traditionally cancer has been treated with DNA targeted chemotherapy which can be very effective but for unpredictable side effects, in certain cases extreme toxicity, in a proportion of the general population. Research on the molecular biology of normal and cancerous cells has revealed several signaling pathways that are either unique or significantly different in cancerous cells. Focus on these differences has revealed some novel potential targets for targeted cancer therapy. Targeted chemotherapy is when an external molecule or a combination of molecules is used to interact preferentially with specific targets present in malignant cells. This type of therapy aims to decrease the risk of toxicity and have a more predictable effect. Hence the inclusion of these novel targeted therapies in current treatment modalities is a major challenge in oncological research [1–5].

A group of targets that has been identified as being significantly different between normal and cancerous cells are protein tyrosine kinases (PTKs). These proteins are involved in the maintenance of cellular homeostasis which can be unregulated or down regulated by a number of different oncogenes [1].

Using the ATP binding site as a molecular template, imatinib maleate was designed as a tyrosine kinase inhibitor and successfully introduced for the treatment of chronic myeloid leukemia (CML) and Gastro-Intestinal Stromal Tumors (GIST) [6–8]. A number of other compounds have been designed which also inhibit various tyrosine kinase receptors, and have been registered for treatment of a number of different diseases, such as gefitinib,

* Corresponding author at: Department of Medical Oncology, VU University Medical Center, PO Box 7057, 1007 MB Amsterdam, The Netherlands.
Tel.: +31 20 4442633; fax: +31 20 4443844.
E-mail address: gj.peters@vumc.nl (G.J. Peters).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.03.010

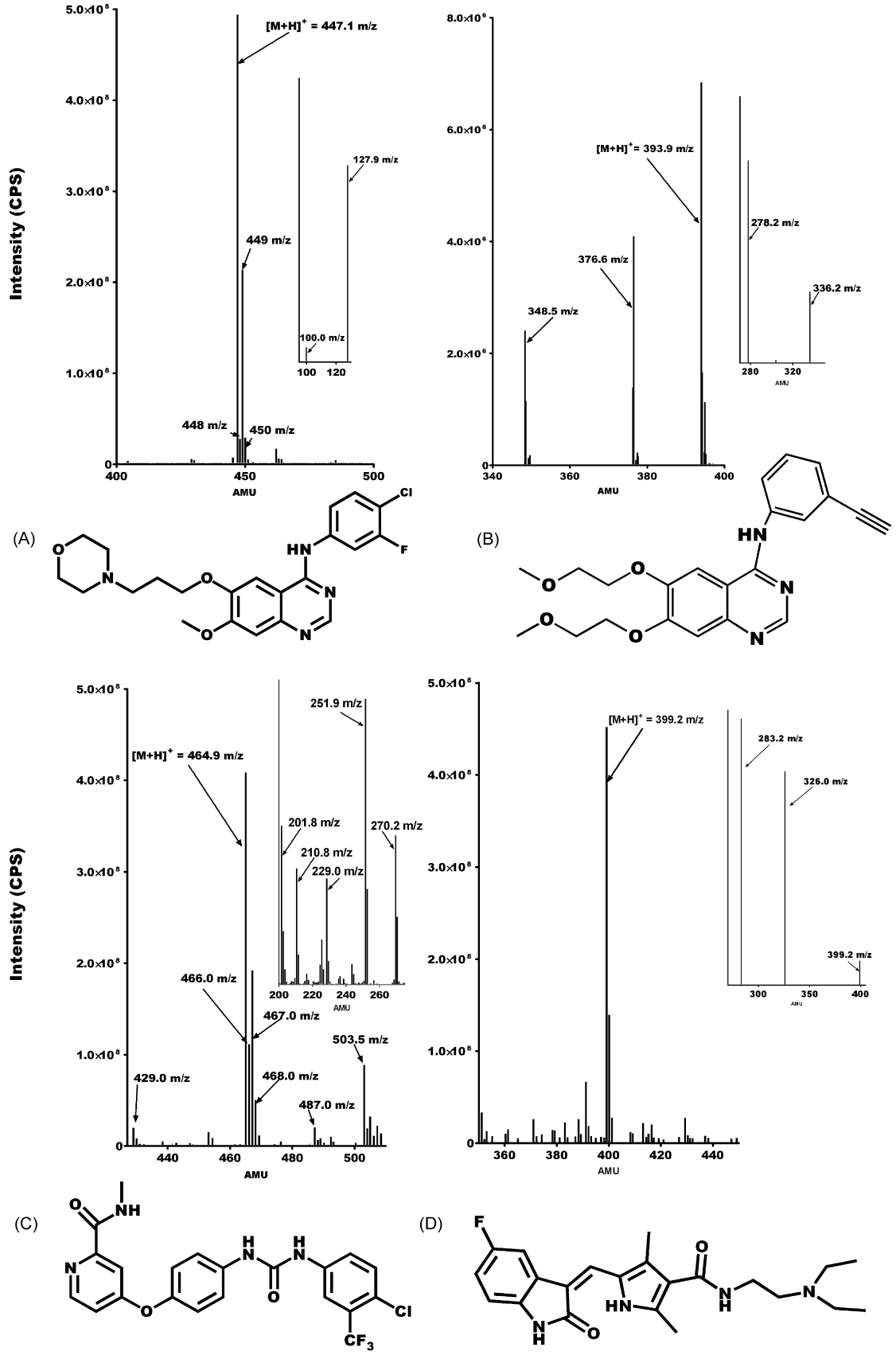

Fig. 1. Structural formula and spectra of Gefitinib (A), Erlotinib (B), Sorafenib (C) and Sunitinib (D) in 20 mm ammonium acetate:methanol:acetonitrile; inset is the product ion spectrum of each $[M+H]^+$ ion showing the characteristic fragmentation.

erlotinib, sorafenib and sunitinib (Fig. 1A–D) [9] illustrate this type of molecule and are all used in the clinical setting or at phase 2 or phase 3 trial status.

Gefitinib and erlotinib are small molecules that compete with ATP for binding sites on the intracellular tyrosine kinase EGFR domain. The autophosphorylation of EGFR is subsequently inhibited, which leads to the interference of downstream signaling. Both gefitinib and erlotinib have been indicated for the treatment of patients with locally advanced or metastatic NSCLC [10–13]; after failure of at least one prior chemotherapy regimen [14], while gefitinib is also registered for first line treatment of patients with an EGFR mutation.

Sunitinib and sorafenib are both small molecules that target multiple receptor tyrosine kinases, both on tumor cells and supporting endothelial cells. Sunitinib is a broad spectrum inhibitor of the VEGF-R (types 1, 2 and 3), PDGF-Rα, PDGF-Rβ, c-KIT and FLT3 [15], while sorafenib is a major inhibitor of C-Raf, B-Raf (mutant and wild type), Ras, MEK and ERK signaling pathways [16,17].

Analytical methodology for the determination of gefitinib, erlotinib, sorafenib and sunitinib is limited by the actual molecular structure of the individual compounds. The chromophore sites of resonance are limited in these structures, decreasing in the intensity of the energy difference between two molecular orbitals ranging from gefitinib, erlotinib, sorafenib to sunitinib. The end result is that sorafenib and sunitinib do not demonstrate significant absorption in the UV region of the spectrum; one of the main techniques behind HPLC detection of molecules. Also, the lack of sensitivity for the detection of gefitinib and erlotinib is such that typical HPLC-UV is not a technique that can be used for low level pharmacokinetic or bio-molecular investigations, although methods have been published for erlotinib [18], sorafenib [19] and sunitinib [20]. In response to this lack of sensitivity the more advanced technique of LCMSMS has been used to detect gefitinib [21–24], erlotinib [25–28], sorafenib [29–32] and sunitinib [33,34], but these methods are all independent and demonstrate little commonality.

From the perspective of a research laboratory, having methodology that is common between differing compounds represents a cost saving in both materials and time. A methodology that is fast, sensitive, selective and reliable also provides the environment for accurate and reproducible research enabling the investigation of PK-PD parameters of the small molecules as single agents and in combination. Therefore, the objective of the present study was to develop one analytical method that could determine concentrations of each of the compounds in a variety of different matrixes. This method was validated in accordance with both European [35] and US federal regulations [36], and it was subsequently shown to be effective in both a clinical and preclinical research setting.

2. Materials and methods

2.1. Materials

Analytical grade solvents such as acetonitrile, formic acid and methanol were supplied by Biosolve BV, The Netherlands. HPLC grade water was supplied via a MilliQ water purification system (Millipore, The Netherlands). Cell culture media RPMI-1640 and DMEM, foetal bovine serum, (FBS), penicillin (50 IU/ml) and streptomycin (50 μg/ml) were from Gibco (Gaithersburg, MD). The reference standard for gefitinib was a donation from Astra Zeneca, (London, UK), erlotinib and its primary metabolite OSI-420 were donations from Roche (Basel, Switzerland), sunitinib and sorafenib were supplied by JS Research Chemicals Trading, (Wedel, Germany).

Table 1
Compound specific operational parameters.

Compound	Q1 mass	Q3 mass	DP	FP	EP	CE	CXP
Gefitinib	447.0	128.0	56	280	10	35	16
Erlotinib	394.0	278.8	66	330	10	45	18
Sorafenib	465.0	251.9	76	370	10	47	16
Sunitinib	399.1	283.0	41	210	10	41	18

Q1 = first quadruple mass, Q3 = second quadruple mass, DP = declustering potential, FP = focusing potential, EP = entrance potential, CE = collision energy, CXP = cell exit potential.

2.2. Equipment

Chromatography was conducted using a Dionex Ultimate 3000 system coupled with an Applied Biosciences SCIEX API 3000 mass spectrometer for detection. The interface between the HPLC and detection systems was a Turbo Spray ionization (TSI) source. The injection system of the Dionex Ultimate 3000 HPLC was fitted with a 1 μl PEEK sample loop and 0.05 mm PEEK transfer tubing. All volumetric transfers were performed with calibrated pipettes into 1.5 ml polypropylene screw cap tubes. Sample analysis was performed using a polypropylene skirted 96-well plate and a resealable sample cover (Thermo Fisher Scientific; NUNC brand; Denmark). Software used for data acquisition and integration was Analyst version 1.42 from Applied Biosciences, in combination with Dionex Chromeleon LC modules; version 6.8 controlled by Dionex Mass link (DMS) version 2.0 software.

2.3. Analytical procedure

2.3.1. Mass spectrometry optimization of compound specific parameters

The optimized TSI conditions were as follows: nebulizing gas flow 11 l/min, curtain gas flow 9 l/min, collision activated dissociation gas flow (CAD) 4 l/min, nebulizer current 3.0 kV, probe temperature 425 °C. Compound specific parameters such as declustering potential (DP), focusing potential (FP), entrance potential (EP), collision cell entrance potential (CE), and collision cell exit potential (CXP) were optimized from a 1 μg/ml stock solution of each individual component (Table 1).

The mass spectrometry conditions of each compound were determined by an infusion at 0.5 ml/h into the TSI source at room temperature using a variety of different solvent conditions (Table 2). A period of 5 min was allowed for equilibration before spectra were collected over the positive and negative Q1 range of 50–2000 *m*/*z* for 3 s. Molecular ions were determined for fragmentation over a positive Q3 range of 50–300 *m*/*z* for 3 s. The solvent with the best response relative to 50% 10 mM formic acid:50% acetonitrile, across all four compounds was selected for initial optimization of the chromatography. Quantification was developed in positive multi reaction monitoring (MRM) mode by the monitoring of the determined transition pairs using the optimized compound specific parameters DP, FP, CE and CXP.

2.3.2. Chromatographic conditions

Chromatographic separation was performed on a Prodigy ODS-3 (3 μm; 100 mm × 2.0 mm) distributed by Phenomenex, The Netherlands. The column was maintained at 35 °C in a fan assisted Advance Professional HPLC Column Oven (Separations; The Netherlands) and isocratically eluted with a mobile phase consisting of 66.6% acetonitrile:25% 20 mM ammonium acetate (pH 7.8):8.3% methanol (v/v). Mobile phase was filtered through a 0.2 μm pore nylon membrane filter and degassed by ultrasonication at 4 °C. Flow rate was set at 200 μl/min and the peaks of interest eluted within 5 min after an injection of 1 μl.

Table 2
Relative detector sensitivity of gefitinib, erlotinib, sunitinib and sorafenib under differing chromatographic solvent conditions.

	Gefitinib		Erlotinib		Sorafenib		Sunitinib	
	p	*n*	*p*	*n*	*p*	*n*	*p*	*n*
Molecular weight	447	445	394	392	465	463	399	397
AmmAc pH 3 + MeOH	0.50	2.57	0.52	3.97	0.84	0.13	0.64	1.11
AmmAc pH 5.5 + MeOH	0.62	2.29	0.50	2.94	0.57	0.10	0.36	1.72
AmmAc pH 8 + MeOH	0.56	2.35	0.12	1.17	0.48	0.15	0.40	1.59
AmmF pH 3 + MeOH	0.70	0.30	0.21	0.79	**0.94**	0.11	0.47	0.35
AmmF pH 5.5 + MeOH	**1.07**	0.32	0.02	0.23	0.49	0.35	0.39	0.00
AmmF pH 8 + MeOH	0.85	0.60	0.00	0.21	0.61	0.15	0.40	0.00
FA + MeOH	0.91	0.70	0.33	0.31	0.43	0.06	0.72	0.23
HAC + MeOH	0.32	0.26	0.13	0.20	0.39	0.07	0.04	0.09
PA + MeOH	0.00	0.20	0.57	0.48	0.21	0.04	0.10	0.04
AmmAc pH3 + ACN	0.68	2.87	0.65	**11.44**	0.49	1.74	0.42	0.97
AmmAc pH 5.5 + ACN	0.70	**4.17**	0.02	0.26	0.50	2.02	0.64	**1.40**
AmmAc pH 8 + ACN	0.60	3.12	0.03	0.16	0.45	2.01	0.52	0.00
AmmF pH 3 + ACN	0.92	1.47	0.26	0.47	0.79	2.52	0.52	0.70
AmmF pH 5.5 + ACN	0.52	0.57	0.10	0.15	0.10	1.01	0.57	0.00
AmmF pH 8 + ACN	0.63	1.03	0.37	1.37	0.66	**2.56**	**0.81**	0.00
FA + ACN	*1.00*	*1.00*	*1.00*	*1.00*	*1.00*	*1.00*	*1.00*	*1.00*
AA + ACN	0.54	0.66	**0.83**	1.15	0.28	0.33	0.31	1.91
PA + ACN	0.42	0.51	0.64	1.05	0.59	0.23	0.46	0.00

p = Positive detector mode, n = negative detection mode; AmmAc = ammonium acetate. AmmF = ammonium formate, FA = formic acid, HAC = acetic acid; PA = propionic acid; MeOH = methanol; ACN = acetonitrile; sensitivity ratio is calculated relative to 10 mM formic acid:acetonitrile infusion (ideal conditions are indicated in bold type for each compound).

2.3.3. Sample collection

All standard/sample collection and preparations were performed on ice. Whole blood samples were taken from volunteers into either heparin or EDTA tubes. Plasma and cell culture medium samples were prepared by centrifugation at $1650 \times g/4\,^{\circ}C$ and stored at $-20\,^{\circ}C$ until required. Cell culture pellets were washed twice with phosphate buffer prior to being snap frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ until analysis. Serum was prepared from whole blood samples taken from volunteers into serum separator tubes and allowed to stand at room temperature for 30–60 min. After centrifugation the serum was stored at $-80\,^{\circ}C$ until required for analysis.

2.3.4. Preparation of standards

Stock solutions of gefitinib, erlotinib, sunitinib and sorafenib were accurately prepared in DMSO at a concentration of approximately 10 mg/ml. DMSO was used since the relative solubility of these compounds is very low in either water or alcohols. Purity and weight variations were adjusted by diluting in ethanol approximately 1:10 to give an accurate stock solution of 1 mg/ml. Subsequent dilutions of gefitinib, erlotinib, sunitinib and sorafenib stock solutions were prepared to give standard combined calibration solutions of 10, 5, 4, 2, 1, 0.5, 0.2, 0.1, 0.05, 0.01 and 0.001 ng/μl. 10 μl of each dilution was added to 100 μl of either control plasma, serum, whole blood or cell culture medium (RPMI or DMEM) for standard preparation.

2.3.5. Preparation of cell culture pellets

Each pellet was thawed by addition of 160 μl of cold phosphate buffer and homogenized by pipette aspiration. 10–20 μl (depending on the pellet size) of the cell suspension was aliquoted for protein quantification and 100 μl aliquoted for gefitinib, erlotinib, sunitinib and sorafenib determination.

2.3.6. Sample and standard extraction

Plasma, serum, whole blood and cell culture medium samples/standard preparations were vortexed briefly prior to 20 μl being aliquoted into a clean labeled tube. To each aliquot 200 μl of acetonitrile was added followed by further vortex mixing. To the cell culture pellet suspension (100 μl) 400 μl of acetonitrile was added followed by further vortex mixing. Samples were allowed to stand on ice for 20 min before vortexing again and subsequent centrifugation at $21{,}000 \times g/4\,^{\circ}C$ for 10 min; 100 μl was transferred to a 96-well plate for LC injection.

2.3.7. Gefitinib, erlotinib, sunitinib and sorafenib cellular accumulation

Approximately 0.2×10^5 cells/cm^2 of a colon adenocarcinoma cell line (WiDr) was prepared in a 6-well plate with RPMI (containing HEPES and 10% fetal bovine serum) as the medium. Cells were in culture with gefitinib (300 nM; 8 μM); erlotinib (300 nM; 8 μM); sunitinib (2 μM) and sorafenib (2 μM) for 2 h at 37 °C/5% CO_2. Cells were harvested by trypsination, washed in phosphate buffer, centrifuged and the pellet snap frozen in liquid nitrogen. Cells pellets were extracted as detailed above and concentration of the respective compounds were subsequently determined.

2.3.8. Analysis of plasma, serum or whole blood from cancer patients

The assay was also applied to plasma and whole blood samples from cancer patients treated with either gefitinib, erlotinib, sunitinib, sorafenib or a combination of these compounds. Plasma was prepared by collecting whole blood in heparinised tubes followed by centrifugation at $1600 \times g/4\,^{\circ}C$. After collection all samples were stored at either $-20\,^{\circ}C$ or $-80\,^{\circ}C$ until analysis. Gefitinib and erlotinib were determined in whole blood samples taken from subjects with non-small-cell lung cancer (NSCLC) during standard maintenance therapy (250 mg/day and 150 mg/day respectively). Erlotinib (150 mg/day) was also determined in combination with sorafenib (2×400 mg/day) in plasmas from NSCLC patients enrolled in an ongoing phase II study. Sunitinib (50 mg/day) and sorafenib (2×400 mg/day) were measured in plasma from renal cancer cell patients receiving the compounds as single agents.

2.3.9. Protein determination

The 10–20 μl aliquot of cell suspension was diluted to 50 μl used for protein measurement with a standardized kit from Bio-Rad. Briefly the Bio-Rad Protein Assay is based on the method of Bradford [37] and is a simple yet accurate method for determin-

ing concentration of solubilized protein. It involves the addition of an acidic dye to the protein solution, and the subsequent measurement of the complex at 595 nm by spectrophotometry. Comparison to a standard curve provides a relative measurement of protein concentration.

2.3.10. Data handling and calculations

A calibration curve for each analyte was prepared using linear least square analysis with $1/x$, $1/x^2$ and without weighting as well as quadratic regression. Parameters used to determine the validity of the linearity were the deviation of the slope, the accuracy of the fit of the line and residuals. Recovery was determined as a percentage of the theoretical concentration in comparison to the calculated concentration from the line of best fit. Dilution conversion factors were used to present the final concentration in terms of μM for plasma, whole blood and cell culture medium, while cell culture pellet concentrations were given in pmol per μg protein.

2.3.11. Validation procedure

Selectivity was determined as the ability of the analytical method to differentiate and quantify an analyte in the presence of other related components and from individual components of the sample matrix. The precision (defined as the reproducibility of measurement of the same sample), accuracy (defined as the variation between theoretical and practical measurements) and robustness (quality of being able to withstand minor stresses, pressures, or changes in procedure or environment) were determined from the variation of duplicate control linearities analyzed on five different days. The recovery of the extraction procedure was calculated by comparing the peak areas of each standard concentration against equivalent absolute standard dilutions. Limit of detection was determined by successive standard dilutions and calculating the signal to noise ratio based on signal standard deviation, the limit set for detection was a signal/noise ratio of >3. The LOQ is the limit at which the difference between two different values can be determined, in practice the limit of quantitation was defined simply as 5 times the LOD.

3. Results

3.1. Mass spectra

Test solvents were chosen based on the typical chromatography used for liquid chromatography tandem mass spectrometry. These consisted of 10 mM aqueous ammonium acetate, ammonium formate, formic acid, acetic acid and propionic acid combined in a ratio of 50:50 with either methanol or acetonitrile. From the collected mass spectra the intensity of the $[M+H]^+$ and the $[M-H]^-$ were normalized against the intensity observed for 10 mM formic acid:acetonitrile (Table 2). Positive mode was generally more stable than negative mode across the four components, although for some individual components negative mode can give an increase in intensity of 2–10 fold compared to positive mode for the same solvent. However, there was no one solvent suitable for all compounds in the negative mode. Therefore, positive mode was selected as the prime detection mode.

In terms of the detection sensitivity, different pH conditions for the various buffers had differing effects on each of the compounds. From the resulting data the best solvent for over all sensitivity was considered to be ammonium formate (pH 5.5) with methanol; with the next options being ammonium acetate (pH 5.5)/acetonitrile or formic acid/acetonitrile. Test chromatography indicated that a mobile phase of lower than pH 4 gave very little retention (less than 1 min for elution). Alternatively aqueous ammonium acetate gave better retention characteristics compared to ammonium formate, which was not really surprising since the buffer range for ammonium formate is from 2.8 to 4.8 (pK_a 3.8) whereas ammonium acetate has a range of 3.8–5.8 (pK_a 4.8), the pK_a for gefitinib and erlotinib are about 5.4 ± 0.1, while that of sunitinib is 8.95 but no value could be found for sorafenib in literature [38]. With reference to these considerations the mass spectrometer sensitivity was optimized using 20 mM ammonium acetate:acetonitrile (50:50; pH 5.5) as the infusion solvent. The optimized parameters for the DP and FP were then used to establish the fragmentation profile for each compound from the $[M+H]^+$ ion (Fig. 1). Further optimization (collision energy and cell exit potential) was performed on the most intense observed fragment, resulting in an optimized MRM transition (Table 1).

Gefitinib demonstrated a clear response at 447.1 m/z which corresponds to the $[M+H]^+$ ion, the isotopic profile of this ion confirmed the presence of a single chloride ion, this corresponded with the predicted profile for gefitinib. No solvent or compound related adducts were observed at higher m/z and in the 50–400 m/z region of the spectra only minor solvent interference was observed. Fragmentation of the 447 m/z produced two main peaks at 127.9 and 100.0 m/z (Fig. 1A), of which the 127.9 m/z gave the better optimized response. On the basis of this the resulting MRM transition was optimized to be 447.1/127.9 (Table 1).

Erlotinib shows three distinct responses at 393.9, 376.6 and 348.5 m/z in the initial spectrum. The 393.9 m/z corresponds to the $[M+H]^+$ ion, while the ions 376.6 m/z and the 348.5 m/z can be related to the loss of an hydroxyl group (−17) or a methoxymethane group (−45) from the molecule. The spectrum was clean of ionization adducts and solvent interference was considered to be minor. Fragmentation of the 393.9 response produced two main peaks at 335.8 and 278.2 m/z (Fig. 1B) with 278.2 m/z being the more intense signal. On the basis of this the resulting MRM transition was optimized to be 393.9/278.2 (Table 1).

The spectrum for Sorafenib had four distinct responses at 503.5, 464.9, 429.0 and 319.1 m/z. The 464.9 m/z corresponds to the $[M+H]^+$ ion, closer examination of the ion cluster observed at 465–469 m/z demonstrated the classical isotopic profile of a compound containing a single chloride ion. However, the 429.0 m/z ion cluster did not show a chloride profile indicating the in-source ionization loss of a chloride ion (−35.5), while the 503.5 ion (which did have a chloride profile) can be related to a stable adduct with aqueous potassium. No solvent interference was observed in the area of interest. The remaining ion is related to an ion observed in the blank solvent but of a higher intensity, and it is known to be linked to $[\text{dioctyl phthalate}+H]^+$ which is a common plasticizer found in PVC. Fragmentation of the 464.9 m/z response produced five fragment peaks at 270.2, 251.9, 229.0, 210.8 and 201.8 m/z (Fig. 1C). The fragmentation to 251.9 was determined to be robust and the most intense, hence, on this basis the resulting MRM transition was optimized to be 465.0/251.9 (Table 1).

Sunitinib demonstrated a $[M+H]^+$ ion at 399.2 m/z with no interference observed for the solvent or any compound related ionization adducts. Fragmentation of the 399.2 response produced two fragment peaks at 326.0 and 283.2 m/z (Fig. 1D), the major being 283.2 m/z. On the basis of this the resulting MRM transition was optimized to be 399.1/283.2 (Table 1).

3.1.1. Specificity

Specificity of each compound was determined in the presence of several common co-commitment medications. No evidence of a response could be observed for any compound following these infusions, indicating a high degree of specificity.

3.1.2. Chromatography

Chromatography was initiated using ammonium acetate 20 mM:acetonitrile (pH 5.5) as determined in the MS sensitivity testing. Under these conditions all compound co-eluted rapidly

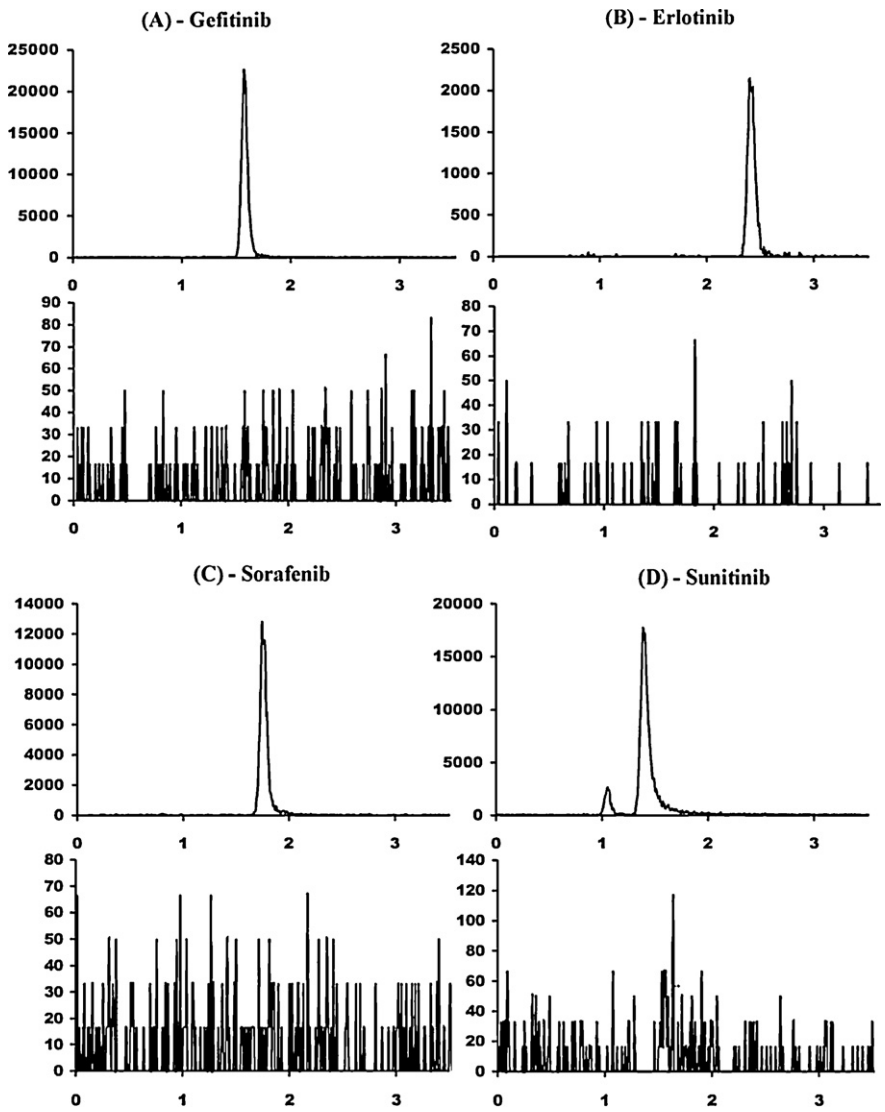

Fig. 2. Representative chromatograms of a MLOQ standard and a blank extracted sample using the optimised MS/MS conditions and developed chromatography.

at 1.1 min post-injection. Buffer adjustments achieved resolved chromatography in under 4 min using 20 mM ammonium acetate (25%):acetonitrile (66.7%):methanol (8.3%) with the pH set to 7.8 with ammonium hydroxide. Parameters for mass spectrometry were re-optimized for this buffer. Examples of a blank and a standard chromatogram are shown in Fig. 2.

3.2. Extraction method

The described extraction method used a simple protein precipitation/dilution technique that did not require further sample clean-up and provided adequate sensitivity for the required purpose. To expand the dynamic range higher it was determined that 1:10, 1:20 or even 1:100 sample dilutions could be used with no loss of resolution, linearity or specificity providing the acetonitrile:water ratio was maintained at 5:1.

3.3. Limit of detection

The detection limit was defined as the lowest concentration that had a signal to noise ratio of 3 or greater. Signal to noise ratio (S/N) was calculated as being the peak intensity divided by the average of the noise preceding the peak elution under optimized chromatographic conditions. The limit of quantification (LOQ) was set at 5 ng/ml when extracted from plasma for gefitinib, erlotinib, sorafenib and sunitinib. The values are well below the detection limits reported for HPLC/UV assays and similar to single drug LCMSMS assay reported in literature.

A parameter that is seldom reported in terms of method validation is the higher limit of quantization (HLOQ). For this series of compounds the HLOQ was determined to be 4000 ng/ml for all compounds when extracted from 10 μl of matrix (dilution factor 1:10). Above 6000 ng/ml the sensitivity of the LCMSMS technique is compromised and peak areas obtained are lower than would be theoretically expected. However, problems were observed if the ratio of acetonitrile:water exceeded 10, because samples were found to evaporated too quickly, even at 5 °C and in a sealed well plate.

3.4. Linearity, accuracy, precision and recovery

For gefitinib, erlotinib, sunitinib and sorafenib the linear regression data indicated a co-efficient of >0.99 between the linear range of 1–4000 ng/ml, $1/x^2$ weighting was proved to be the line of best fit for all components. Linearity was preserved for $n = 7$ analysis performed over a 5 day period by two different operators for plasma (r^2: 0.999 ± 0.005), whole blood (r^2: 0.999 ± 0.008), serum

Table 3
Accuracy, precision and recovery parameters calculated for LLOQ (5 ng/ml); MLOQ (200 ng/ml) and HLOQ (400 ng/ml).

	Accuracy ± precision (%)		Recovery (%)	
	Medium	Blood	Medium	Blood
Gefitinib				
LLOQ	111.2 ± 6.6	112.6 ± 12.0	86.2 ± 5.0	101.2 ± 2.7
MLOQ	100.2 ± 3.8	104.6 ± 4.6	101.0 ± 0.8	103.7 ± 3.5
HLOQ	100.5 ± 5.6	99.3 ± 3.1	107.0 ± 0.5	102.9 ± 1.3
Erlotinib				
LLOQ	113.5 ± 8.1	103.7 ± 5.1	105.3 ± 5.3	96.0 ± 3.0
MLOQ	101.6 ± 3.7	105.9 ± 3.6	107.6 ± 0.0	107.5 ± 0.8
HLOQ	100.5 ± 5.7	98.6 ± 3.0	109.3 ± 0.9	104.0 ± 0.8
Sunitinib				
LLOQ	111.3 ± 6.0	106.7 ± 7.2	78.7 ± 2.5	81.7 ± 3.0
MLOQ	100.8 ± 4.3	109.2 ± 1.9	74.3 ± 2.9	83.6 ± 2.5
HLOQ	100.2 ± 5.8	97.5 ± 3.7	78.0 ± 1.6	81.0 ± 1.9
Sorafenib				
LLOQ	108.7 ± 6.7	104.8 ± 11.6	105.5 ± 3.8	90.6 ± 3.2
MLOQ	93.9 ± 3.0	107.3 ± 2.9	104.1 ± 3.0	94.1 ± 1.5
HLOQ	101.9 ± 6.6	99.9 ± 2.8	103.7 ± 1.0	92.0 ± 1.48

Average medium values are reported as a combined result for RPMI and DMEM since results were almost identical. Plasma, serum and whole blood were also combined since the results observed did not significantly differ from each other. All values quoted are calculated from $n = 7$ standard points determined over 5 separate days.

(r^2: 0.999 ± 0.006) and RPMI/DMEM medium (r^2: 0.998 ± 0.005). Validation was subsequently conducted in cell culture medium (DMEM) and in plasma. Table 3 illustrates the accuracy (%) of the LLOQ, MLOQ and HLOQ for each compound. For all standards tested the accuracy was observed to be within 100 ± 15%, conforming to all regulatory specifications.

Precision was calculated as the coefficient of variation of the mean, all values were within the 15% specification set for biological analysis by the regulatory authorities (Table 3). Recovery for gefitinib, erlotinib and sunitinib was calculated by direct comparison of neat diluted stock standard solution and matrix extracted standard preparations and was close to 100 ± 10% for almost all concentration levels for all compounds. However, sunitinib demonstrated a tendency to return a lower recovery at the LLOQ in blood (Table 3) which was associated with auto-sampler carryover and column memory effects. This problem was resolved by the careful manipulation of needle height and puncture depth as well as using a washing solvent of 100% acetonitrile in the auto-sampler. Recovery for sorafenib was calculated using RPMI extracted standard dilutions since it was determined that sorafenib was unstable in aqueous dilutions (not shown).

3.5. Stability

The stability of each compound was determined for stored samples (−20 °C and −80 °C) and for extracted samples (4 °C) while awaiting injection. Fig. 3 shows graphically that up to 72 h after extraction, the samples remain stable at 4 °C when derived from medium; for blood derived samples it can be observed that all compounds are stable until 48 h and demonstrate a non significant (<10%) decrease in concentration after 72 h. However this decrease is consistent with all standards and samples and does not affect the final result. Sample analysis is under 5 min for each injection, therefore, 100 samples can analyzed in under 10 h, hence a minor degradation in 72 h does not present a problem in terms of assay robustness.

A series of standard preparations in plasma, serum and whole blood were stored at −20 °C and −80 °C and analyzed at one week, one month and three months. The linear regression observed was within specifications of r^2: 0.99 for individual analysis and for the combined responses for all extractions ($n = 3$).

As has already been indicated sorafenib demonstrated a significant instability when prepared in aqueous solutions of greater than 50% water. Within 2 h of preparation such standard solutions were observed to have degraded by 90% (not shown). However, when sorafenib dilutions were prepared in ethanol and assay standard preparations were made in a matrix containing 2–10% protein the resulting standards proved to be far more stable giving regression parameters within specification (not shown). This was reflected in the accuracy of the concentration of plasma and serum samples determined at different times and at different dilutions.

3.6. Clinical and research application

Applications of the developed assay were tested under a variety of conditions, i.e. tumor cells exposed to differing doses of individual drugs, and plasma, serum or whole blood from subjects undergoing chemotherapeutic treatment with the compounds in question, individually or in combination.

3.6.1. Gefitinib, erlotinib, sunitinib and sorafenib cell accumulation

The intracellular concentrations of the individual drugs after a timed exposure (2 h) at concentrations shown earlier to give a 50% growth inhibition [39] were determined from WiDr colon cancer cell pellets stored at −80 °C. Gefitinib was clearly visible and quantifiable after exposure to both 300 nM and 8 μM (0.20 and 5.21 nmol per 10^6 cells). Erlotinib demonstrated a significantly lower concentration than the other three compounds, lower than the limit of quantification for this assay, after exposure to 300 nM and 8 μM (0.006 and 0.023 nmol per 10^6 cells). However, the levels observed were greater than the limit of detection. The low levels were believed to be a function of the biological system and specific uptake and efflux properties of the compound rather than the extractability or sensitivity of the assay. Sorafenib and sunitinib were also clearly visible in WiDr cells after exposure to 2 μM (21.3 and 7.9 nmol per 10^6 cells).

3.6.2. Analysis of gefitinib and erlotinib in whole blood, serum or plasma from cancer patients

The results obtained from patients treated individually with gefitinib or erlotinib demonstrate some unforeseen complications. The plasma concentrations were in the low nM range for both gefitinib and erlotinib (0.004 ± 0.003 nM and 0.008 ± 0 nM respectively), literature suggests that these are lower than expected results. While the plasma samples needed a 1:5 dilution in order to

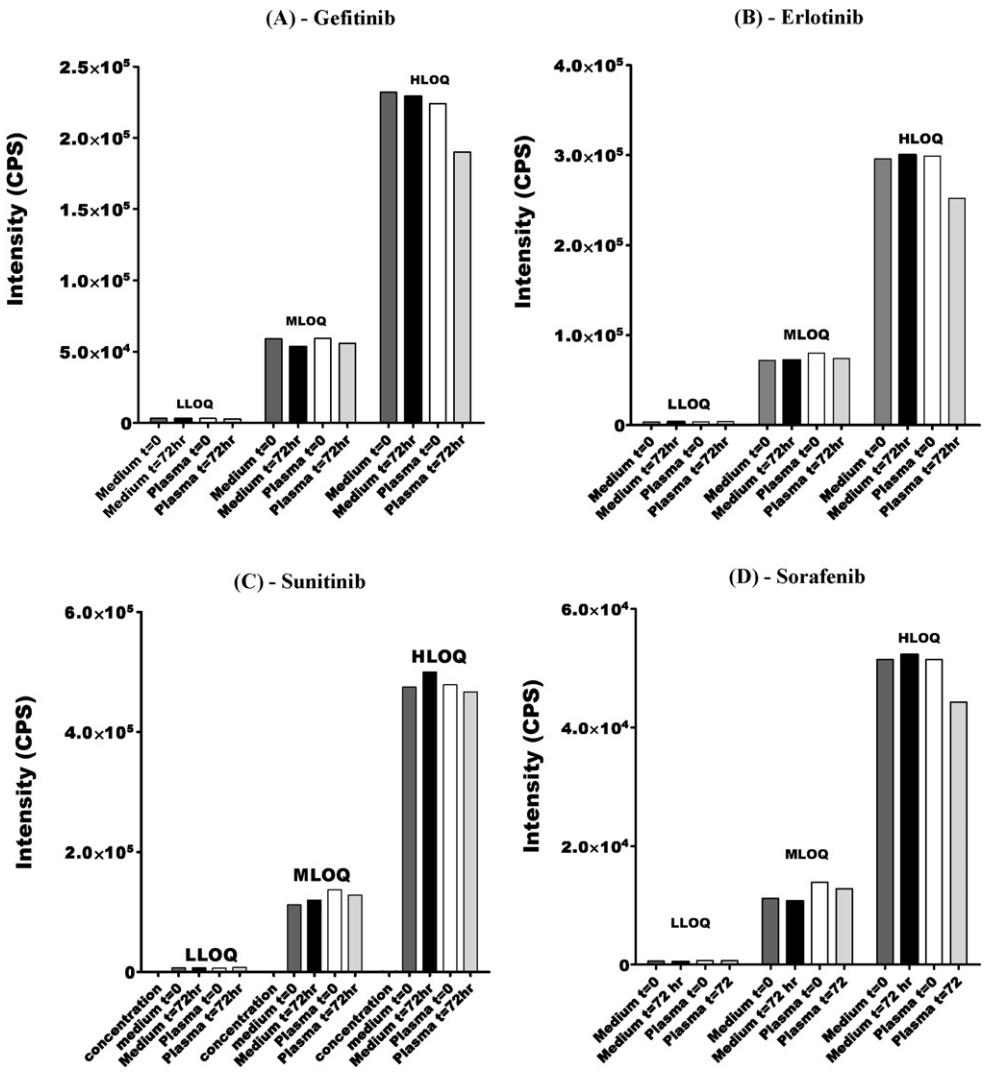

Fig. 3. Stability of each compound when extracted from plasma or cell culture medium.

have them fall within the linear range, the whole blood of the same samples required a 1:100 dilution for concentrations to fall into the linear range of the assay. Whole blood concentrations were in the medium to high μM range for both gefitinib and erlotinib (5.8 ± 3.0 and 58.7 ± 43.4 μM respectively). This difference between plasma and whole blood was thought to be due to differences in blood compartmentalization; this difference is currently being investigated further.

3.6.3. Erlotinib and sorafenib from steady state plasma

In 5 subjects measurable quantities of both erlotinib and sorafenib in the week one and week three samples were clearly detectable within the linear range of the assay after a 1:10 dilution was performed. The pre-dose sample shows no evidence of either erlotinib or sorafenib while after one week plasma concentration of erlotinib was 0.3 ± 0.2 μM (median 0.2 μM). After three weeks the overall average of erlotinib had decreased to 0.2 ± 0.2 μM (median 0.2 μM), statistically not significant ($p = 0.1515$). In contrast, sorafenib was present in 6 fold higher concentrations with a week one average of 1.8 ± 0.9 μM (median 1.7 μM) which is consistent with the 5 fold higher daily dosing level for sorafenib compared to erlotinib. Following 3 weeks of dosing sorafenib concentrations increased to 2.1 ± 1.0 μM but was not statistically significant ($p = 0.1872$) compared to 1 week of dosing. Serum samples from the same time points as the plasma analyzed above had approximately 6 fold higher concentration for both sorafenib and erlotinib. This is consistent with the previous findings that whole blood concentration is significantly higher than plasma concentrations.

3.6.4. Sunitinib from steady state plasma

In 5 subjects a measurable quantity of sunitinib in samples taken at 24 h and three weeks after start of continuous therapy were clearly detectable, as well as within the linear range of the assay after a 1:10 dilution was performed. The pre-dose sample showed no evidence of sunitinib while the samples at six weeks (after two weeks wash out) showed levels at the LLOQ for this assay. After 24 h plasma concentrations of 45.7 ± 19.8 nM were observed, following approximately 3 weeks of dosing the plasma concentration had risen to 128.5 ± 69.2 nM; this did not represent a statistically significant difference ($p = 0.0845$) but patient numbers were very low ($n = 5$). In direct contrast to gefitinib, erlotinib and sorafenib plasma and serum samples for the same time points demonstrated concentrations in the same range.

3.7. Sample dilution effects

Above 4000 ng/ml integrated areas were observed to decrease with increasing concentration. For example, concentrations of

Table 4
A summary of current published methodologies for the determination of gefitinib, erlotinib, sorafenib and sunitinib individually by LC–MS/MS or by HPLC-UV techniques.

Reference	Year	Drug	Sensitivity (minimum–maximum) (ng/ml)	Detection	Column	Mobile phase	Isocratic/gradient
[21]	2004	Gefitinib	0.5–1000	+LCMS/MS MRM 447.2–127.8	Phenomenex Synergi 4 μm Max C12 75 mm × 2.0 mm	Acetonitrile–1% formic acid [30:70, v/v]	Isocratic
[22]	2002	Gefitinib	1.5–400	+LCMS/MS MRM 447.2–128	Inertsil ODS3 150 mm × 4.6 mm	Acetonitrile–Ammonium acetate (1%, w/v) [80:20, v/v]	Isocratic
[24]	2005	Gefitinib	1–1000	+LCMS/MS MRM 447.1–128	Waters X-Terra MS 50 mm × 2.1 mm	Acetonitrile–0.1% formic acid [70:30, v/v]	Isocratic
[a]	2009	Gefitinib Erlotinib	10–5000	UV λ 348 nm λ 348 nm	Phenomenex Luna 5 μm C18 150 mm × 4.6 mm	Acetonitrile–ammonium acetate (20 mM) [45:55, v/v initial condition]	Gradient
[25]	2003	Erlotinib	100–4500	UV λ 348 nm	Waters Nova-Pak 4 μm C18 150 mm × 3.9 mm	Acetonitrile:water (pH 2.0 with TFA)	Isocratic
[18]	2005	Erlotinib	12.5–4000	UV λ 345 nm	Waters Symmetry 5 μm C18 150 mm × 4.6 mm	Acetonitrile:0.05 M potassium phosphate buffer (pH 4.8 with 0.2% TEA) [42:58, v/v]	Isocratic
[26]	2007	Erlotinib	10–4000	+LCMS/MS MRM 394.1–278	Phenomenex Luna 5 μm C18 150 mm × 4.6 mm	Acetonitrile–ammonium acetate (5 mM) [45:55, v/v]	Isocratic
[28]	2003	Erlotinib	10–10,000	+LCMS/MS MRM 394–278	Waters X-Terra MS 50 mm × 2.1 mm	Acetonitrile–0.1% formic acid [70:30, v/v]	Isocratic
[34]	2008	Sunitinib	0.2–500	+LCMS/MS MRM 399–283.0	Waters X-Terra MS 3.5 μm ODS 50 mm × 2.1 mm	Acetonitrile–0.1% formic acid [70:30, v/v]	Isocratic
[33]	2004	Sunitinib	10.5–10,500	+LCMS/MS MRM 399–326.0	Waters Symmetry shield 3.5 μm C8 50 mm × 2.1 mm	Acetonitrile–15 mM ammonium formate (pH 3.25) [26:74, v/v]	Isocratic
[19]	2009	Sorafenib	0.5–20	UV λ 255 nm	Ultrasphere 5 μm ODS	Acetonitrile–ammonium acetate (20 mM) [45:55, v/v]	Gradient
[29]	2004	Sorafenib	80–2000	UV λ 254 nm	Waters Symmetry μm C18 150 mm × 4.6 mm	100% acetonitrile B–0.2% acetic acid	Gradient
[32]	2009	Sorafenib	10–5000	+LCMS/MS MRM 465.1–252.0	Polaris 3 3 μm C18-A 50 mm × 2 mm	Acetonitrile–0.1% formic acid	Gradient
[31]	2007	Sorafenib	7.3–7260	+LCMS/MS MRM 465.1–252.0	Waters X-Terra MS 3.5 μm ODS 50 mm × 2.1 mm	Acetonitrile:10 mM ammonium acetate (pH 3.5) [65:35, v/v]	Isocratic
[30]	2008	Sorafenib	5–2000	+LCMS/MS MRM 464.9–252.0	Waters Symmetry shield μm C8 50 mm × 2.1 mm	Acetonitrile–0.1% formic acid [65:35, v/v]	Isocratic

[a] Unpublished method under validation by the authors.

6000 ng/ml or more gave a calculated concentration that fell within the standard curve range of 0–4000 ng/ml. This represented a significant chance of error in the determination of serum, plasma and medium concentrations. Chromatography and peak shape analysis did not give any indication of the observed error. Hence the risk remained that sample concentrations could be under estimated by a factor of 1000 or more. It was observed that a dilution of such high concentration samples (1:10 or 1:100) gave a calculated concentration higher than the undiluted sample. It was by this means only that real sample concentrations were established, it was accepted that for these compounds that two analyses should always be performed with two different dilutions, thereby, giving a means of determining any "over concentration" errors that might occur. In this situation more typical HPLC-UV analysis can offer an advantage over LC–MSMS methodology, since sensitivity is not an issue at these concentrations.

4. Discussion

Targeted therapies are increasingly given to cancer patients not only individually or in combinations with cytotoxic drugs but also in combination with each other. In order to analyze interactions of these drugs or elucidate relations with clinical parameters (e.g. clinical outcome and toxicity) sensitive, selective and accurate assays are required. Preferably an assay should be able to determine in a single analysis all the drug concentrations required from a single sample. The present paper describes a single straight forward assay validated for four targeted compounds currently registered for the treatment of various tumors.

The assay was validated for a number of different matrixes; quantification was all within specifications dictated by the guidelines of agencies such as the Federal Drug Agency (FDA) of the USA or the European medicine safety committee (EMEA). Atypically, next to plasma and cell pellets matrixes, serum, whole blood and blood cells were also investigated; with all matrixes similar results were obtained underlying the universal application of the developed assay.

The assay compared favorably with several LCMSMS assays developed for each of the individual drugs (Table 4), and is much more sensitive then all referenced HPLC-UV methodology. However, a straightforward HPLC-UV assay does still provide an essential technique for the monitoring of patients using high dose chemotherapy since these concentrations are well within the μM range (data not shown).

Although the assay allowed reliable measurement of all the specified drugs in a variety of matrixes, one major complication was observed because of the saturation of the system. LCMSMS technique relies on the successful counting of ions on the detec-

tion plate after filtering of individual ions generated in the source via the quadrupole system. Several factors can detrimentally affect the accuracy of this count. The first is that the number of ions being counted has a finite limit, exceeding this limit means that a proportion of the ions arriving at the detection source are not seen at all. The result is an under estimation of the total and is not reflected in any of the chromatographic representations used in quantitative analysis. A second consideration is that when excessive ions are generated in the source there is a physical limit to the number successfully being able to pass through the orifice to the quadrupole's. Such "crowded" conditions also promote aggregation of ions into larger masses and the fragmentation of ions in source prior to the first quadrupole. All these factors should be considered when determining the dynamic range of any developed methodology [40].

The results have clearly shown that the basic principle behind the extraction (protein precipitation with acetonitrile) is valid for a wide range of dilution factors (1:5–1:500). These dilutions increase the effective dynamic linear range from 1 to 4000 ng/ml to 1–50 μg/ml. The extraction method also lends itself favorably to sample concentration techniques whereby the solvent is evaporated to dryness and reconstituted in a smaller volume, increasing the sample concentration in the injection solvent. Hence, the lower limit of detection and quantification could be extended to pmol or fmol levels relatively easily. This was not investigated for this validation since this degree of sensitivity was not required for the clinical and preclinical samples tested.

The present method allowed the measurement of significant drug accumulation into tumor cells, enabling a better understanding whether sufficient drug would be present intra-cellularly in order to adequately inhibit the tyrosine kinases. Initial results in 50 patients also indicates that the steady state plasma concentrations of erlotinib seem to decrease in a proportion of the population over the course of treatment when in combination with sorafenib, whereas, this does not seem to be the case for sorafenib in the same population [41]. These data are in agreement with results from a previous phase I study of gefitinib–sorafenib combination [42]. In contrast, preliminary investigation into drug interactions in cell culture has not shown any significant differences in the erlotinib and sorafenib combination compared to individual drug administration [43]. The present assay allows a more detailed analysis of such cellular and tumoral accumulation.

5. Conclusions

The assay as developed conforms to all standards set for a validated method in USA and Europe. It has been shown to be a fast, cost effective and robust method for the analysis of clinical samples in a variety of matrixes. There exists the potential for this assay to be extended to cover other tyrosine kinase inhibitors of similar structure and size such as imatinib, lapatinib, vandetanib, vatalanib and canertinib, among a growing list of many structurally related compounds with increasing clinical application.

References

[1] C. Montagut, J. Settleman, Cancer Lett. 283 (2009) 125.
[2] F. Kyle, J. Spicer, Cancer Imaging 8 (2008) 199.
[3] W.J. Chng, L.G. Lau, N. Yusof, B.M. Mow, Cancer Control 12 (2005) 91.
[4] R. Longo, F. Torino, G. Gasparini, Curr. Pharm. Des. 13 (2007) 497.
[5] J. Baselga, Science 312 (2006) 1175.
[6] F. Roszkiewicz, R. Garidi, I. Vaida, B. Royer, A. Parcelier, J.P. Marolleau, G. Damaj, Pharmacology 84 (2009) 38.
[7] M. Tolomeo, F. Dieli, N. Gebbia, D. Simoni, Anticancer Agents Med. Chem. (Oct) (2009) 853.
[8] F. Duffaud, C.A. Le, Target Oncol. 4 (2009) 45.
[9] D. Pytel, T. Sliwinski, T. Poplawski, D. Ferriola, I. Majsterek, Anticancer Agents Med. Chem. 9 (2009) 66.
[10] T.E. Stinchcombe, M.A. Socinski, Proc. Am. Thorac. Soc. 6 (2009) 233.
[11] E.S. Kim, V. Hirsh, T. Mok, M.A. Socinski, R. Gervais, Y.L. Wu, L.Y. Li, C.L. Watkins, M.V. Sellers, E.S. Lowe, Y. Sun, M.L. Liao, K. Osterlind, M. Reck, A.A. Armour, F.A. Shepherd, S.M. Lippman, J.Y. Douillard, Lancet 372 (2008) 1809.
[12] F.A. Shepherd, P.J. Rodrigues, T. Ciuleanu, E.H. Tan, V. Hirsh, S. Thongprasert, D. Campos, S. Maoleekoonpiroj, M. Smylie, R. Martins, K.M. van, M. Dediu, B. Findlay, D. Tu, D. Johnston, A. Bezjak, G. Clark, P. Santabarbara, L. Seymour, N. Engl. J. Med. 353 (2005) 123.
[13] Z.T. Zhou, X.H. Xu, Q. Wei, M.Q. Lu, J. Wang, C.H. Wen, Cancer Chemother. Pharmacol. 64 (2009) 1123.
[14] G. Giaccone, Clin. Cancer Res. 10 (2004) 4233s.
[15] D.B. Mendel, A.D. Laird, X. Xin, S.G. Louie, J.G. Christensen, G. Li, R.E. Schreck, T.J. Abrams, T.J. Ngai, L.B. Lee, L.J. Murray, J. Carver, E. Chan, K.G. Moss, J.O. Haznedar, J. Sukbuntherng, R.A. Blake, L. Sun, C. Tang, T. Miller, S. Shirazian, G. McMahon, J.M. Cherrington, Clin. Cancer Res. 9 (2003) 327.
[16] X. Tong, L. Yang, J.C. Lang, M. Zborowski, J.J. Chalmers, Cytometry B Clin. Cytom. 72 (2007) 310.
[17] D. Strumberg, Drugs Today (Barc.) 41 (2005) 773.
[18] W. Zhang, L.L. Siu, M.J. Moore, E.X. Chen, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 814 (2005) 143.
[19] B. Blanchet, B. Billemont, J. Cramard, A.S. Benichou, S. Chhun, L. Harcouet, S. Ropert, A. Dauphin, F. Goldwasser, M. Tod, J. Pharm. Biomed. Anal. 49 (2009) 1109.
[20] B. Blanchet, C. Saboureau, A.S. Benichou, B. Billemont, F. Taieb, S. Ropert, A. Dauphin, F. Goldwasser, M. Tod, Clin. Chim. Acta 404 (2009) 134.
[21] F. Bai, L.C. Iacono, B. Johnston, C.F. Steward, J. Liquid Chromatogr. Relat. Technol. 27 (2004) 2743.
[22] H.K. Jones, L.E. Stafford, H.C. Swaisland, R. Payne, J. Pharm. Biomed. Anal. 29 (2002) 221.
[23] G. Guetens, H. Prenen, G. de Boeck, W. van Dongen, E. Esmans, F. Lemiere, A.T. van Oosterom, P. Schoffski, E.A. de Bruijn, J. Chromatogr. A 1082 (2005) 2.
[24] M. Zhao, C. Hartke, A. Jimeno, J. Li, P. He, Y. Zabelina, M. Hidalgo, S.D. Baker, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 819 (2005) 73.
[25] E.R. Lepper, S.M. Swain, A.R. Tan, W.D. Figg, A. Sparreboom, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 796 (2003) 181.
[26] A.R. Masters, C.J. Sweeney, D.R. Jones, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 848 (2007) 379.
[27] L. Signor, E. Varesio, R.F. Staack, V. Starke, W.F. Richter, G. Hopfgartner, J. Mass Spectrom. 42 (2007) 900.
[28] M. Zhao, P. He, M.A. Rudek, M. Hidalgo, S.D. Baker, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 793 (2003) 413.
[29] S. Afify, U.R. Rapp, P. Hogger, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 809 (2004) 99.
[30] L. Jain, E.R. Gardner, J. Venitz, W. Dahut, W.D. Figg, J. Pharm. Biomed. Anal. 46 (2008) 362.
[31] M. Zhao, M.A. Rudek, P. He, F.T. Hafner, M. Radtke, J.J. Wright, B.D. Smith, W.A. Messersmith, M. Hidalgo, S.D. Baker, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 846 (2007) 1.
[32] R.W. Sparidans, M.L. Vlaming, J.S. Lagas, A.H. Schinkel, J.H. Schellens, J.H. Beijnen, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 877 (2009) 269.
[33] S. Baratte, S. Sarati, E. Frigerio, C.A. James, C. Ye, Q. Zhang, J. Chromatogr. A 1024 (2004) 87.
[34] P. Minkin, M. Zhao, Z. Chen, J. Ouwerkerk, H. Gelderblom, S.D. Baker, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 874 (2008) 84.
[35] Y. Vander Heyden, A. Nijhuis, J. Smeyers-Verbeke, B.G.M. Vandeginste, D.L. Massart, J. Pharm. Biomed. Anal. 24 (2001) 723.
[36] Food and Drug Administration of the USA, in: Centre for Drug Evaluation and Research (CDER) (Ed.), U.S. Department of Health and Human Sciences, 2001.
[37] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[38] P. Ballard, R.H. Bradbury, C.S. Harris, L.F. Hennequin, M. Hickinson, J.G. Kettle, J. Kendrew, T. Klinowska, D.J. Ogilvie, S.E. Pearson, E.J. Williams, I. Wilson, Bioorg. Med. Chem. Lett. 16 (2006) 4908.
[39] C. Lemos, I. Kathmann, E. Giovannetti, C. Calhau, G. Jansen, G.J. Peters, Br. J. Cancer 100 (2009) 1120.
[40] R.L. Foltz, R.W. Edom, J. Mass Spectrom. Soc. Jpn. 46 (1998) 235.
[41] J.G. Kuhn, M. Gilbert, P. Wen, T. Oughesy, J. Cooper, V. Puduvalli, L. DeAngelis, L. Lieberman, K. Lambon, M. Prados, J. Clin. Oncol. 27 (2009) 2500.
[42] A.A. Adjei, J. Thorac. Oncol. 3 (2008) S160–S163.
[43] E. Giovannetti, R. Danesi, E.F. Smit, R.J. Honeywell, N. Losekoot, K. Yarzadah, R. Ruijtenbeek, H.M. Verheul, G.J. Peters, Proc. AACR 50 (2009) 423 (#1760).