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Development of a high-performance liquid chromatographic–mass spectrometric method for the determination of cellular levels of the tyrosine kinase inhibitors lapatinib and dasatinib

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ABSTRACT

A highly sensitive and selective liquid chromatography tandem mass spectrometry (LC–MS/MS) method has been developed to quantify cellular levels of the tyrosine kinase inhibitors (TKIs) dasatinib (Sprycel™) and lapatinib (Tykerb™, Tyverb™). Cellular samples were extracted with a *tert*-butyl methyl ether:acetonitrile (3:1, v/v):1 M ammonium formate pH 3.5 (8:1, v/v) mixture. Separation was achieved on a Hyperclone BDS C18 (150 mm × 2.0 mm 3 μm) column with isocratic elution using a mobile phase of acetonitirile–10 mM ammonium formate, pH 4 (54:46, v/v), at a flow rate of 0.2 mL/min. The TKIs were quantified using a triple quadrupole mass spectrometer which was operated in multi-reaction-monitoring mode employing positive electrospray ionisation. The limit of detection and limit of quantification for lapatinib was determined to be 15 and 31 pg on column, respectively. The limit of detection and quantification for dasatinib was 3 and 15 pg on column, respectively. The method allowed for sensitive and accurate determination of cellular levels of dasatinib and lapatinib. In addition, we examined the potential for this method to be utilised to quantitate other TKIs, using gefitinib, erlotinib, imatinib and sorafenib as examples. In principle, these agents were also quantifiable by this method, however, no drug specific validation studies were undertaken with these TKIs. The data indicates that in the cancer cell-line model, DLKP, significantly more lapatinib accumulates in cells in comparison to dasatinib. Additionally, over-expression of the membrane protein drug transporter, P-glycoprotein (P-gp) a common cancer drug resistance mechanism, greatly reduces the cellular accumulation of dasatinib but not of lapatinib.

1. Introduction

The conventional chemotherapy drugs, used in the treatment of many forms of advanced cancer, attack proliferating healthy and cancerous cells leading to potentially serious side effects including myelosuppression, hair loss and gastrointestinal problems. The emerging goal of cancer pharmacology research is the development and optimal application of targeted therapies. Targeted therapies include drugs which are specifically active in cancer cells thereby giving increased activity while having reduced toxicity and side effects.

In many cases, the abnormal proliferation characteristics of cancer are driven by growth factor receptor-mediated signalling. Receptor tyrosine kinases play a critical role in relaying these proliferation, differentiation and/or anti-apoptotic signals which are generated by growth factor receptor activation [1]. The characterisation of the role of these enzymes has led to the development of pharmaceutical inhibitors of kinase functionality and such inhibitors are now emerging with some success from oncology clinical trials into mainstream cancer treatment [2,3].

Tyrosine kinase inhibitors (TKIs) are a class of small molecule drugs that block the intracellular signals which drive proliferation in many malignant cells by specifically inhibiting the kinase function of individual intracellular pathways involved in receptor-mediated growth signalling [4].

Dasatinib (BMS-354825, Sprycel™) is a thiazole-based ATP-competitive, dual Src/Abl kinase inhibitor[5], approved for the treatment of imatinib resistant and imatinib-intolerant patients across all phases of chronic myelogenous leukemia (CML) [6].

Lapatinib (GW572016, Tykerb™), a 4-anilinoquinazoline compound, is a small molecule dual inhibitor of the tyrosine kinase functionality of the epidermal growth factor receptor (EGFR or ErbB1) and HER-2/*neu* (erbB2) receptors of the epidermal growth

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Table 1
Comparison of analytical methods for lapatinib or dasatinib in biological matrices.

Analytes determined	Biological matrix	Sample clean-up	Analytical method	Run time	Sensitivity (ng/mL)	% recovery	Ref.
Lapatinib	Human plasma	Turbulent flow on-line extraction	Isocratic elution, LC–MS/MS	<0.4 min	–	–	[9]
Lapatinib	Human plasma	On-line extraction	LC–MS/MS	3 min	LOQ: 15 ng/mL	75	[10]
Imatinib, dasatinib, nilotinib	Human plasma	Protein precipitation	Gradient elution, LC–MS/MS	20 min	LOQ: 78.1 ng/mL LOQ: 62.5 ng/mL LOQ: 61.5 ng/mL	95–114	[14]
Dasatinib, imatinib, nilotinib	Mouse plasma	Protein precipitation	Hydrophilic interaction LC–MS/MS	–	ng/mL range – data not shown	–	[15]
Imatinib, nilotinib, dasatinib, sunitinib, sorafenib, lapatinib	Human plasma	Protein precipitation	Step-wise gradient elution, LC–MS/MS	20 min	LOQ: 1 ng/mL LOQ: 1 ng/mL LOQ: 1 ng/mL LOQ: 1 ng/mL LOQ: 10 ng/mL LOQ: 5 ng/mL	93.3	[16]

factor (EGF) family [7] approved for use in breast cancer treatment [8].

As with most approaches used to quantify pharmaceuticals, analytical methods for measuring tyrosine kinase inhibitor levels have focussed on each agent in isolation and generally have been applied to pharmaceutical analysis or plasma determination of the agent.

To date, there are two reported methods for the LC–MS analysis of lapatinib [9,10] and a small number of analytical methods have been reported for dasatinib based on HPTLC or HPLC [11] or radioactive labelling methods [11,12] and a LC–MS method for plasma determination of this agent [13–16]. A summary of the published methods for the analysis of lapatinib or dasatinib in biological matrix has been compared in Table 1.

We report the first validated method that allows for the sensitive determination of cellular levels of either TKI agent. This method may also potentially have broader applicability for the sensitive determination of several other TKI agents.

Resistance to the therapeutic actions of cancer drugs is a significant clinical challenge [17]. In particular, the over-expression of cellular drug efflux pumps, such as P-glycoprotein, P-gp, in tumour cells is thought to play a significant role in the resistance phenotype [18]. Research now indicates that many TKIs may interact with drug efflux pumps and, in some cases, these pumps may play a role in resistance to these newer anti-cancer agents [19]. Polli et al. have demonstrated that lapatinib interacts with P-gp [20] and previous work by our group and others indicates that lapatinib inhibits P-gp action [21]. It has been unclear if P-gp over-expression could alter the cellular accumulation of pharmacologically relevant concentration of lapatinib. Hiwase et al. have also demonstrated that a P-gp inhibitor can increase the accumulation of dasatinib in a resistant leukemic cancer cell model using radiolabelled drug [12].

In this study we sought to utilise the specificity and sensitivity of LC–MS/MS coupled with liquid–liquid extraction (LLE) to provide a rapid, routine, non-radioactive assay to examine the cellular pharmacokinetics of TKIs. Using lapatinib and dasatinib as examples, we examined the impact of a well known drug transporter resistance mechanism, P-gp, on the cancer cell accumulation of these agents.

Table 2
The optimal fragmentor voltages and collision energy settings for the determination of the TKI drugs employed in this study.

Name	Precursor ion	Optimum fragmentor voltage	Product ion	Optimum collision energy
Lapatinib	581	200	365[a]	40
Dasatinib	488	120	401[a]	50
			231	30
Gefitinib	447.2	160	128[a]	30
Erlotinib	393.9	160	278[a]	35
			336	25
Imatinib	494.2	120	394[a]	25
			217	25
			99	30
Sorafenib	465	160	252[a]	35
			326	20

[a] Quantifier ion.

2. Experimental

2.1. Chemicals and solvents

Lapatinib ditosylate, dasatinib, gefitinib, erlotinib HCl, imatinib mesylate and sorafenib tosylate were purchased from Sequoia Chemicals, Pangbourne, UK.

Water and acetonitrile (ACN), MS grade, formic acid ammonium salt (ammonium formate), formic acid, *tert*-butyl methyl ether (*t*BME), dichloromethane and ethyl acetate were purchased from Sigma–Aldrich, Dublin.

2.2. Standards and solutions

Primary stock solutions of lapatinib and dasatinib were prepared at 75 μg/mL in ACN. The other TKIs imatinib, gefitinib, erlotinib and sorafenib were prepared as 50 μg/mL stock solutions in ACN. Working stock solutions were prepared fresh daily to a concentration of 10 μg/mL in acetonitrile. The internal standard (IS) for dasatinib analysis was 500 ng/mL lapatinib while the internal standard for lapatinib was 500 ng/mL dasatinib.

2.3. Instrumentation

The chromatographic separation employed an Agilent (Ireland) 1200 Rapid Resolution LC system consisting of a degasser, binary pump, a thermostated column compartment and autosampler. Mass spectrometric detection was performed with an Agilent 6410 triple quadrupole system in multi-reaction-monitoring (MRM)

mode interfaced with an electrospray ionisation source in positive mode.

All cell culture work was carried out in a Holten Maxi-safe laminar flow air cabinet. A Labinco (The Netherlands) vortex, Stuart Scientific (UK) blood tube mixer and Thermo (Ireland) centrifuge were used during sample pre-treatment. A Genevac EZ-2 (Ipswich, UK) was used to evaporate solvent from extracted samples.

2.4. Chromatographic and mass spectrometric conditions

Chromatographic separation was achieved using a Hyperclone BDS C18 column (150 mm × 2.0 mm i.d., 3 μm) with a SecurityGuard C18 guard column (4 mm × 3.0 mm i.d.) both from Phenomenex, UK. A mixture of acetonitrile–10 mM ammonium formate pH 4 (54:46, v/v) was used as mobile phase, at a flow rate of 0.2 mL/min. The column temperature was maintained at 20 °C and the temperature of the autosampler was maintained 4 °C. The complete chromatographic run time of each sample was 10 min, which separated dasatinib and lapatinib from each other with retention times 2.3 and 5.1 min, respectively. Peaks were quantified using Agilent Masshunter Software.

The mass spectrometer was operated using an ESI source in the positive ion detection mode. The ionisation temperature was 350 °C, gas flow rate was 11 L/min and nebulizer pressure was 345 kPa. Nitrogen was used as the ionisation source gas and ultrapure nitrogen as the collision cell gas.

Analysis was performed in MRM mode with the following transitions: m/z 581 → m/z 365 for lapatinib, and m/z 488 → m/z (231 and 401) for dasatinib where 401 m/z was the quantifier ion, with a dwell time of 200 ms. Additional studies were also undertaken for other TKIs and these transitions and their optimal detector settings are listed in Table 2. The quantifier ions are indicated in the table though all transitions were monitored with the qualifier ions adding specificity.

Quantification was based on the integrated peak area as determined by the Masshunter quantification analysis software which quantitates the peak areas of the MRM transitions of each analyte.

2.5. Liquid–liquid extraction (LLE) procedure

To the complete cell pellet (approx 50 μL), 100 μL of internal standard was added to the extraction tube, along with 200 μL of 1 M ammonium formate pH 3.5 buffer and 1.6 mL of extraction solvent *t*BME/ACN (3:1, v/v). The extraction tubes were vortexed and mixed on a blood tube mixer for 15 min. The samples were centrifuged at 3200 × *g* for 5 min. The organic layer was removed with a glass pasteur pipette and 1.1 mL of solvent was transferred to conical bottomed glass LC autosampler vials (Chromacol). The vials were evaporated to dryness using a Genevac EZ-2 (Ipswich, UK) evaporator at ambient temperature, without light. The samples were reconstituted in 40 μL of acetonitrile with 20 μL injected automatically by the autosampler.

2.6. Optimisation of sample pre-treatment

To determine the optimum system for liquid–liquid extraction three commonly used immiscible solvents were tested: ethyl acetate; dichloromethane and *tert*-butyl methyl ether. Results are outline in Table 3. Also, the optimum pH of the extraction buffer was investigated.

Samples were extracted as outlined in Section 2.5; however 50 μL of cell suspension in water and 100 μL of analyte was added to the extraction tubes. For optimisation of the solvent system, identical samples were extracted and the solvent system was varied.

For optimisation of the extraction buffer pH, identical extractions were carried out using *t*BME/ACN (3:1, v/v) as the solvent system and varying the pH of 1 M ammonium formate extraction buffer.

2.7. Lapatinib and dasatinib samples

To 50 μL of cell suspension 100 μL of internal standard and 100 μL of analyte varying in concentration from 1 to 2000 ng/mL was added to an extraction tube, yielding an in-tube mass of 50 ng of internal standard and 0.1–200 ng of analyte. These were extracted according to the method outlined in Section 2.5.

To determine the limits of detection and limits of quantification the concentration range was extended to a lower analyte concentration of 0.01 ng/mL, which gives an in-tube mass of 1 pg. This extended the assay range below the LOD and LOQ to accurately confirm the LOD and LOQ.

2.8. Other TKI samples

A mixture of 5 μg/mL of gefitinib, sorafenib, erlotinib, imatinib, dasatinib and lapatinib was prepared from primary stock solutions. From these stocks of each drug 500, 50 and 5 ng/mL solutions were prepared in acetonitrile. 200 μL of the drug mixture was placed in an extraction tube giving 100, 10 and 1ng of each drug, respectively. To this tube, 50 μL of cell suspension, 200 μL of extraction buffer and 1.6 mL of *t*BME:ACN (3:1, v/v) extraction solvent was added. The mixture was extracted in the same manner as the cell samples (outlined in Section 2.5).

The LC–MS method was altered to include the MRM transitions for all the molecules present and the run time was extended to 15 min, though the retention time for the latest eluting molecule, sorafenib, was 8.6 min.

2.9. Cell samples

DLKP [22] and the drug resistant variant, DLKP-A [23], lung cancer cell-lines were cultured in DMEM (Dulbecco's Modified Eagle's Medium):Hams F12 50:50 supplemented with 5% Foetal Calf Serum (Lonza).

Triplicate T25 cm^2 Costar cell culture flasks were seeded with 5 mL of 5×10^4 cells/mL and given one day to attach to the flask surface. The cells were exposed to the 2 μM, 1 μM, 500 nM and 100 nM concentrations of drug in the culture medium for 2 h. After this time the media was removed and the cells were washed in cold PBS to remove any traces of growth media, trypsinised and transferred to 10 mL polypropylene extraction tubes (Sarstedt). These were centrifuged at 200 g, the waste media removed and resuspended in 1 mL of PBS—a small aliquot removed for cell count. The

Table 3
The percentage recovery of dasatinib and lapatinib obtained with different extraction solvents.

Solvent	Dasatinib % recovery	% RSD	Lapatinib % recovery	% RSD
Ethyl acetate	58	11	29	19
Tert-Butyl methyl ether	71	9	98	21
Dichloromethane	77	5	53	10
Ethyl acetate:acetonitrile (3:1)	84	9	61	39
Tert-Butyl methyl ether:acetonitrile (3:1)	92	11	107	24
Dichloromethane:acetonitrile (3:1)	81	19	90	21

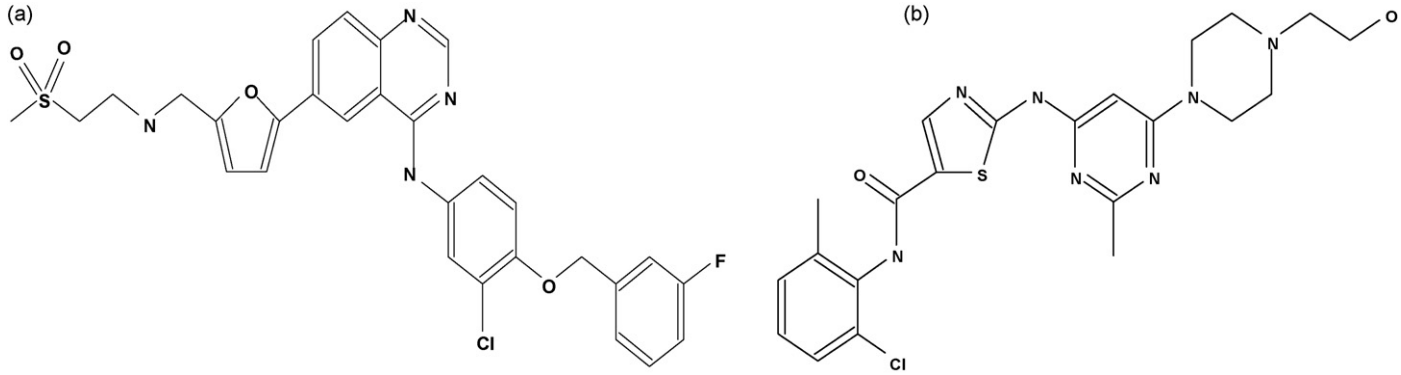

Fig. 1. (a) The structure of lapatinib and (b) the structure of dasatinib.

tubes were then centrifuged again, the PBS supernatant removed and the cell pellet frozen at −20 °C for later extraction. The complete cell pellet was extracted according to the procedure outlined in Section 2.5, though 100 μL of analyte was not added to the extraction tube.

All results are reported as mean and standard deviation (SD) of the mass per million cells in triplicate flasks. Mass per million cells was calculated as the mass of drug measured divided by the counted number of cells and multiplied by one million.

3. Results and discussion

3.1. Method development

3.1.1. Optimisation of sample pre-treatment

3.1.1.1. Choice of internal standard (IS). Deuterated analogues were not available to us and as the agents are recently released, we could not identify routinely available close structural analogues for use as internal standards. Several of the TKIs have broadly similar physiochemical properties and are more widely available to researchers hence we chose to employ lapatinib as the IS for dasatinib and vice versa. Although evidence suggests differences in their properties detected by the MS, the use of an internal standard in an isocratic environment (where properties such as ionisation efficiency remain largely constant) provided an acceptable level of reproducibility across the concentration ranges employed.

3.1.1.2. Solvent optimisation. Due to the high binding affinity of TKI agents to intracellular targets, and the complex nature of cell culture samples, liquid–liquid extraction (LLE) was chosen in preference to other forms of drug extraction [24]. Based on work by Oostendorp et al. [25], for the liquid–liquid extraction of imatinib, the extraction efficiencies for dasatinib and lapatinib in various solvents alone and in combination with ACN were tested, with the summary findings presented in Table 3. A combination of acetonitrile and *t*-butyl methyl ether (*t*BME) was determined to be the optimum solvent system for both dasatinib and lapatinib in combination with extraction buffer pH 3.5. The combination of *t*BME with acetonitrile gave the optimal compromise of separating the drugs of interest from their intracellular binding sites while keeping extraction of potential interfering substances from cells to a minimum; *t*BME also gave the best drug recovery across a number of different pH values.

3.1.1.3. Extraction buffer optimisation. To determine the optimum extraction system for the extraction of lapatinib and dasatinib it was also necessary to examine the effect of pH of the aqueous extraction buffer. Due to its compatibility with mass spectrometry 1 M ammonium formate was selected as the aqueous buffer. The aqueous phase was tested across a pH range to determine an optimum in combination with *t*BME/ACN (3:1, v/v) extraction solvent (Fig. 1).

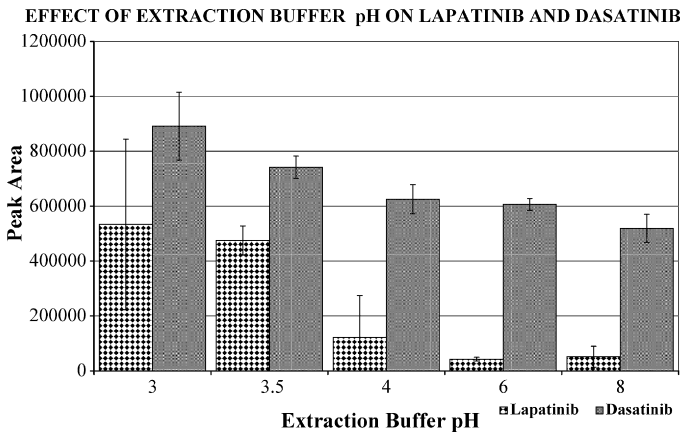

Fig. 2. The effect of extraction buffer pH on the peak area of extracted lapatinib and dasatinib. Extraction of in-tube mass of 50 ng (data illustrates the mean and standard deviations of triplicate estimates).

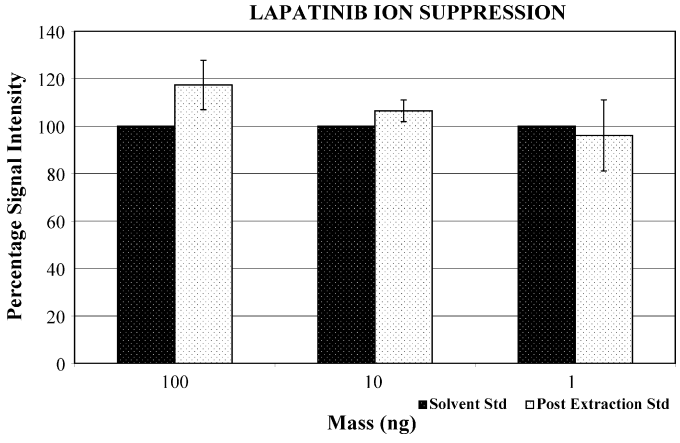

Fig. 3. Demonstration of the effect of ion suppression on lapatinib recovery. The peak area of solvent standards were normalised to 100% and the post-extraction standards were expressed as a function of solvent standard (data illustrates the mean and standard deviations of triplicate estimates).

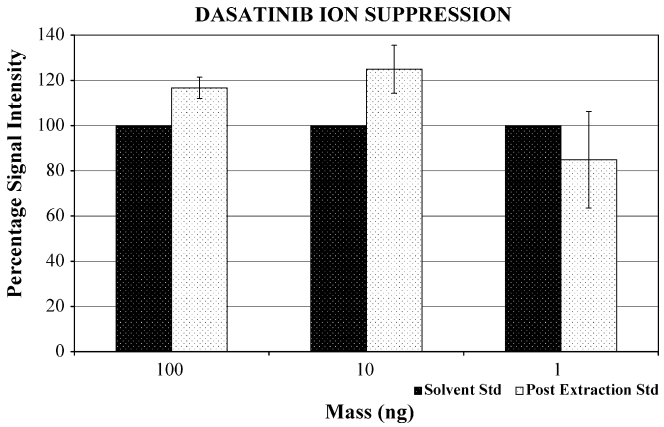

Fig. 4. Demonstration of the effect of ion suppression on dasatinib recovery. The peak area of solvent standards were normalised to 100% and the post-extraction standard were expressed as a function of solvent standard (data illustrates the mean and standard deviations of triplicate estimates).

As shown in Fig. 2 the optimum extraction buffer pH was determined to be pH 3.5 as this gave greater recovery than pH 4 but also gave lower standard deviations and noise in the recovery estimates that were evident at pH 3.

3.1.2. Optimisation of chromatography conditions

10 mM ammonium formate buffer was chosen as the aqueous mobile phase. Even though the pK_a of dasatinib is 5.8 and the pK_a of lapatinib is 6.6, pH 4 was chosen as the optimum mobile phase pH as pH 3 gave poorer lapatinib peak shape with a greater asymmetry factor than pH 4.

Optimal resolution of dasatinib and lapatinib peaks from each other and from the void volume was achieved with 54% ACN in the mobile phase when run isocratically at 0.2 mL/min.

Additional variations in mobile phase flow rate and organic content were examined. While it was possible to elute the agents earlier, we noticed an increased in ion suppression effects as the dasatinib peak eluted earlier. This was due to elution of biological artefacts originating from the cell extract which occur immediately after the column void volume. The final conditions chosen were therefore a compromise which ensured adequate and timely separation without exogenous interference.

3.1.3. Optimisation of mass spectrometry settings

Mass spectrometry settings for each drug were optimised by injecting 250 ng/mL of drug and by flow injection analysis the precursor ion, optimum fragmentor voltage, optimum collision energy and product ion was determined. Table 2 outlines the optimum settings identified for lapatinib and dasatinib as well as a number of other TKIs which were examined in this study. To improve specificity, all detector transitions were used; the most abundant product ion was used as the quantifier ion while any additional product ions were used as qualifier ions.

3.1.3.1. Ion suppression. The potential impact of ion suppression on the quantification was assessed by comparison of the results obtained with standard dilutions of each drug in acetonitrile (solvent standards) against standard dilutions which were added to dried extracted cell blanks (post-extraction standards), as outline by Zirrolli et al. [26]. 20 μL of each concentration was injected.

Ion suppression was calculated as peak area of the analyte of interest in the post-extraction standard compared to the solvent standard. Data is expressed as a function of the initial mass of drug in the tube. The findings are outlined in Figs. 3 and 4. Under the

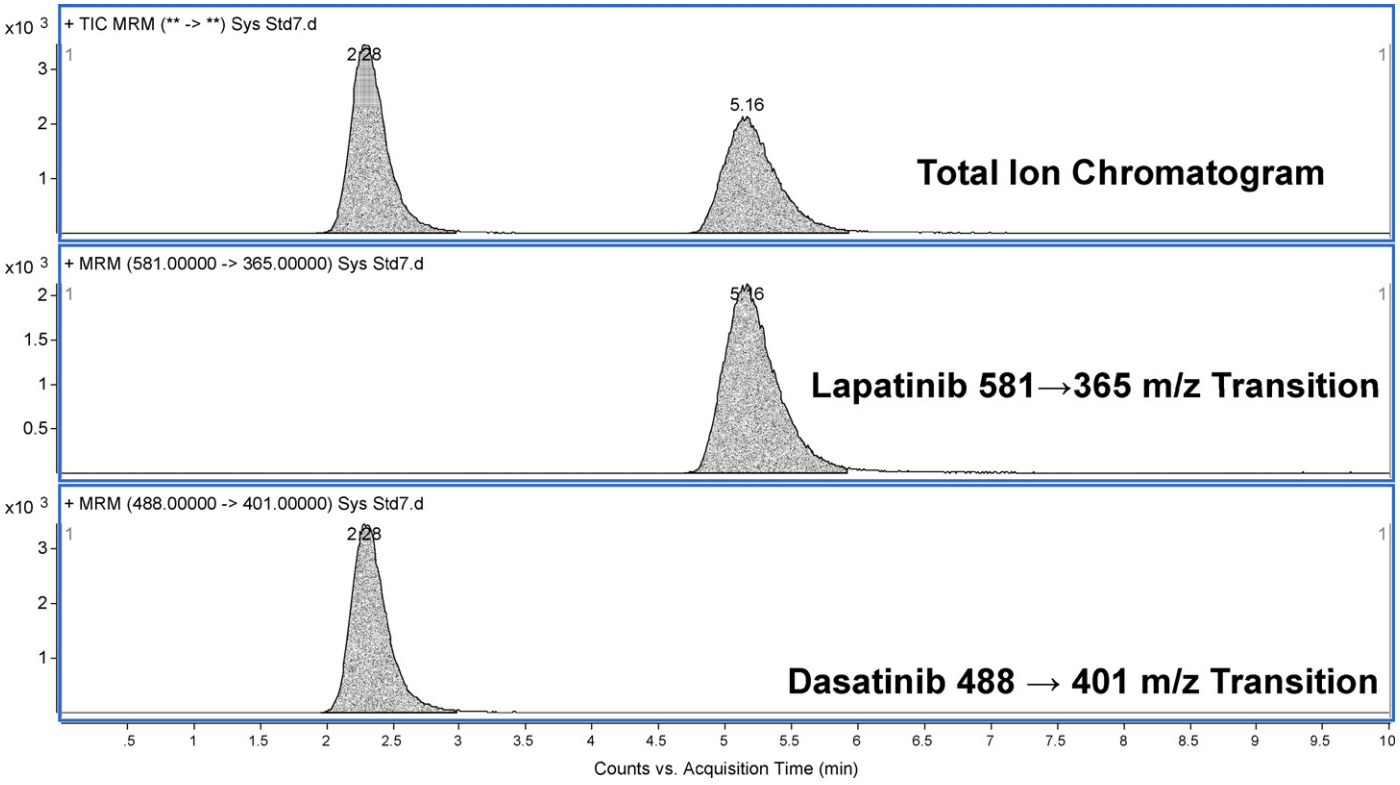

Fig. 5. Representative chromatogram of lapatinib and dasatinib separation. The total ion current for lapatinib and dasatinib in MRM mode, and one extracted MRM spectrum for each analyte.

Table 4a
Intra-day analysis for precision and accuracy of lapatinib and dasatinib ($n = 3$).

Spiked mass (ng)	Lapatinib mean mass observed	Lapatinib % accuracy	Lapatinib % RSD	Dasatinib mean mass observed	Dasatinib % accuracy	Dasatinib % RSD
0.10	0.12	120.5	5.4	0.10	104.6	9.1
0.25	0.24	95.5	8.1	0.26	102.3	12.6
0.50	0.55	110.8	7.9	0.49	97.9	12.9
10	9.1	91.0	2.7	9.1	90.7	11.4
50	55.4	110.8	1.9	53.7	107.4	10.3
200	209.7	104.8	2.8	200.9	100.4	9.2

Table 4b
Inter-day analysis for accuracy and precision of lapatinib and dasatinib ($n = 4$).

Spiked mass (ng)	Lapatinib mean mass observed (ng)	Lapatinib % accuracy	Lapatinib % RSD	Dasatinib mean mass observed (ng)	Dasatinib % accuracy	Dasatinib % RSD
0.10	0.12	120.3	16.6	0.10[a]	97.9	9.6
0.25	0.25	99.0	6.1	0.25[a]	100.9	2.5
0.50	0.56	111.6	7.6	0.49	98.3	2.8
10	8.7	87.0	8.9	10.3	102.8	6.8
50	53.0	105.9	8.0	54.8	109.7	6.0
200	217.1	108.5	16.9	181.2	90.6	10.7

[a] $n = 3$.

conditions tested, ion suppression effects were found to be minimal.

3.2. Method validation

The overall LC–MS method was validated for the following performance parameters—linearity and range, intra-day precision (repeatability) and inter-day precision (intermediate precision), accuracy, sensitivity (LOD and LOQ), recovery and sample stability according to the guidelines described by Ermer [27]. An illustrative chromatogram is provided in Fig. 5. MRM allowed individual determination of each drug necessary with the use of internal standard-based quantification.

3.2.1. Linearity and range

Regression analysis was used to assess the linearity between the peak area ratios (analyte/IS) and the analyte concentration.

The calibration curves for dasatinib and lapatinib were linear over the range of 0.1–200 ng in tube and recovery was linear. Since the TKIs resolved well from each other and were extracted with high efficiency one TKI was used as the internal analytical standard for the other agent to reduce error. 50 ng (100 μL of a 500 ng/mL stock) of internal standard was added to each tube. Whilst the calibration curves were linear, over such a broad range, the bias of the regression line tends to make the determination of lower drug concentration values much less accurate; hence it was decided to use a log–log plot of the peak area ratio versus the mass of drug in all calculations.

The log–log plot gave typical correlation coefficients (R^2) values of >0.99.

Table 5a
Lapatinib intra assay recovery ($n = 3$).

Mass (ng)	Mean % recovery	% RSD
0.1	112.4	21.8
10	98.6	15.6
100	85.8[a]	14.2

[a] $n = 2$.

Table 5b
Dasatinib intra assay recovery ($n = 3$).

Mass (ng)	Mean % recovery	% RSD
0.1	99.2	16.1
10	90.7	5.4
100	104.4	14.6

3.2.2. Precision and accuracy

Intra-day precision and accuracy was assessed over the mass range (0.1–200 ng) by extraction and analysis of triplicate spiked samples on the same day (Table 4a).

Inter-day precision and accuracy was assessed over the mass range (0.1–200 ng) by extraction and analysis of triplicate spiked samples over four days (Table 4b).

The percentage relative standard deviation (% RSD) was employed as a measure of precision. The percentage accuracy was determined by dividing the average calculated drug concentration by that of the spiked known concentration.

In all cases, the RSD values were less than 17%, with the majority being less that 10%, and the average percentage accuracy was 103%.

3.2.3. Selectivity and sensitivity

The limit of detection (LOD) was defined as the mass of drug which gave a signal-to-noise ratio of 3:1. The limit of quantification (LOQ) was defined as the mass of drug which gave a signal-to-noise ratio of 5:1 [28].

Given these criteria, the LOD for lapatinib was determined to be 50 pg of lapatinib in the extraction tube which equated to 15 pg on column with a RSD of 19%. The LOQ for lapatinib was 100 pg which equates to 31 pg on column with an RSD of 13%.

The LOD for dasatinib was determined to be 10 pg, which equated to 3 pg on column with an RSD of 15%. The LOQ for dasatinib was determined to be 50 pg which equates to 15 pg on column with an RSD of 8%. These values for LOD and LOQ enable pharmacokinetic monitoring of both drugs in cell samples.

The signal-to-noise ratio was calculated by the Masshunter qualitative analysis software. Calculations were based on the peak area of each drug individually not on the peak area ratio.

The LC–MS method easily resolved both TKIs from each other and due to the high sensitivity of mass spectrometric detection and the low background when quantifying based on the molecular transitions of the ions in the collision cell (Multiple Reaction Monitoring, MRM) gave excellent selectivity and the very low LOD and LOQs necessary to comfortably quantify a broad range of drug levels found in the cancer cells.

Table 6a
Dasatinib freeze–thaw stability ($n = 3$).

Mass (ng)	First freeze–thaw cycle % recovery	Second freeze–thaw cycle % recovery	Third freeze–thaw cycle % recovery	Fourth freeze–thaw cycle % recovery
0.1	100	98.3	105.7	101.7
0.5	100	104.1	91.1	100.5
50	100	120.4	107.7	111.2
200	100	84.9	94.8	90.3

Table 6b
Lapatinib freeze–thaw stability ($n = 3$).

Mass (ng)	First freeze–thaw cycle % recovery	Second freeze–thaw cycle % recovery	Third freeze–thaw cycle % recovery	Fourth freeze–thaw cycle % recovery
0.1	100	98.2	97.8	84.8
0.5	100	102.1	101.3	89.8
50	100	109.2	102.1	81.4
200	100	92.5	98.1	88.5

3.2.4. Recovery/extraction efficiency

The extraction efficiency of the procedure was determined by comparing the peak areas of the extracted analytes with those from non-extracted samples. Recoveries are at low, medium and high concentrations were evaluated. Recoveries for both drugs were good and ranged from 86 to 112%. Results are shown in Tables 5a and 5b.

3.2.5. Stability

The stability of lapatinib and dasatinib in the presence of cells was determined over four freeze–thaw cycle at concentrations of 1, 5, 500 and 2000 ng/mL, as shown in Tables 6a and 6b. On day one triplicate standards were extracted and the remaining samples were frozen at −20°C. The standards were then thawed, extracted and refrozen each day for a further three days.

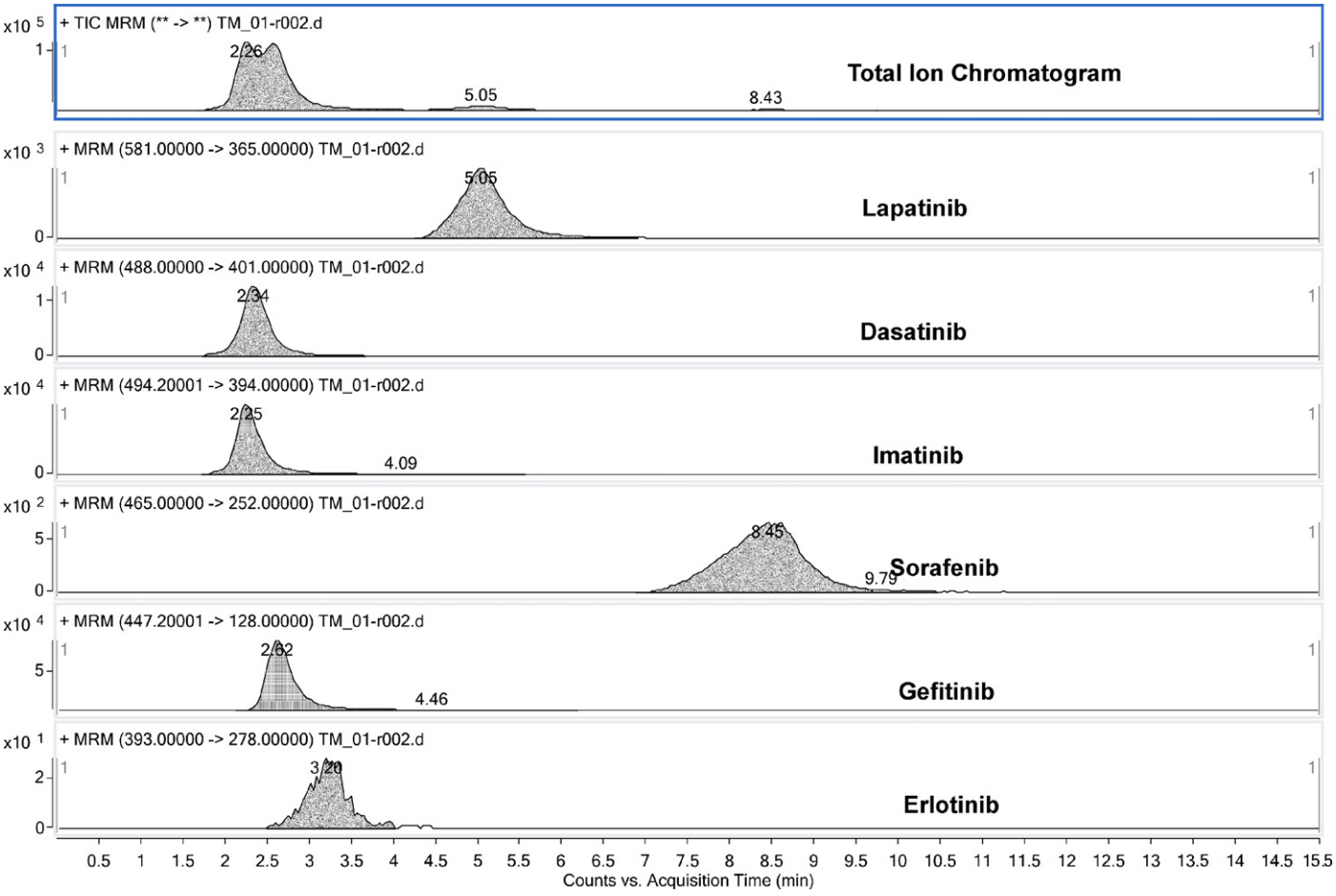

Fig. 6. Total ion chromatogram and extracted MRM transitions of 6 tyrosine kinases inhibitors (lapatinib, dasatinib, erlotinib, gefitinib, imatinib and sorafenib), and one extracted transition for each analyte following LLE-LC–MS.

The data generally indicate that both agents were stable over the course of the experiment. However, it was noted that instability was seen in lapatinib on Day 4 of the freeze–thaw assay, suggesting that this agent is slightly less stable under these conditions .

3.3. Method application

3.3.1. Multiple tyrosine kinase inhibtors

The similarity in the physiochemical properties (especially lipophylicity) of many TKIs prompted us to undertake a brief examination of the potential for the method to analyse multiple other TKIs. Gefitinib, erlotinib, imatinib and sorafenib were used as representative agents.

Fig. 6 shows the trace and extracted MRM for six TKIs in a single sample. The figure illustrates that the drugs all extracted under these conditions and although the chromatographic separation did not resolve all the TKIs from each other, MRM determination enabled individual detection and resolution of each agent. Therefore, in principle, the method may be applicable for simultaneous determination of these agents; however, further validation studies would clearly be necessary, in particular to clarify if all agents were extracted efficiently under the experimental conditions outlined.

3.3.2. Cell sample analysis

The overall assay was applied to biological cell samples to determine the differences in uptake of dasatinib and lapatinib by DLKP and P-gp over-expressing, DLKP-A cell-lines, to examine if P-gp expression had any impact on the cellular pharmacokinetics of these drugs. With a two-hour exposure period, the accumulation of each agent appeared to be proportionally but non-linearly associated with the amount of drug administered. As is evident from a comparison of the *y*-axes of the graphs, very significant differences in the accumulation of each agent were evident, with much higher levels of lapatinib found in the sensitive and resistant cells (see Fig. 7) as compared to dasatinib (see Fig. 8).

The impact of P-gp can be seen for each analyte by comparing the accumulation in the parental and resistant cell-line. Comparison of the accumulation between DLKP and DLKP-A illustrated that P-gp over-expression had a considerable impact on the amount of dasatinib accumulated, suggesting that P-gp may significantly decrease the accumulation of dasatinib. However, lapatinib accumulation in the same cell-lines showed only minor differences in accumulation between the parental cell-line and the P-gp over-expressing variant. Illustratively, at 2 μM after two hours exposure 80 ng (±16 ng) of dasatinib per million cells was accumulated in

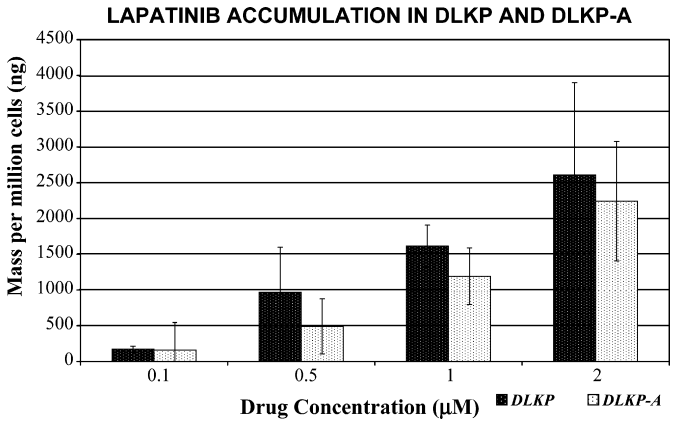

Fig. 7. Lapatinib accumulation after two hours in DLKP and DLKP-A cell-lines, comparing the level of lapatinib accumulated in the parental cell-line, DLKP, and the P-gp over-expressing cell-line, DLKP-A. Data illustrates the mean and standard deviation of six estimates.

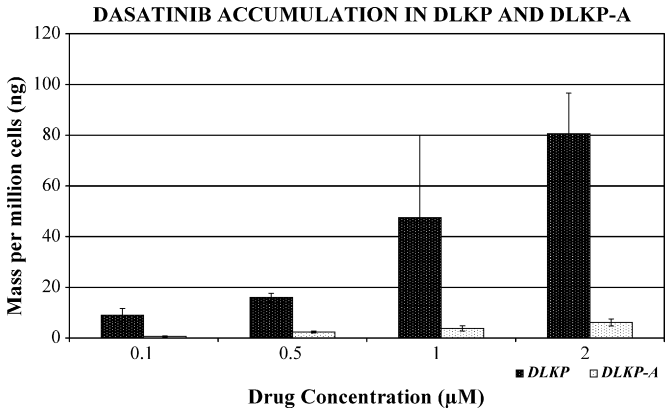

Fig. 8. Dasatinib accumulation after two hours in DLKP and DLKP-A cell-lines, comparing the level of dasatinib accumulated in the parental cell-line DLKP and the P-gp over-expressing cell-line DLKP-A. Data illustrates the mean and standard deviation of six estimates.

DLKP cell-line, whereas only 6 ng (±1 ng) per million cells (or 8% of the parental amount) was detected in DLKP-A. However, at 2 μM after two hours exposure 26010 ng (±994 ng) of lapatinib per million cells was accumulated in DLKP and 2242 ng (±789 ng) (or 86%) lapatinib was accumulated in DLKP-A.

4. Conclusions

A novel and sensitive assay has been developed and validated for the determination of cellular levels of the tyrosine kinase inhibitors lapatinib and dasatinib based on LLE coupled to LC–MS/MS.

The use of a liquid–liquid extraction clean-up stage gives the simplicity of application making the method accessible to other researchers undertaking large numbers of analyses without the need for extra equipment or expensive clean-up columns, while an isocratic elution scheme gives a simple, robust and reproducible chromatographic method.

Our experimental approach has yielded superior levels of sensitivity and the specificity of the analytical method may also allow for broader applicability to the determination of a number of other TKI agents and from other biological matrices, e.g. patient serum.

This method has been applied to cancer cell-line models and used to examine potential mechanisms of pharmacokinetic resistance in cancer cell models. There are significant differences in the overall cellular uptake of lapatinib and dasatinib, the biological implications of this difference is unclear but clearly this has the potential to impact efficacy. Lapatinib is not actively effluxed from P-gp over-expressing cancer cells, while P-gp activity significantly decreases cellular levels of dasatinib suggesting that this agent is a substrate for P-gp and over-expression of this pump in cancer cells could have a role in resistance to the agent.

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References

[1] N. Steeghs, J.W. Nortier, H. Gelderblom, Ann. Surg. Oncol. 14 (2007) 942.
[2] A. Arora, E.M. Scholar, J. Pharmacol. Exp. Ther. 315 (2005) 971.
[3] D.S. Krause, R.A. Van Etten, N. Engl. J. Med. 353 (2005) 172.
[4] S.R. Hubbard, J.H. Till, Annu. Rev. Biochem. 69 (2000) 373.

[5] L.J. Lombardo, F.Y. Lee, P. Chen, D. Norris, J.C. Barrish, K. Behnia, S. Castaneda, L.A. Cornelius, J. Das, A.M. Doweyko, C. Fairchild, J.T. Hunt, I. Inigo, K. Johnston, A. Kamath, D. Kan, H. Klei, P. Marathe, S. Pang, R. Peterson, S. Pitt, G.L. Schieven, R.J. Schmidt, J. Tokarski, M.L. Wen, J. Wityak, R.M. Borzilleri, J. Med. Chem. 47 (2004) 6658.
[6] P. Ramirez, J.F. DiPersio, Oncologist 13 (2008) 424.
[7] W. Xia, R.J. Mullin, B.R. Keith, L.H. Liu, H. Ma, D.W. Rusnak, G. Owens, K.J. Alligood, N.L. Spector, Oncogene 21 (2002) 6255.
[8] C.E. Geyer, J. Forster, D. Lindquist, S. Chan, C.G. Romieu, T. Pienkowski, A. Jagiello-Gruszfeld, J. Crown, A. Chan, B. Kaufman, D. Skarlos, M. Campone, N. Davidson, M. Berger, C. Oliva, S.D. Rubin, S. Stein, D. Cameron, N. Engl. J. Med. 355 (2006) 2733.
[9] S. Hsieh, T. Tobien, K. Koch, J. Dunn, Rapid Commun. Mass Spectrom. 18 (2004) 285.
[10] F. Bai, B.B. Freeman III, C.H. Fraga, M. Fouladi, C.F. Stewart, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 831 (2006) 169.
[11] L. Wang, L.J. Christopher, D. Cui, W. Li, R. Iyer, W.G. Humphreys, D. Zhang, Drug Metab. Dispos. 36 (2008) 1828.
[12] D.K. Hiwase, V. Saunders, D. Hewett, A. Frede, S. Zrim, P. Dang, L. Eadie, L.B. To, J. Melo, S. Kumar, T.P. Hughes, D.L. White, Clin. Cancer Res. 14 (2008) 3881.
[13] A.V. Kamath, J. Wang, F.Y. Lee, P.H. Marathe, Cancer Chemother. Pharmacol. 61 (2008) 365.
[14] 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.
[15] Y. Hsieh, G. Galviz, Q. Zhou, C. Duncan, Rapid Commun. Mass Spectrom. 23 (2009) 1364.
[16] 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. (2009).
[17] R. O'Connor, Anticancer Res. 27 (2007) 1267.
[18] R. O'Connor, M. Clynes, P. Dowling, N. O'Donovan, L. O'Driscoll, Exp. Opin. Drug Metab. Toxicol. 3 (2007) 805.
[19] C. Ozvegy-Laczka, J. Cserepes, N.B. Elkind, B. Sarkadi, Drug Resist. Update 8 (2005) 15.
[20] J.W. Polli, J.E. Humphreys, K.A. Harmon, S. Castellino, M.J. O'Mara, K.L. Olson, L.S. John-Williams, K.M. Koch, C.J. Serabjit-Singh, Drug Metab. Dispos. 36 (2008) 695.
[21] D.M. Collins, R. O'Connor, N. O'Donovan, M. Clynes, J. Crown, Ann. Oncol. 17 (2006) 57.
[22] S. McBride, P. Meleady, A. Baird, D. Dinsdale, M. Clynes, Tumour Biol. 19 (1998) 88.
[23] C.P. Duffy, C.J. Elliott, R.A. O'Connor, M.M. Heenan, S. Coyle, I.M. Cleary, K. Kavanagh, S. Verhaegen, C.M. O'Loughlin, R. NicAmhlaoibh, M. Clynes, Eur. J. Cancer 34 (1998) 1250.
[24] M. Jemal, Biomed. Chromatogr. 14 (2000) 422.
[25] R.L. Oostendorp, J.H. Beijnen, J.H. Schellens, O. Tellingen, Biomed. Chromatogr. 21 (2007) 747.
[26] J.A. Zirrolli, E.L. Bradshaw, M.E. Long, D.L. Gustafson, J. Pharm. Biomed. Anal. 39 (2005) 705.
[27] J. Ermer, J. Pharm. Biomed. Anal. 24 (2001) 755.
[28] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, J. Pharm. Sci. 81 (1992) 309.