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Liquid chromatography–tandem mass spectrometric assay for the tyrosine kinase inhibitor afatinib in mouse plasma using salting-out liquid–liquid extraction

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ABSTRACT

A quantitative bioanalytical liquid chromatography–tandem mass spectrometric (LC–MS/MS) assay for afatinib, an irreversible inhibitor of the ErbB (erythroblastic leukemia viral oncogene homolog) tyrosine kinase family, was developed and validated. Plasma samples were pre-treated using salting-out assisted liquid–liquid extraction (SALLE) with acetonitrile, magnesium chloride and a stable isotopically labeled internal standard. After dilution, the extract was directly injected into the reversed-phase liquid chromatographic system. The eluate was transferred into the electrospray interface with positive ionization and compounds were detected in the selected reaction monitoring mode of a triple quadrupole mass spectrometer.

The assay was completely validated for plasma in a 0.5–500 ng/ml calibration range with $r^2 = 0.995 \pm 0.002$ ($n = 6$) using linear regression with the inverse square of the concentration as the weighting factor for the calibration. Within-run precisions ($n = 18$) were 2.7–11.7% and between-run (3 runs; $n = 18$) precisions 3.0–14.5%. Accuracies were between 96–109% for the whole calibration range. The drug was sufficiently stable under all relevant analytical conditions. Finally, the assay was successfully applied to determine plasma drug levels and study pharmacokinetics after oral administration of afatinib to female FVB mice.

1. Introduction

Afatinib (BIBW 2992, Gilotrif®; Fig. 1) is an orally administered Tyrosine Kinase Inhibitor (TKI) for the treatment of patients with distinct types of metastatic non-small cell lung carcinoma (NSCLC). The U. S. Food and Drug Administration (FDA) granted approval in July 2013 for the first-line treatment of patients with metastatic NSCLC whose tumors have epidermal growth factor receptor (EGFR) exon 19 deletions or exon 21 (L858R) substitution mutations [1]. The European Committee for Medicinal Products for Human Use (CHMP) issued a positive opinion in July 2013 for granting a marketing authorization [2]. Afatinib is a highly selective, irreversible inhibitor of EGFR and human epidermal growth-factor receptor (HER)-2 [3]. EGFR and HER-2 are members of the receptor tyrosine kinase (RTK) superfamily [4] and gene amplification leading to overexpression of these receptors is associated with higher EGFR pathway signaling activity, increased proliferation of cancer cells and reduced apoptosis [5]. The overexpression of EGFR and HER-2 is often found in human cancers such as gliomas, carcinomas of the breast, lung, ovaries, bladder and NSCLC. It has recently been shown that afatinib is effective for a subset of these cancers expressing an overexpression of EGFR and HER-2 [6–8] making it an attractive candidate for further clinical research.

Abbreviations: AUC, area under the plasma concentration–time curve; CHMP, Committee for Medicinal Products for Human Use; EGFR, epidermal growth factor receptor; FDA, Food and Drug Administration; FVB, Friend Leukemia Virus; HER, human epidermal growth-factor receptor; NSCLC, non-small cell lung cancer; SALLE, salting-out assisted liquid–liquid extraction; TKI, tyrosine kinase inhibitor.

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Afatinib is an inhibitor and substrate for both, the *P*-glycoprotein (*P*-gp; ABCB1) and Breast Cancer Resistance Protein (BCRP; ABCG2) drug transporters based on *in vitro* data [1]. The drug is eliminated slowly ($t_{1/2}$ = 23 h after the first dose in human) and the main elimination route is in feces as parent drug [2]. Because no prominent systemic metabolites are formed from afatinib, monitoring only the parent drug in pharmacokinetic studies should be sufficient.

Recently, two bioanalytical methods for afatinib in human plasma were reported using Liquid Chromatography with Diode Array detection [9,10]. Unfortunately, the method of Xiang et al [10] shows poor sensitivity (600 ng/ml). The range (5–250 ng/ml) investigated by Fouad et al. [9] however, is appropriate for therapeutic drug monitoring, but this assay using micro solid-phase extraction pretreatment may have limited selectivity due to the UV detection. For enhanced sensitivity and selectivity the use of SRM detection with tandem mass spectrometry is preferred as previously applied for afatinib in phase I [11–13], phase II [14] and metabolic [15] studies. Unfortunately, details of this assay were, other than the use of solid-phase extraction, electrospray ionization and a deuterated analogue as internal standard (IS) [15], never reported. Therefore, we developed and validated a new sensitive LC–MS/MS assay for small volumes of mouse plasma (10 μl), using a simple extraction method as an indispensable tool for pharmacokinetic and drug transporter studies in mice.

2. Experimental

2.1. Chemicals

Afatinib (>99%) and $^{13}C_6$-afatinib (>99%; ^{13}C-enrichment 99.5%) were obtained from Alsachim (Strasbourg, France). Water (LC–MS grade), methanol (HPLC grade) and acetonitrile (HPLC-S grade) were obtained from Biosolve (Valkenswaard, The Netherlands). Water, not used as eluent, was home purified by reversed osmosis on a multi-laboratory scale. Formic acid and ammonium acetate were of analytical grade originating from Merck (Darmstadt, Germany). Other chemicals were of analytical grade and were obtained from Sigma-Aldrich (Steinheim, Germany). Female CD-1 (cluster of differentiation 1) mouse lithium-heparin plasma, pooled and from individual animals, was supplied by Seralab Laboratories (Haywards Heath, West Sussex, UK).

2.2. Equipment

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

2.3. LC–MS/MS conditions

10 μl partial loop injections were made on an Aquity UPLC® BEH C18 column (30 × 2.1 mm, d_p = 1.7 μm, Waters, Milford, USA) with an Aquity UPLC® BEH C18 VanGuard pre-column (Waters, 5 × 2.1 mm, d_p = 1.7 μm). The column temperature was maintained at 40 °C and the auto-sampler at 4 °C. Gradient elution was used at 0.6 ml/min with a gradient of solvent A containing 0.1% (v/v) ammonium hydroxide in water and solvent B being acetonitrile. After injection, the percentage of solvent B was increased linearly from 35 to 50% (v/v) during 1 min. Next, the column was flushed with 100% (v/v) B for 0.25 min and finally, the column was reconditioned at the starting conditions (35% (v/v) B) for 0.75 min until starting the next injection. The whole eluate was transferred into the electrospray probe, starting at 0.6 min after injection by switching the MS divert valve until 1.3 min after injection. The electrospray was tuned in the positive ionization mode by introducing 0.6 ml/min of a solvent mixture containing 50% (v/v) of 0.1% (v/v) formic acid and 50% (v/v) acetonitrile and 5 μl/min of 10 μg/ml of afatinib. A 3000 V spray voltage, 380 °C capillary and 316 °C vaporizer temperatures and nitrogen sheath, ion sweep and auxiliary gasses set at 60, 8 and 55 arbitrary units, respectively, were used for the positive electrospray ionization. The skimmer voltage was −6 V. The SRM mode was used with argon as the collision gas at 1.5 mTorr. The mass resolutions were set at 0.7 full width at half height (unit resolution) for

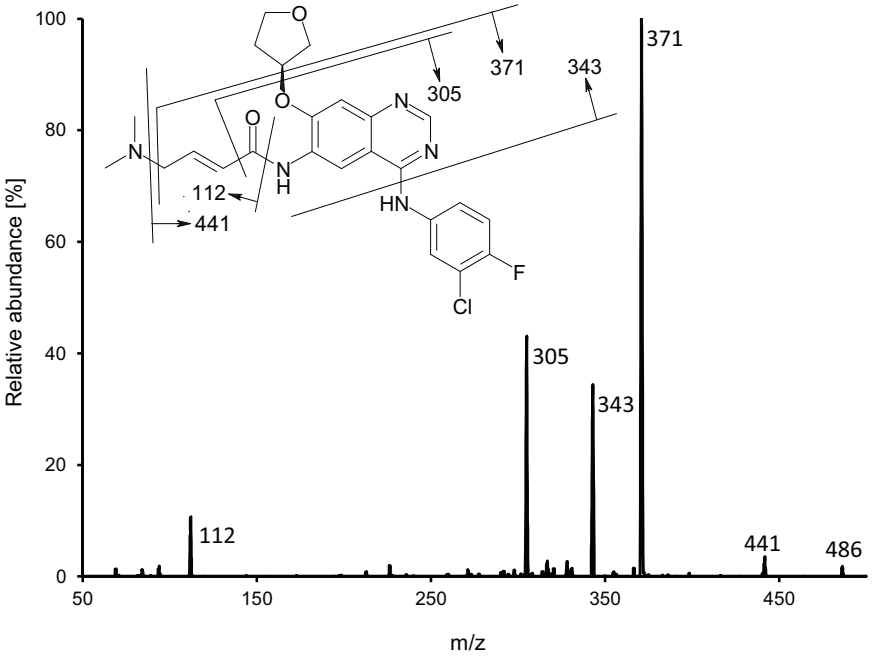

Fig. 1. Chemical structure and product spectrum, formed by collision-induced dissociation of the protonated molecule of afatinib; *m/z* 486.1@-30 V. Dissociation pathways of the reactions have been proposed.

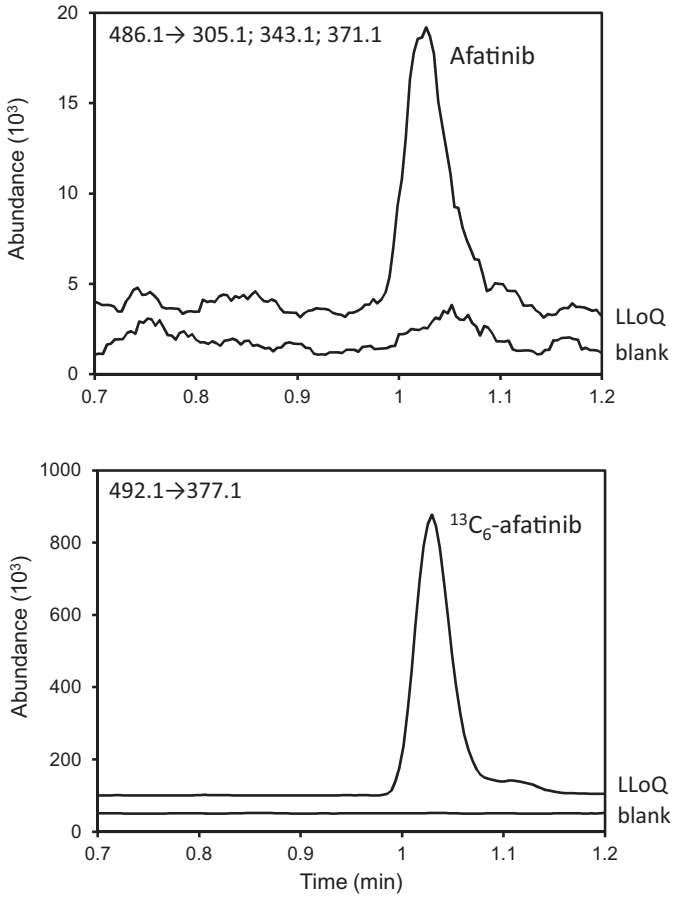

Fig. 2. SRM chromatograms of afatinib and the IS in plasma extracts: blank and LLoQ (0.5 ng/ml) spiked plasma. An artificial off set was given to all traces.

both separating quadrupoles and all dwell times at 0.05 s, the tube lens off set was 108 V for all transitions. Afatinib was monitored at m/z 486.1 → 305.1; 343.1; 371.1 at −36, −37 and −29 V collision energies, respectively, and $^{13}C_6$-afatinib at m/z 492.1 → 377.1 at −29 V.

2.4. Sample pre-treatment

To a 10 μl plasma sample (female mouse; lithium heparin), pipetted into a polypropylene micro-tube (0.5 ml), 5 μl of 3 M magnesium chloride in water and 25 μl of 40 ng/ml $^{13}C_6$-afatinib (IS; internal standard) in acetonitrile were added. After vortex mixing vigorously for *ca.* 20 s, the liquid phases were separated by centrifuging at 10,000 × *g* for 2 min at 15 °C. Twenty μl of the clear supernatant were transferred into a glass injection vial and supplemented with 150 μl 25% (v/v) methanol, followed by vortex mixing the closed vial shortly.

2.5. Validation

A laboratory scheme based on international guidelines was used for the validation procedures [16,17].

2.5.1. Calibration

A stock solution of 1 mg/ml afatinib was prepared in methanol and a $^{13}C_6$-afatinib stock solution was prepared at 0.2 mg/ml in methanol. Stock solutions were stored in 1.5-ml polypropylene tubes at −30 °C. The afatinib stock solution was diluted to a 20 μg/ml working solution in 50% (v/v) methanol and further diluted to the highest 500 ng/ml calibration sample in pooled female lithium heparin mouse plasma, stored in polypropylene micro-tubes at −30 °C. Additional calibration samples were prepared daily at 250, 125, 25, 12.5, 2.5, 1.25 and 0.5 ng/ml by dilution of the 500 ng/ml calibration solution with blank mouse plasma. All calibration samples were processed in duplicate for each daily calibration. Least-squares linear regression was employed to define the calibration curve using the ratios of the peak area of the analyte and the IS. The reversed square of the concentration ($1/x^2$) served as the weighting factor.

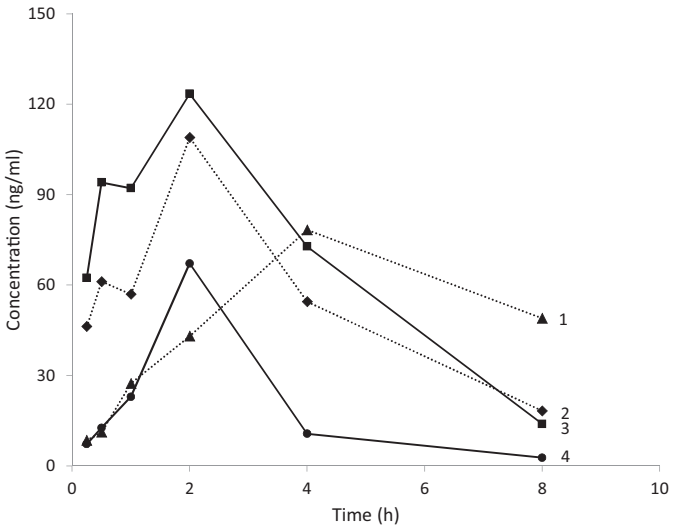

Fig. 3. Pharmacokinetic plot of afatinib in mouse (n = 4) plasma after oral administration of a single dose of 10 mg/kg afatinib.

2.5.2. Precision and accuracy

A second stock solution of afatinib at 0.5 mg/ml was used to prepare a 10 μg/ml working solution in 50% (v/v) methanol that was further diluted to obtain validation (quality control; QC) samples in pooled mouse female lithium heparin mouse plasma at 400 (QC-high), 20 (QC-med), 1.5 (QC-low) and 0.5 ng/ml (QC-LLoQ). Samples were stored in 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 per QC). Relative standard deviations were calculated for both the within- and between-run precisions.

2.5.3. Selectivity

Six individual mouse plasma samples were processed to test the selectivity of the assay. Samples were processed without afatinib and without IS (double blank), without afatinib (blank) and with afatinib at the LLoQ level (0.5 ng/ml), supplemented with the IS.

2.5.4. Recovery and matrix effect

The extraction recovery was determined (n = 4) by comparing processed plasma samples (QC-high, -med, -low; the same samples as used for precision and accuracy) with reference afatinib solutions in blank pooled plasma extract added at the same levels.

The matrix effect was assessed using a post column infusion experiment. A mixture of 200 ng/ml afatinib in 50% (v/v) methanol at 5 μl/min was mixed post-column with the eluent while blank extracted samples were injected without using the divert valve. Six individual blank extracts were injected and responses at the afatinib retention time (1.0 min) were compared to blank injections of 50% (v/v) methanol. Further, inter-lot (n = 6) relative matrix effects in plasma were assessed at the QC-low level by processing each sample and by comparing the relative peak area of drug added to blank extract with the relative peak area of drug in solvent.

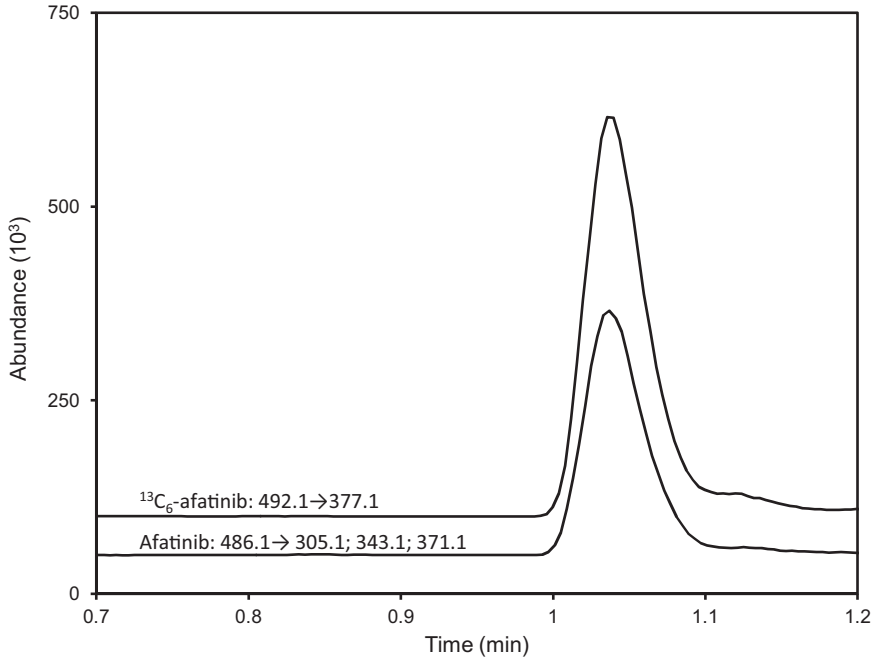

Fig. 4. SRM chromatogram of afatinib (27.4 ng/ml) and the IS in a plasma extract. The sample was taken 1 h after oral administration of 10 mg/kg afatinib to mouse 4. An artificial off set was given to both traces.

2.5.5. Stability

The stability of afatinib was investigated in QC-high and -low plasma samples stored in polypropylene tubes. Quadruplicate analysis of these samples, stored in separate tubes, was performed after storage at 20 °C (ambient temperature) for 24 h, three additional freeze–thaw cycles (thawing at 20 °C during *ca.* 2 h and freezing again at −30 °C for at least one day), and storage at −30 °C for 2 months, respectively. Furthermore, an analytical run was re-injected after additional storage of the extracts at 4 °C for four and seven nights, respectively, to test the stability at these conditions in the auto-injector.

Finally, the responses of afatinib from the stock solutions in methanol after 6 h at 20 °C ($n = 2$) and after 2 months at −30 °C ($n = 3$) were compared to fresh stock solutions with LC–MS/MS after appropriate dilution of the samples and adding IS. In addition, the working solutions in 50% (v/v) methanol were compared to fresh solutions after 2 months storage at −30 °C ($n = 3$).

2.6. Mouse samples

Wild-type (FVB (Friend Leukemia Virus, strain B) genetic background) female mice ($n = 4$) were housed and handled as reported previously [18] and were treated with 10 mg/kg afatinib orally. The 1 mg/ml afatinib solution was obtained by dissolving the drug in 50% polysorbate 80 and 50% ethanol (1:1, v/v) to a concentration of 40 mg/ml, followed by 40-fold dilution in 5% (v/v) glucose in water to obtain the solution for administration. Blood samples (≤50 μl) were collected in lithium heparin-containing microvettes via the tail vein at 0.25, 0.5, 1, 2, and 4 h after oral administration of the drug. After 8 h mice were anesthetized with isoflurane and blood was drawn by cardiac puncture. After centrifugation at 8000 × g for 5 min at 4 °C, plasma samples were stored at −30 °C prior to analysis.

Pharmacokinetic parameters were calculated; area under the plasma concentration-time curve ($AUC_{0\text{-}8}$) using the trapezoidal rule for the 8 h of the experiment and terminal half-life ($t_{1/2}$) using the two latest time points.

Incurred study samples with sufficient sample volume (18 out of 24) were reanalyzed 1.5 months after the first analysis.

3. Results and discussion

3.1. Method development

ESI-MS/MS settings were optimized for afatinib to obtain maximal sensitivity; a product spectrum of afatinib is presented in Fig. 1. Ammonium hydroxide showed higher responses than formic acid and acetonitrile resulted in narrower peaks than methanol when used in the eluent.

A standard short universal reversed-phase column was used suited for fast and high pH applications because of its trifunctional bonding and with small particles for efficient separations. The use of gradient elution showed a slight improvement of peak shape and precisions of retention time and detector responses. Strongly retained plasma constituents like phospholipids were removed from the column using a high organic flush at the end of each analytical run in order to prevent long term suppression effects of the ionization.

Sample pretreatment was initially tested using protein precipitation with acetonitrile but resulted in ion suppression that may

Table 1
Assay performance data of afatinib resulting from four validation (QC, $n = 18$ each) samples in 3 analytical runs.

Nominal concentration (ng/ml)	Within day precision [%]	Between day precision [%]	Accuracy [%]
400	3.9	4.6	105.6
20	2.7	3.0	108.9
1.5	9.7	10.1	108.0
0.5	11.7	14.5	95.6

Table 2
Stability data (recovery [%]; ±S.D.; $n = 4$) of afatinib in murine lithium heparin plasma, reporting the percentage of the initial concentration.

Condition	QC-high	QC-low
24 h at ambient temperature	93.4 ± 1.0	92.6 ± 4.5
3 freeze–thaw cycles	98.2 ± 3.6	99.7 ± 5.3
2 months at −30 °C	95.0 ± 4.9	97.3 ± 4.7

affect accuracy and sensitivity of the assay. A more selective but also simple alternative is salting-out assisted liquid–liquid extraction (SALLE), a method gaining interest in recent years [19,20] for the application in bioanalytical LC–MS/MS assays. So far, acetonitrile has almost exclusively been used as extraction liquid for these bioanalytical applications. Sodium chloride, magnesium sulfate, ammonium acetate and magnesium chloride were tested as salts at 1 M calculated on total water volume, to obtain phase separation. Magnesium was chosen for the highest recovery and precision obtained for afatinib; further, a small matrix effect interfering with afatinib detection was observed using ammonium acetate. A stable isotopically labeled internal standard of afatinib, with six 13-carbon atoms being present in the chloro-fluoro-phenyl ring, could be obtained for optimal correction of the variation in extraction recovery and ionization.

3.2. Validation

Because maximum clinical drug levels reported were *ca.* 200 ng/ml [11] and a range with a magnitude of 1000 was expected to be a suited and achievable range, the 0.5–500 ng/ml range was chosen for validation, SRM chromatograms of blank and LLoQ spiked samples are depicted in Fig. 2.

3.2.1. Calibration

The relative response of afatinib showed linearity in the whole investigated range. For 6 calibrations the concentrations were back-calculated from the ratio of the peak areas (analyte and IS), using the calibration curves of the run in which they were included. No deviations from the average of each level higher than 3.4% were observed (data not shown), indicating an good suitability of the linear regression model [16,17]. The average of the reproducible regression parameters of the linear regression functions ($n = 6$) were $y = 0.0007\ (\pm 0.0019) + 0.0210\ (\pm 0.0006)\ x$ with a regression coefficient of 0.995 ± 0.002. Here, x is the afatinib concentration (ng/ml) and y is the drug response relative to the IS.

3.2.2. Precision and accuracy

Assay performance data of the validation samples at four concentrations are reported in Table 1. Within-run and between-run variations lower than 15% were observed and deviations of the accuracies were lower than 9%. The precision and accuracy therefore met the required ±15% variation (±20% for the LLOQ) [16,17].

3.2.3. Selectivity

The analysis of six independent blank mouse plasma samples showed no interfering peaks in the SRM traces for afatinib; the double blank samples showed also no interference of the labeled IS. Blank afatinib responses at the signal noise level were $10 \pm 7\%$ ($n = 6$) of the LLoQ response, meeting the required 20% [17], and double blank IS responses $0.11 \pm 0.05\%$ of the normal response. The signals at the LLoQ level (0.5 ng/ml; $n = 6$) corresponded to 0.45 ± 0.08 ng/ml afatinib, demonstrating the applicability of the investigated LLoQ level [16]. The selectivity is superior compared to existing LC–UV methods as expected [9,10].

Table 3
Pharmacokinetic data of 4 mice after oral administration of 10 mg/kg afatinib.

	1	2	3	4	Average ± S.D.
AUC_{0-8} (ng min ml^{-1})	552	441	425	162	395 ± 165
c_{max} (ng/ml)	123.5	109.0	78.4	67.2	94.5 ± 26.2
t_{max} (h)	2	2	2	4	
$t_{1/2}$ (h)	1.7	2.5	5.9	2.0	3.0 ± 1.9

3.2.4. Recovery and matrix effect

Extraction recoveries showed no losses for afatinib and ranged from 101 to 104% (data not shown) in plasma. Using the post-column infusion experiment, no matrix effects ($n = 6$, inter-lot) could be observed in the 0.5–1.3 min time window. The inter-lot ($n = 6$) relative matrix factor at the QC-low level was $101.8 \pm 6.8\%$. Overall, the absence of significant extraction losses and matrix effects contributed to a successful validation of the assay with a relative matrix effect variability <15% [17].

3.2.5. Stability

The stability of afatinib in female mouse lithium heparin plasma after different storage procedures is presented in Table 2. The drug showed sufficient stability under all conditions. Re-injection of extracted plasma calibration and QC samples after additional storage at 4 °C for one and two weeks, respectively, resulted again in successful performances without any loss of precision and accuracy. The number of QC—failures out of 24 was 4 or 5 in all runs, thus QC failures remained below a 33% frequency as required [16,17] during one week.

Recoveries of afatinib in stock and working solutions were all within ±5%: 100.8% (stocks after 6 h at 20 °C; $n = 2$), 104.3% (stocks after 2 months at −30 °C; $n = 3$) and 98.8% (working solutions after 2 months at −30 °C; $n = 3$), respectively.

3.3. Mouse study

After a successful validation procedure, the new assay was used to investigate afatinib plasma levels in a pilot study during 8 h after administration of a single oral dose of 10 mg/kg to FVB mice ($n = 4$). Levels in the range 1.8–124 ng/ml were assayed and are shown in separate pharmacokinetic plots (Fig. 3). The lower levels could not have been assessed using one of the existing LC–UV methods [9,10]. For one of the samples the SRM chromatogram is shown in Fig. 4. Pharmacokinetics (Table 3) showed variable results ($AUC_{0-8} = 395 \pm 165$ ng min ml^{-1}) with mouse 4 showing a low AUC_{0-8} (162 ng min ml^{-1}). Mouse 1 showed slow absorption ($t_{max} = 4$ h compared to 2 h for the other mice) prohibiting proper calculation of the $t_{1/2}$ for this animal. For the other animals $t_{1/2} = 2.1 \pm 0.4$ h ($n = 3$), which is fast compared to human. A longer evaluation time is therefore advised for future pharmacokinetic experiments with this mouse strain.

Incurred sample reanalysis 1.5 months later than initial analysis ($n = 18$) resulted in a response of $89.4 \pm 10.2\%$ compared to the initial response with 3 samples exceeding ±20% where 5 is allowed [17].

4. Conclusions

The current study reports the first sensitive fully validated assay for the quantification of afatinib in plasma. The LC–MS/MS assay includes a fast and simple sample pre-treatment procedure, SALLE, applicable to small mouse plasma samples (10 μl). Results showed values of accuracy, precision, recovery and stability compliant to international guidelines [16,17]. The new assay was successfully used for the analysis of plasma afatinib levels after administration of a single oral dose of 10 mg/kg to FVB mice.

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