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# Liquid chromatography-tandem mass spectrometric assay for the light sensitive tyrosine kinase inhibitor axitinib in human plasma

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ABSTRACT

A bioanalytical assay for the new tyrosine kinase inhibitor axitinib was developed and validated. In addition, the light mediated *trans* to *cis* isomerization of this drug was investigated. For the quantitative assay, human plasma samples were pre-treated under light protection using protein precipitation with acetonitrile containing erlotinib as the internal standard. The extract was diluted with water and injected into the chromatographic system. The system consisted of a trifunctional bonded octadecyl silica column with isocratic elution using formic acid in a water-methanol mixture. The eluate was transferred into an electrospray interface with positive ionization and the analyte was detected and quantified using the selected reaction monitoring mode of a triple quadrupole mass spectrometer. The assay was validated in a 0.2–200 ng/ml concentration range, the lowest level of this range being the lower limit of quantification. Within day precisions were 2.5–6%, between day precisions 4–9% and accuracies were between 91 and 106% for the whole calibration range. Light protected axitinib showed no isomerization and was shown to be chemically stable under all relevant conditions. Finally, the assay was successfully applied for a mouse tissue distribution study using mouse samples diluted with human plasma.



## 1. Introduction

Axitinib (AG013736, Fig. 1A) is a selective orally active tyrosine kinase inhibitor (TKI) with anti-angiogenic effects. *In vitro*, the compound has been shown to inhibit angiogenesis, vascular permeability and blood flow [1,2]. In phase I and II clinical studies, axitinib showed antitumor activity against different advanced tumors [3], including renal cell carcinoma (RCC) [4,5], pancreatic cancer [6] and thyroid cancer [7]. At the moment, two phase III clinical studies with axitinib are ongoing [8,9]. In one study axitinib is combined with gemcitabine for the treatment of advanced pancreatic cancer [9], and in the other phase III trial axitinib is tested as a second line therapy for metastatic RCC in comparison to sorafenib [8]. In both studies 5 mg of axitinib twice daily (BID) is used as a starting dose.

Pharmacokinetic evaluations of axitinib, important during initial drug development, are still scarce [10]. The assay in that first pharmacokinetic study was, however, reported very concisely and used liquid chromatography–tandem mass spectrometry (LC/MS/MS) in combination with (laborious) liquid–liquid extraction [10]. Therefore, the development and validation of a simple, fast and sensitive bioanalytical assay for axitinib in human plasma was conducted and reported in this paper before starting a new clinical trial.

Validated bioanalytical assays using LC/MS/MS for the first TKIs have been reported frequently since 2002 [11–32], with very recently the introduction of assays for multiple components of this class of drugs [29,31,32]. Most often, these assays have been validated for plasma of human subjects [11–15,17–20,22,24–26,29–31]. For erlotinib [11,12], imatinib [19–21], sunitinib [27] and sorafenib [23] a metabolite could be quantified simultaneously with the parent drugs. In most methods the protonated parent molecule is used in the selected reaction monitoring (SRM) mode using a triple quadrupole (QqQ) system, with a quadrupole–linear ion trap combination [15] as the only

*Abbreviations:* BID, twice daily; DFC, 5′-deoxy-5-fluorocytidine; dFdU, 2′,2′-difluorodeoxyuridine; DFU, 5′-deoxy-5-fluorouridine; DMSO, dimethyl sulfoxide; i.v., intravenous; LLQ, lower limit of quantification; mepepy, 1-(pyridine-4-yl)-2-(N-methylpyrrol-2-yl)ethane; PP, protein precipitation; QqQ, triple quadrupole; RCC, renal cell carcinoma; SRM, selected reaction monitoring; TKI, tyrosine kinase inhibitor; QC, quality control.

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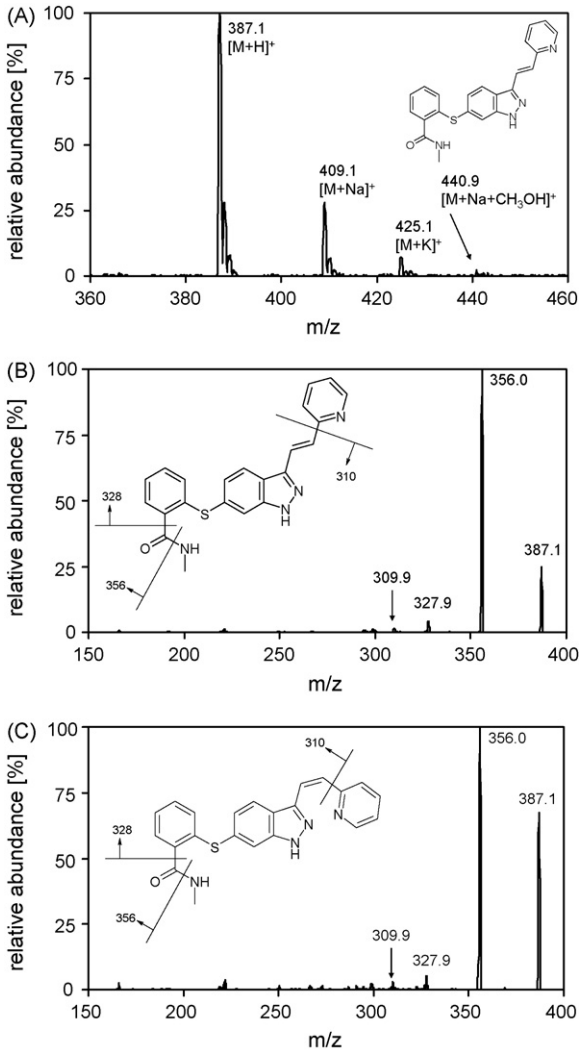


**Fig. 1.** Electrospray spectrum of axitinib (A) and product spectra at *m*/*z* 387.1 @ −18 V for both, *trans*- (B) and *cis*-axitinib (C), mass spectrometric conditions are as used in the reported bioanalytical assay without using SRM.

exception. This assay of Guetens et al. [15] was also the only method using a capillary LC system. Two new methods used a single quadrupole system in the single ion monitoring mode [30,31] for dasatinib, nilotinib and imatinib.

Apart from an application using a pentafluorophenyl column [22] and one using a hydrophilic interaction chromatography system [32] all investigators used an octa or octadecyl modified silica phase with at least end-capping as prevention of unwanted interaction of the basic TKI drugs with the free silanol groups of the silica. Typically, methanol is used as organic modifier in the eluent for all imatinib single drug assays [16–21] and the nilotinib assay [30], while acetonitrile is used for other TKI drugs: erlotinib [11,12], gefitinib [13–15], lapatinib [22,29], vandetanib [28], sunitinib [26,27,29], sorafenib [23–25,29], dasatinib [29,31,32] and nilotinib [29–32]. To control the pH of the eluent and to improve ionization of the analytes, formic acid [12,14,19,22–26,29–32] and ammonium acetate [11,13,15,16,18,20–22,24,29,32] are both frequently used, with ammonium formate [17,27] and acetic acid [28] being incidental alternatives.

Lower limits of quantification (LLQs) for TKIs in the sub-ng/ml range (0.1–0.5 ng/ml) were obtained using liquid–liquid extraction with methyl-*t*-butyl ether [13,15,26,27]. Other pre-treatment methods, including protein precipitation (PP) with acetonitrile [12,14,16,18–20,23–25,29,30,32] or acetonitrile–methanol [31], liquid–liquid extraction with hexane-ethyl acetate [11,17] or pentane–ethyl acetate [11,17,28] and solid-phase extraction on C18 [22] or C8 [21] sorbents, resulted in LLQs in the 1–100 ng/ml range.

Recently, axitinib was reported to form the sulfoxide metabolite and a glucuronide conjugate as circulating metabolites and hydroxy-axitinib in excreta in human ADME studies [33], unfortunately no further details were reported. Therefore, and also because no axitinib metabolites were commercially available, we focused on developing and validating a bioanalytical LC/MS/MS assay for the axitinib parent compound. A QqQ system and PP as a simple pre-treatment technique were used to obtain sensitivity in the sub-ng/ml range for this new TKI. The higher sensitivity compared to other TKI drugs using this approach may partially result from the use of a new generation LC system and column with higher pressures and sub-2 μm particles (Ultra Performance LC), not being used earlier for TKI drugs.

## 2. Experimental

### 2.1. Chemicals

Axitinib (≥99%) and erlotinib hydrochloric acid were obtained from Sequoia Research Products (Pangbourne, UK) and LC-MS grade water, methanol of HPLC quality and acetonitrile of HPLC-S gradient grade quality were from Biosolve (Valkenswaard, The Netherlands). Water not used as eluent was home-purified by reversed osmosis on a multi-laboratory scale. Formic acid was of analytical grade and originated from Merck (Darmstadt, Germany) and analytical grade dimethyl sulfoxide (DMSO) was supplied by Acros Organics (Geel, Belgium). Human EDTA plasma (pooled and from individual donors) was from Innovative Research (Southfield, MI, USA).

### 2.2. Equipment

The LC/MS/MS equipment consisted of an Accela pump and autoinjector and a TSQ Quantum Ultra quadrupole mass spectrometer with heated electrospray ionization (Thermo Fisher Scientific, San Jose, CA, USA). Data were recorded and the system was controlled using the Thermo Fisher Xcalibur software (version 2.07).

### 2.3. LC/MS/MS conditions

Partial-loop injections (5 μl) were made on an Acquity UPLC® BEH C18 column (30 × 2.1 mm, $d_p$ = 1.7 μm, Waters, Milford, USA), protected by the corresponding VanGuard pre-column (Waters, 5 × 2.1 mm). The column temperature was maintained at 40 °C and the autoinjector at 4 °C. The eluent comprised a mixture of methanol (45%, v/v) and 0.1% (v/v) formic acid in water (55%, v/v) that was pumped at 0.6 ml/min. The whole eluate was transferred into the electrospray probe, starting at 0.4 min after injection by switching the MS inlet valve, until the end of the analytical run at 1.2 min. The heated electrospray was tuned in the positive ionization mode by introducing 0.5 ml/min of a mixture of acetonitrile (50%, v/v) and 0.1% formic acid in water (50%, v/v) and 5 μl/min of 10 μg/ml axitinib. The highest response was obtained with a 4000 V spray voltage, a 350 °C capillary temperature and vaporizer temperature and the nitrogen sheath, ion sweep and auxiliary gasses set at 50, 8 and 50 arbitrary units, respectively; the skimmer voltage was set off. The SRM mode was used with argon as the collision gas at 1.0 mTorr. The tube lens off set was 106 V for both compounds. Axitinib was monitored at *m*/*z* 387.1 → 356.1 at −18 V collision energy and a 0.3 s dwell time, erlotinib at *m*/*z* 394.1 → 278.1 at −30 V collision energy and a 0.1 s dwell time. The mass resolutions were set at 0.7 full width at half height (unit resolution) for both separating quadrupoles.

### 2.4. Sample pre-treatment

Liquid handlings were all performed in a corner of the laboratory with reduced light intensity. To a volume of 100 μl human plasma sample, pipetted into a 1.5 ml amber colored polypropylene tube, 150 μl of 20 nM erlotinib in acetonitrile was added. The tube was then closed and shaken vigorously for ca. 5 s using vortex-mixing. After centrifugation of the sample at $10 \times 10^3 \times g$ at ambient temperature for 1 min, 125 μl of the supernatant was pipetted into a 250 μl glass insert placed in an amber colored autoinjector vial. Before closing the vial, 125 μl of water was added and finally, 5 μl of the mixture was injected onto the column.

### 2.5. Validation

A laboratory scheme based on international guidelines [34–36] was used for the validation procedures.

#### 2.5.1. Calibration

Stock solutions of axitinib at 0.5 and 2 mg/ml in amber colored polypropylene tubes and erlotinib (IS) at 10 mM were prepared in DMSO. The stock solutions were stored at −30 °C.

The 0.5 mg/ml stock solution of axitinib was diluted to a 200 ng/ml calibration sample in pooled human EDTA plasma stored in amber colored polypropylene tubes at −30 °C. Additional calibration samples were prepared daily at 50, 10, 2.5, 0.5 and 0.2 ng/ml by dilution with the blank matrix. The 0.2, 0.5 and 200 ng/ml calibration samples were processed in duplicate for each daily calibration, whereas the levels in between were processed only once. Least-squares double-logarithmic regression was employed to define the calibration curves using the ratios of the peak area of axitinib and the IS.

#### 2.5.2. Precision and accuracy

The 2 mg/ml stock solution of axitinib was used to obtain validation (quality control (QC)) samples in pooled human EDTA plasma at 150 (QC-high), 10 (QC-med), 0.5 (QC-low) and 0.2 ng/ml (QC-LLQ). The QC samples were stored in amber colored polypropylene tubes at −30 °C. Precisions and accuracies were determined by sextuple analysis of each QC in three analytical runs on three separate days for all QCs (total: $n = 18$). Relative standard deviations were calculated for both the within day precision (repeatability) and the between day precision (reproducibility).

#### 2.5.3. Selectivity

Six individual EDTA plasma samples were processed to test the selectivity of the assay. These samples were processed with and without IS and with IS after spiking axitinib at the LLQ level (0.2 ng/ml). Additionally, 20 compounds were tested for potential interference in the axitinib assay. These compounds can be categorized in generally used drugs (methadone, sulfamethoxazole, trimethoprim, ranitidine, caffeine, acetaminophen, oxazepam, rifampicin, pantoprazole, dexamethasone and metoclopramide), anticancer drugs, currently used as co-administered drugs in clinical studies of axitinib (capecitabine, gemcitabine, cisplatin, carboplatin, paclitaxel and docetaxel) [37] and a few of their metabolites (5′-deoxy-5-fluorocytidine (DFC), 5′-deoxy-5-fluorouridine (DFU) and 2′,2′-difluorodeoxyuridine (dFdU)). To each of five blank plasma samples four of these compounds were spiked at a level near the upper therapeutic concentration (Table 1) [38] and these samples were processed, both as blanks and spiked with axitinib at the LLQ level, using the reported assay.

#### 2.5.4. Recovery

The extraction efficiency was determined in quadruplicate by comparing processed samples (QC-high, -med and -low) with extracts of drug-free human EDTA plasma spiked with the analytes at these levels. Ionization efficiency (ion suppression) was assessed by comparing the spiked blank extracts with reference material solutions in water–acetonitrile (3/1; v/v) at the three validation levels (QC-high, -med and -low). The extraction and ionization efficiencies of the IS were assessed using identical procedures at the erlotinib concentration used in the assay.

**Table 1**
Samples with potentially co-administered drugs and their metabolites used for selectivity assessment and the levels near the upper therapeutic concentrations [38] used.

| Sample | Compounds (concentration [μg/ml]) |
|---|---|
| 1 | Methadone (2); sulfamethoxazole (50); trimethoprim (10); ranitidine (2). |
| 2 | Caffeine (15); acetaminophen (20); oxazepam (2); rifampicin (10). |
| 3 | Capecitabine (10); DFC (10); DFU (10); pantoprazole (10). |
| 4 | Dexamethasone (2); metoclopramide (2); gemcitabine (50); dFdU (50). |
| 5 | Cisplatin (3); carboplatin (10); paclitaxel (9); docetaxel (5). |

#### 2.5.5. Stability

The stability of axitinib was investigated in QC-high and -low plasma samples stored in amber colored polypropylene tubes. Quadruplicate analysis of these samples was performed after storage at ambient temperature (24 h at 23 °C), three additional freeze-thaw cycles (thawing at ambient temperature during ca. 1 h and freezing again at −30 °C for at least 1 day), and storage at −30 °C for 2 months, respectively. Furthermore, validation runs were re-injected after additional storage of the extracts at 4 °C for 2 weeks.

### 2.6. Mouse samples

Mice were housed and handled according to guidelines of the Netherlands Cancer Institute complying with the Dutch legislation. Animals used in this study were male wild-type mice of FVB:Ola1 genetic background, between 9 and 15 weeks of age. Animals were kept in a temperature-controlled environment with a 12 h light/12 h dark cycle and received a standard diet (AM-II, Hope Farms, Woerden, The Netherlands) and acidified water (to pH 2.4–2.5 using hydrochloric acid to suppress bacterial growth) *ad libitum*.

Wild-type mice were treated with a dose of 2 mg/kg of axitinib by intravenous (i.v.) administration in the tail vein. Axitinib solution was obtained by dissolving the drug in a mix (1:1, v/v) of Tween 80 and ethanol (100%), followed by dilution with 0.9% (w/v) sodium chloride in water. Blood samples were collected in heparinized amber tubes via cardiac puncture 30 min after administration of the drug. Samples were kept on melting ice. After centrifugation at $2100 \times g$ for 6 min at 4 °C, plasma was pipetted into amber tubes and stored at −30 °C. In addition, liver, small intestine, kidney, spleen and brain were collected after cardiac puncture and stored in tubes wrapped in aluminium foil on dry ice. All mouse tissues were homogenized in different volumes of ice-cold 4% (w/v) bovine serum albumin solution in water using a Polytron PT1200 blender (Kinematica, Littay, Switzerland) for 1 min at position 23. The volumes used for liver or small intestine (with content) were 5 ml, for kidney 3 ml, for brain 1 ml and for spleen 0.5 ml. The homogenates were stored at −30 °C in amber tubes. Mouse samples were diluted with pooled human EDTA plasma in a 1:20 (v/v) ratio for plasma and kidney, 1:5 for liver, small intestine and spleen and 1:1 for brain samples and processed using the reported method. From the final tissue samples 1 μl was injected into the LC/MS/MS instead of the original 5 μl volume.

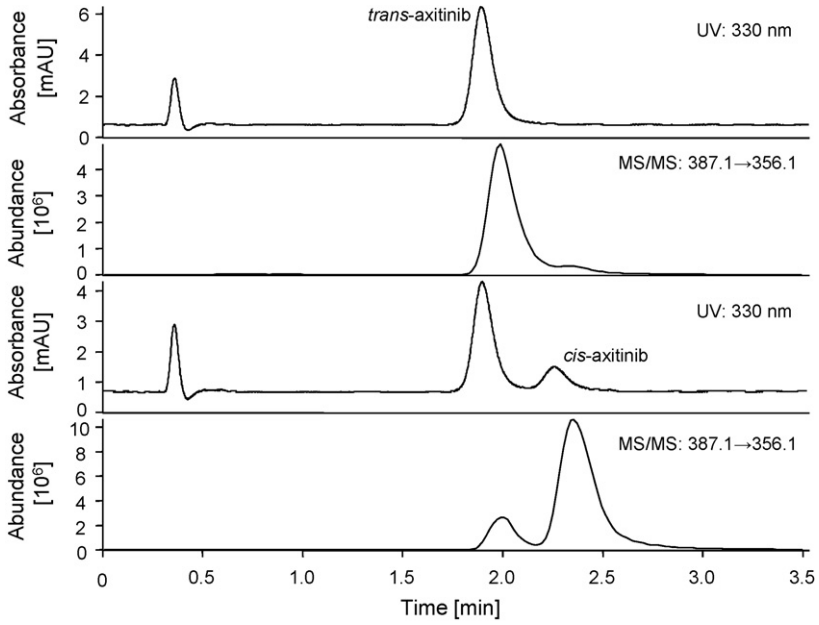


**Fig. 2.** UV and SRM chromatograms of 1 μg/ml axitinib before (both top chromatograms) and after (both bottom chromatograms) a 4.5-h exposure to light on the laboratory bench top. Ten microliters were injected on a Vision HT C18-P column (30 × 2 mm, $d_p$ = 1.5 μm) with a corresponding 5 mm pre-column (both from Grace), both maintained at 30 °C. The eluent (0.3 ml/min) comprised 45% (v/v) of 100 mM ammonium acetate in water and 55% (v/v) methanol. The LC(UV)/MS/MS equipment consisted of a DGU-14A degasser, a CTO-10Avp column oven, a Sil-HTc autosampler, two LC10-ADvp-μ pumps, a SPD10-Avp spectrophotometric UV–vis detector (all from Shimadzu, Kyoto, Japan) and a Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer with electrospray ionization (Thermo Fisher).

## 3. Results and discussion

### 3.1. Method development

Because of the expected high selectivity and sensitivity of the MS/MS detection in combination with an LC system using sub 2 μm particles, a simple pre-treatment procedure was explored as the first option. PP with acetonitrile, also used for several other TKI assays [12,14,16,18–20,23–25], showed high extraction recoveries for both analytes during initial method development experiments. Water was added to the extract to obtain sharp, symmetric peaks because the eluent contained less organic modifier than the extract.

During electrospray ionization, axitinib and erlotinib both formed the corresponding protonated molecules as their most abundant ions, just as has been experienced for all TKIs so far [11–32], including erlotinib [11,12], the present IS. An electrospray mass spectrum and a product spectrum of axitinib are shown in Fig. 1A and B, including explanation of the fragments.

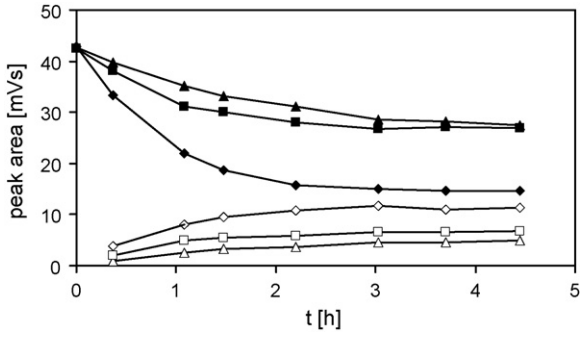


**Fig. 3.** LC–UV response (330 nm) of both axitinib isomers (filled symbols (▲; ■; ♦) for *trans*-axitinib and open symbols (△; □; ◊) for *cis*-axitinib) after exposure to light in the laboratory during the day. Triangles (▲; △): 20 μg/ml axitinib in methanol–water (1/3; v/v); squares (■; □): 1 μg/ml axitinib in methanol–water (1/3; v/v); diamonds (♦; ◊): 20 μg/ml axitinib in DMSO. Samples at the 20 μg/ml level were diluted to 1 μg/ml with methanol–water (1/1; v/v) prior to injection in the LC system reported in the caption of Fig. 2.

Initial chromatograms of axitinib directly showed two chromatographic peaks on a Vision HT C18-P column (30 × 2 mm, $d_p$ = 1.5 μm, Grace, Deerfield, IL, USA). Because further experiments showed almost identical product spectra for both peaks (Fig. 1B and C), the presence of two isomers was assumed. This observed isomerization of axitinib in solution showed to be light induced and was expected to be a *trans* to *cis* reaction. Such a process has already been shown and thoroughly investigated for the structural analogue 1-(pyridine-4-yl)-2-(N-methylpyrrol-2-yl)ethane (mepepy) [39]. For axitinib the LC–UV response at 330 nm was monitored during bench top storage in transparent polypropylene reaction tubes for 4.5 h in two different solvents, DMSO and water–methanol (3/1; v/v). Results are shown in Figs. 2 and 3. Fig. 3 shows equilibriums between both isomers after 3–4 h at different ratios: ca. 67% *cis* isomer in DMSO and ca. 40% in water–methanol (3/1; v/v). Isomerization is somewhat faster (less than a factor 2) at the lower concentration (1 vs. 20 ng/ml) in water–methanol (3/1; v/v) and the *cis* isomer shows less than half of the UV-absorbance compared to the original *trans* isomer of axitinib. A reduced UV absorbance for the *cis* isomer was also found for analogue mepepy [39]. The increased SRM response for the *cis* isomer in Fig. 2 was caused by selective suppression of *trans*-axitinib ionization by ammonium acetate, this effect could be shown by reducing the concentration of ammonium acetate in the eluent (Fig. 4). The chosen alternative, however, to obtain an optimal SRM response of *trans*-axitinib was replacing the ammonium acetate by formic acid because with this eluent both isomers eluted in reversed order on the Acquity column. In this reversed elution order the analytical run time was reduced by avoiding waiting for the peak of the *cis* isomer.

Light induced isomerization was also observed for the TKI sunitinib and two of its analogues (data not shown), as could be expected based on their structural resemblance with mepepy and axitinib and on some remarks about the light sensitivity of sunitinib in previous studies [27,40]. Haouala et al. [29] recently reported an assay for sunitinib and five other drugs of the same class without

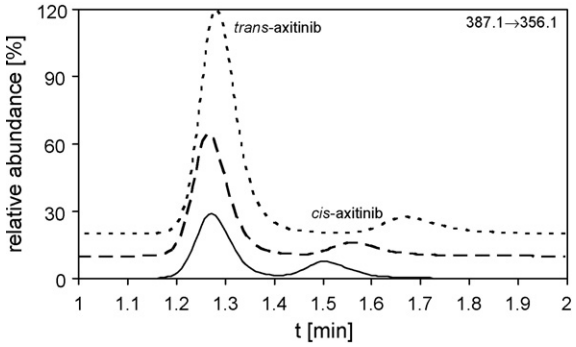


**Fig. 4.** SRM chromatograms (with an artificial response shift) of 25 ng/ml axitinib shortly exposed to light. Ten microliters were injected on a Vision HT C18-P column (30 × 2 mm, $d_p$ = 1.5 μm) with a corresponding 5 mm pre-column (both from Grace, Deerfield, IL, USA), both maintained at 40 °C. The eluent (0.5 ml/min) comprised 45% (v/v) water and 55% (v/v) methanol. Total ammonium acetate concentrations were 45 mM (straight line (——)), 10 mM (broken line (— — —) and 2.5 mM (dotted line (-------)), respectively. Mass spectrometric conditions were as reported in Section 2.

light protection. For quantification, the sum of the SRM responses of both separated sunitinib isomers was used, including the labeled internal standard. We did not consider this option for axitinib because we did not observe equal MS responses for both isomers.

### 3.2. Validation

SRM chromatograms of axitinib and the IS are depicted in Fig. 5, showing chromatograms of blank and LLQ-spiked samples.

#### 3.2.1. Calibration

A 0.2–200 ng/ml range was chosen to trigger expected levels in the range 1–100 ng/ml for the 5 mg BID regimen [10]. The relative response of axitinib deviated from a linear function with a small significance ($P$ = 0.04 for a 1-tailed normal distribution of the average double-logarithmic slope ($n$ = 6) compared to 1); therefore, the double-logarithmic function was used for the assay calibration. For all calibration samples (54 samples in 6 calibration curves), the concentrations were back-calculated from the ratio of the peak area (of analyte and IS) using the calibration curves of the run in which they were included; no deviations of the averages of each level higher than 4.2% were observed (Table 2), indicating the suitability of the regression model. The average regression parameters of the double-logarithmic regression functions ($n$ = 6) were $\log(y) = -1.27(\pm 0.13) + 0.963(\pm 0.017)\log(x)$ with a regression coefficient of 0.9993 ± 0.0005, the functions show reproducible calibration parameters.

#### 3.2.2. Precision and accuracy

Assay performance data from the validation samples at 4 concentrations are reported in Table 3. Between day variations and deviations of the accuracy lower than 9% were observed for all levels. Therefore, the upper limit of the calibration range could be assigned to the upper limit of quantification, and precisions and deviations of the accuracy met the required ±15% (±20% for the LLQ) [34–36].

**Table 2**
Back-calculated concentrations of axitinib from the calibration samples using a linear double-logarithmic calibration ($n$ = 6).

| Nominal Concentration [ng/ml] | Concentration found [ng/ml] | Precision [%] | Accuracy [%] |
|---|---|---|---|
| 0.2 | 0.201 | 7.3 | 100.5 |
| 0.5 | 0.502 | 5.9 | 100.4 |
| 2.5 | 2.44 | 6.9 | 97.4 |
| 10 | 10.42 | 8.1 | 104.2 |
| 50 | 48.6 | 8.3 | 97.1 |
| 200 | 202 | 8.3 | 101.0 |

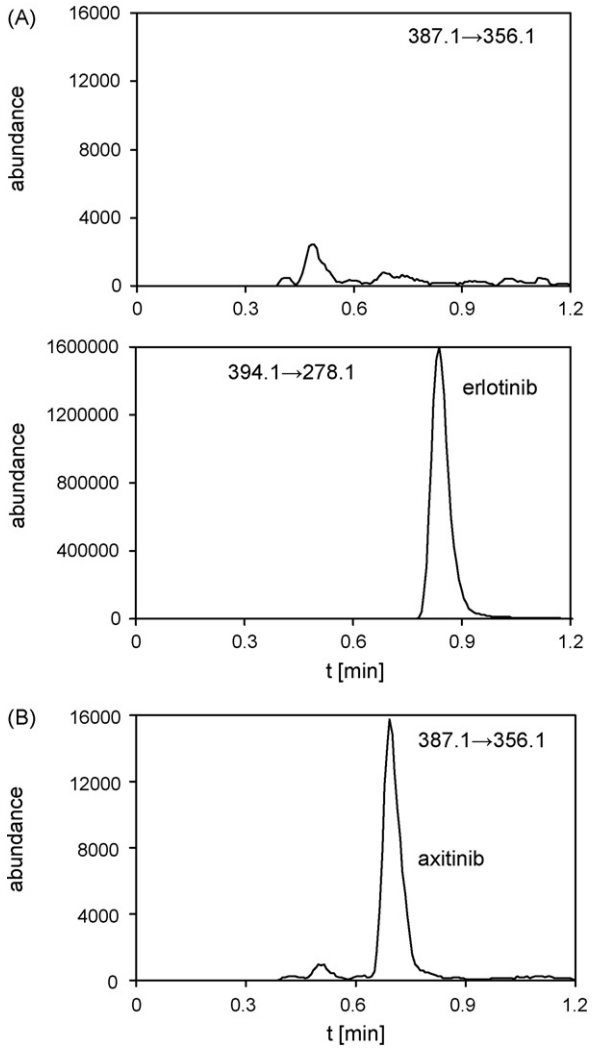


**Fig. 5.** SRM chromatograms of (A) blank plasma and (B) plasma spiked with 0.2 ng/ml axitinib.

#### 3.2.3. Selectivity

The analysis of six batches of blank samples showed no interfering peaks in the SRM traces for axitinib and erlotinib in human EDTA plasma. Blank responses could not be distinguished from the detector noise for both axitinib and IS, and were below 3% of the LLQ response of axitinib and below 0.2% of the regular signal of erlotinib. The average responses of the LLQ-spiked blank samples ($n$ = 6, ±S.D.) were 0.193 ± 0.007 ng/ml axitinib. None of the 20 potentially interfering compounds, spiked to five blank samples, showed an additional response in the SRM traces used. The average responses

**Table 3**
Assay performance data of axitinib resulting from 18 validation (QC) samples in 3 analytical runs.

| Nominal concentration [ng/ml] | Within day precision [%] | Between day precision [%] | Accuracy [%] |
|---|---|---|---|
| 150 | 2.8 | 3.8 | 106.4 |
| 10 | 2.5 | 3.9 | 96.9 |
| 0.5 | 4.8 | 8.8 | 96.2 |
| 0.2 | 6.1 | 7.4 | 91.1 |

**Table 4**
Extraction and ionization efficiency (recovery [%], ±S.D., $n = 4$) of the assay for axitinib and erlotinib (IS).

| Concentration [ng/ml] | Extraction | Ionization |
|---|---|---|
| 150 | 83.0 ± 2.9 | 110.0 ± 4.8 |
| 10 | 83.9 ± 5.3 | 116.3 ± 5.9 |
| 0.5 | 93.4 ± 3.7 | 108.9 ± 11.4 |
| IS | 100.4 ± 3.7 | 97.0 ± 4.9 |

**Table 5**
Stability data (recovery [%]; ±S.D.; $n = 4$) of axitinib in human EDTA plasma, reporting the percentage of the initial concentration.

| Condition | QC-high | QC-low |
|---|---|---|
| 24 h at ambient temperature | 97.0 ± 6.4 | 92.3 ± 5.2 |
| 3 freeze-thaw cycles | 95.5 ± 8.0 | 94.6 ± 8.6 |
| 2 months at −30 °C | 103.3 ± 2.6 | 98.6 ± 3.2 |

of the LLQ-spiked blank samples each containing four additional compounds ($n = 5$, ±S.D.) were 0.175 ± 0.015 ng/ml axitinib. Therefore, with relative standard deviations in these experiments below 20%, the LLQ of the assay is 0.2 ng/ml axitinib in human plasma [34–36].

### 3.2.4. Recovery

The recovery experiments showed only small extraction losses (<20%) for axitinib and no loss of erlotinib (Table 4). Ion suppression was not observed for both compounds and only a minor enhancement may be present (Table 4). Therefore, the validation was also successful on this point [34–36].

### 3.2.5. Stability

Recoveries of axitinib in plasma after different storage procedures are shown in Table 5. The recoveries in the range of 92–103% clearly indicate the absence of significant degradation in the human plasma under all studied storage conditions. A slight increase could be observed for *cis*-axitinib during storage of QC-high plasma samples at both 24 h at ambient temperature and 2 months at −30 °C; however, its response remained below 0.1% of the original *trans*-axitinib response (Fig. 6).

Re-injection of calibration and validation samples after additional storage at 4 °C for 2 weeks resulted again in successful performances and does therefore ensure the opportunity to store the diluted extracts long enough before final analysis.

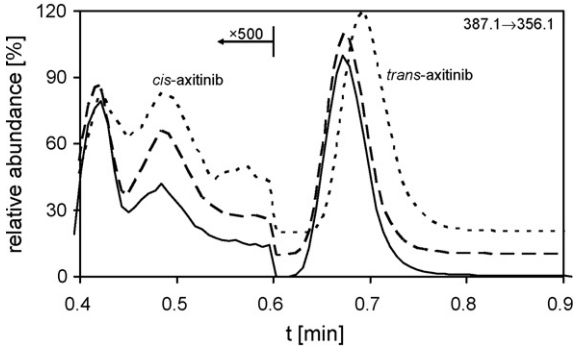


**Fig. 6.** SRM chromatograms (with an artificial response shift) of the initial QC-high sample (straight line (——)) and after storage for 24 h at ambient temperature (broken line (— — —)) or for 2 months at −30 °C (dotted line (- - - - - - -)). The response is magnified a factor 500 until 0.6 min.

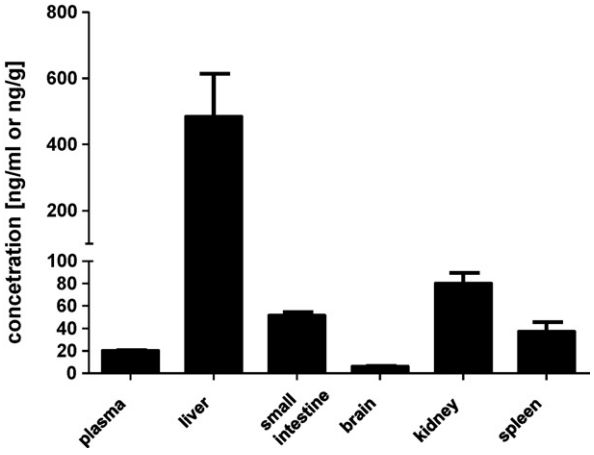


**Fig. 7.** Mouse tissue and plasma distribution 30 min after i.v. administration of 2 mg/kg axitinib to wild-type mice ($n = 4$). Concentrations of axitinib are represented as ng/ml for the plasma samples and as ng/g for the tissue samples.

### 3.3. Mouse samples

Unfortunately, to show the applicability of the new assay after the successful validation procedure, plasma samples from human pharmacokinetic studies were not yet available. Alternatively, the tissue distribution of axitinib 30 min after i.v. administration in wild-type mice is reported in this paper. Results of the animal experiments are shown in Fig. 7. Axitinib showed to be distributed mostly to the liver, and to a lesser extent to small intestine, kidney, spleen and brain. Injection volume and dilution factors of tissue homogenates were chosen after performing post-column infusion experiments with a 5 μl/min flow of 0.5 μg/ml axitinib and 2 μM erlotinib in water–methanol (1:1, v/v). Reduction of the injection volume to 1 μl still resulted in successful processing of calibration and QC samples and also resulted in the absence of significant ion suppression for the tissue samples sufficiently diluted with human EDTA plasma (data not shown).

## 4. Conclusions

The first validated assay for axitinib has now been reported completely for human plasma samples. The LC/MS/MS assay uses a simple sample pre-treatment method and meets commonly accepted criteria for precision, accuracy, recovery and stability [34–36]. The sensitivity of the method for axitinib is better compared to bioanalytical assays for other TKI drugs using the same pre-treatment method [12,14,16,18–20,23–25,29–32] by at least a factor 5. The new assay will be a valuable tool for clinical studies in the future.

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