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Bioanalysis of erlotinib, its O-demethylated metabolites OSI-413 and OSI-420, and other metabolites by liquid chromatography-tandem mass spectrometry with additional ion mobility identification

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ABSTRACT

Erlotinib is a first-generation epithelial growth factor receptor inhibitor used in the treatment of non-small cellular lung cancers. Our previously published method on a Thermo TSQ Quantum Ultra triple quadrupole mass spectrometer for the quantitation of erlotinib, OSI-420, and OSI-413 and some other kinase inhibitors was transferred to a more sensitive Sciex QTRAP5500 system. Both methods showed comparable performance in the previous range (5–5000 and 1–1000 ng/mL for erlotinib and OSI-420) with comparable accuracies and precisions (98.9–106.2 *vs* 98.7.0–104.0, and 3.7–13.4 *vs* 4.6–13.2), and a high level of agreement between the methods ($R^2 = 0.9984$ and 0.9951) for the quality control samples. The new system however was also capable of quantifying lower concentrations of both erlotinib and OSI-420 (0.5 and 0.1 ng/mL) with sufficient accuracy and precision. Along with the increased sensitivity we included the semi-quantitative determination of additional erlotinib metabolites M2, M3, M5, M6, M7, M8, M9, M10, M11, M12, M16 (hydroxy-erlotinib), M17, M18, M19, M20, M21 in a 0.1–1000 ng/mL range to the method. With a simple crash, dilute, and shoot sample preparation with acetonitrile and a 4.5 min analytical run time the method outperformed most other published methods in speed and simplicity and was suitable for TDM. Further, enhancement of the understanding of the pharmacokinetics of erlotinib and its metabolites was demonstrated.

1. Introduction

Erlotinib is an epidermal growth factor receptor (EGFR) inhibitor used for treatment of patients with locally advanced or metastatic non-small cell lung cancer, either with activating mutant EGFR, or without in case of progression after chemotherapy. The recommended dose is 150 mg once daily, but regimens are adjusted for inter-individual variation caused by genetic factors, such as variability in metabolizing enzymes and other factors such as drug-drug or drug-food interactions and smoking, making it a suitable candidate for therapeutic drug monitoring (TDM) [1].

Several quantitative bioanalytical liquid chromatography-tandem mass spectrometric (LC-MS/MS) assays for tyrosine kinase inhibitor erlotinib and both isomeric *O*-desmethyl metabolites of erlotinib, OSI-

Abbreviations: CCS, collisional cross section; CI, Confidence interval; CYP, Cytochrome P450; DMSO, Dimethyl sulfoxide; DT, drift tube; EGFR, Epidermal Growth Factor Receptor; IM, ion mobility; ISTD, internal standard; LC-MS/MS, Liquid Chromatography-tandem mass spectrometry; PK, Pharmacokinetic(s); (R)SD, (Relative) standard deviation; TDM, therapeutic drug monitoring; UHPLC, Ultra-High-Performance Liquid Chromatography.

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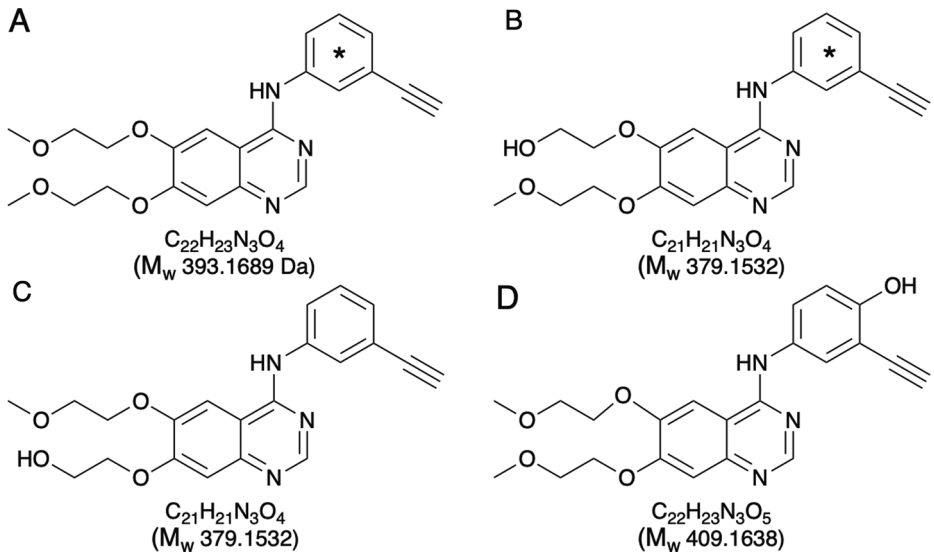

Fig. 1. Structures of A) erlotinib, isomeric desmethyl metabolites B) OSI-420 and C) OSI-413, and D) hydroxy-erlotinib metabolite M16. The location of the $^{13}C_6$-label is denoted with an asterisk (*).

413 and OSI-420, have been developed and validated [2–4]. These assays usually used one of the isomers as a reference, and semi-quantitatively determined other isomers on the calibration of one of the desmethyl metabolites (OSI-420). Several other, mostly older, methods are, however, not capable to distinguish between both isomeric *O*-desmethyl metabolites of erlotinib, OSI420 and OSI413 (Fig. 1) [5–10]. A reliable distinction between both major metabolites may be relevant in the study of averse drug effects of erlotinib. Next to the desmethyl isomers, erlotinib is metabolized extensively to a number of other, mostly less abundant metabolites, listed in Table 1. This metabolism occurs mainly by Cytochrome P450 (CYP) 3A4 and, to a lesser extent, by CYP1A2 and its extrahepatic isoform CYP1A1 [11]. Secondary metabolism through glutathione conjugation also occurs [12], as well as acetylation of desmethyl-erlotinib [9,10]. The desmethyl metabolites OSI-420 and OSI-413 (Fig. 1) are not distinguishable by conventional methods, such as (high-resolution) mass spectrometry or NMR without reference materials. Therefore, to confirm the identity of OSI-420 and OSI-413, a second method, after LC-MS/MS, to distinguish and possibly identify both structures was sought. Drift tube ion mobility (DTIM) mass spectrometry collisional cross section (CCS) measurements were evaluated for such purpose. An interlaboratory study showed low RSDs and biases, potentiating DTIM-MS generated CCS values as a molecular identifier for molecules with a high degree of similarity [13].

Our previous method used a Thermo TSQ Quantum Ultra, equipped with a low-pressure quaternary gradient UHPLC [2]. OSI-420 calibration was used for the quantification of OSI-413 as well. We wanted to transfer this method to a new, more sensitive, Sciex QTRAP5500 system, with a binary high-pressure gradient UHPLC separation and demonstrate the universality of the previously validated method, as well as making use of the gain in sensitivity to semi-quantitatively determine the plasma levels of several other, low-abundant, metabolites, namely M2, M3, M5, M6, M7, M8, M9, M10, M11, M12 (didesmethyl-erlotinib), M16 (hydroxy-erlotinib), M17, M18, M19, M20, M21. Acetyl-desmethyl-erlotinib was only found in urine so far [9,10] and was not included in our assay for plasma samples. Absolute quantification and subsequent validation of all metabolites was (economically) not feasible. Only a few were commercially available, often at high costs for just one milligram. A work-around for this is the semi-quantitative determination by calibration using another (similar) compound. The commercially available didesmethyl-erlotinib and hydroxy-erlotinib were added as reference compounds to evaluate the validity of this approach. It was considered what changes to the reference method could impact the absolute response of the analyte or analytes of interest [14]. As this is difficult to assess due to some fundamental changes in instrument configuration, accuracy, precision, matrix effect and selectivity experiments were performed as a cross validation.

2. Materials and methods

2.1. Chemicals

Erlotinib (as hydrochloric acid) was supplied by Sequoia Research Products (supplier 1, Pangbourne, UK). OSI-420 (as hydrochloric acid, 95.9%), $^{13}C_6$-erlotinib (as hydrochloric acid, >98%, >99% ^{13}C), and $^{13}C_6$-OSI-420 (as hydrochloric acid, >98.0%, >99% ^{13}C), were obtained from Alsachim (supplier 2, Strasbourg, France). OSI-420 was also purchased from SelleckChem (supplier 3, Munich, Germany) as well for confirmation of the structure. OSI-413 was purchased from TLC Pharmaceutical Standards (supplier 4, Ontario, Canada). 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 was of analytical grade originating from Merck (Darmstadt, Germany) and ammonium hydroxide (A.C.S. Reagent) from Sigma-Aldrich (Steinheim, Germany). Pooled human lithium-heparin plasma was supplied by Seralab Laboratories (Haywards Heath, West Sussex, UK), individual drug-free human plasma and pooled whole blood were obtained from Innovative Research Inc. (Novi, MI, USA). Pooled human liver microsomes were purchased from Corning Gentest (Tewksbury, MA, USA). All other chemicals were purchased from either Sigma Aldrich (St. Louis, MO) or Merck (Darmstadt, Germany).

2.2. LC-MS/MS equipment

System 1 (reference) consisted, as reported previously [2], of an Accela quaternary low-pressure gradient ultra-high-performance liquid chromatography (UHPLC) pump and auto-sampler and a TSQ Quantum Ultra triple 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). UHPLC-MS/MS system 2 (comparator) consisted of a Sciex QTRAP® 5500 triple quadrupole mass spectrometer (Sciex, Ontario, Canada), and a binary high-pressure gradient UHPLC

Table 1
Erlotinib and its metabolites in humans, and their exact (mono-isotopic) masses, mass-shifts, and single protonated *m/z* values [2,4].

Compound	Isomer	Reaction	Exact mass (Da)	Mass-shift (Da)	*m/z* [M+H]+
Erlotinib		Parent compound	393.1689	–	394.2
OSI-420	OSI-413	Desmethyl isomer (M14)	379.1532	−14.0157	380.2
OSI-413	OSI-420	Desmethyl isomer (M13)	379.1532	−14.0157	380.2
M2	*	Acetylene oxidation to carboxylic acid + demethylation	413.1587	+19.9898	414.2
M3	M20	Ring-oxidation + Glucuronidation (M16-glucuronide)	585.1959	+192.0270	586.2
M5	*	De-(methoxyethyl) ation	335.1270	−58.0419	336.1
M6		Phenylacetylene oxidation to carboxylic acid	427.1743	+34.0054	428.2
M7		Phenylacetylene oxidation to alcohol + ring-oxidation	429.1900	+36.0211	430.2
M8	M10	Demethylation + glucuronidation (M13-glucuronide)	555.1853	+162.0164	556.2
M9		Ring-oxidation + sulphate (M16-sulphate)	489.1206	+95.9517	490.1
M10	M8	Demethylation + glucuronidation (M14-glucuronide)	555.1853	+162.0164	556.2
M11	*	Methoxyethyl oxidation to carboxylic acid	393.1325	−0.0364	394.1
M12		Di-demethylation	365.1376	−28.0313	366.1
M16		Ring-oxidation	409.1638	+15.9949	410.2
M17	M21	Demethylation + hydroxylation	395.1481	+1.9792	396.2
M18	*	De-(methoxyethyl) ation + glucuronidation (M5-glucuronide)	511.1955	+118.0266	512.2
M19		Phenylacetylene oxidation to acetophenone + ring-oxidation	427.1743	+34.0054	428.2
M20	M3	Ring-oxidation + Glucuronidation (M16-glucuronide)	585.1959	+192.0270	586.2
M21	M17	Demethylation + hydroxylation	395.1481	+1.9792	396.2

* Potential isomeric mixture (pair).

system, consisting of two LC-30AD pumps, a SIL30-ACmp auto-sampler, a CTO-20AC column oven, and a DGU-20A5R degasser, all from Shimadzu (Kyoto, Japan). Data were recorded, and the system was controlled using the Sciex Analyst software (version 1.6.2).

2.3. Data processing

For chromatographic data, Thermo Fisher Xcalibur software (version 2.07), Sciex Analyst 1.6.2, and Sciex MultiQuant 3.0.1 software were used. For further data-processing Microsoft Excel 2016 for Mac (version 15.25), the statistical software R, version 3.4.3 [15], RStudio, version 1.1.423 [16], and GraphPad Prism 7 for Mac OS X, (version 7.0d) were used.

Non-parametric Passing-Bablok regression was used for method comparison and verification of metabolites. The Passing Bablok method does not assume error-free x-values and is more resistant to outliers compared to ordinary least squares or Deming regression [17].

2.4. Chromatography and MS/MS detection

Chromatographic conditions as well as positive electrospray ionization (ESI) settings are shown in Table 2 for comparison of both systems. Compound dependent MS/MS parameters for system 1 are listed in Table 3. Settings for system 1 were optimized as described in the original method [2]. System 2 for all 19 compounds was a binary gradient system, so solvents B and C were combined to 50/50 (v/v) acetonitrile and methanol, used as solvent B, to obtain a similar gradient program but using weaker eluting starting conditions to sufficiently retain the more polar metabolites. For system 2, compound specific MS/MS parameters are listed in Table 4. The precursor *m/z* value for erlotinib in the first quadrupole was shifted to the ^{13}C-isotope (*m/z* 395.1; $[M + H + 1]^+$) to avoid saturation of the continuous electron multiplier, and thus improve linearity. $^{13}C_6$-erlotinib was used as an internal standard (ISTD) for erlotinib, and $^{13}C_6$-OSI-420 was used as ISTD for OSI-420 and OSI-413 on both systems as well as for all other compounds on system 2. The method on system 2 was expanded with transitions for 16 additional metabolites (M2, M3, M5-12, M16-21). The metabolite transitions were adopted from the publications of Svedberg et al. [3], Ling et al. [11], and Li et al. [18] Chromatographic and MS/MS-parameters were optimized using 1 μM erlotinib incubated with human liver microsomes for 2 h, supplemented with a NADPH regenerating system for oxidative metabolites, and from a pooled human plasma sample of 5 patients for the conjugated metabolites, and for other metabolites that were not formed in the microsomal system.

2.5. Drift tube ion mobility - mass spectrometry collision cross section measurements

For DTIM-MS CCS measurements, a 6560 Ion Mobility Q-TOF (Agilent Technologies, Santa Clara, CA, USA) was utilized for all experimental CCS measurements. The ion mobility instrument configuration consisted of a DT length of approximately 80 cm. DT and trap funnel pressures were 4.00 and 3.85 Torr nitrogen, at a DT temperature of 30.0 °C. Trap fill and release times were 20 ms and 150 μs, and maximum drift time was 60 ms. Data acquisition was done at a frame rate of 0.9 IM frames/s and an IM transient rate of 18 IM transients/frame. All CCS measurements were obtained using nitrogen as the buffer gas.

2.6. Confirmation of structures

To verify whether OSI-420 of supplier 2 was the intended metabolite, OSI-420 was acquired from two different suppliers (2 and 3), and OSI-413 from supplier 4. The compounds were first analyzed using the bioanalytical LC-MS/MS method, using LC to separate both isomers. Next, LC was coupled to ion mobility to distinguish between both isomers, the ion mobility measurements were supported by theoretical CCS values calculated by using the structures of OSI-420 and OSI-413 crafted in ChemDraw Pro 16.0, considering three protonation sites. The protonated structures were constructed using a modified method of Campuzano et al. [19]. First, Avogadro 1.2.0 was used to create energy

Table 2
Instruments, materials, and optimized source parameters for systems 1 [1] and 2.

Instruments/parameters	System 1 (reference) [1]			System 2		
UHPLC	Accela Classic quaternary low-pressure gradient			Shimadzu Nexera X2 binary high-pressure gradient		
MS/MS	TSQ Quantum Ultra			Sciex QTRAP® 5500		
Controlling software	Thermo Fisher Xcalibur			Sciex Analyst 1.6.2		
Quantitative software	Thermo Fisher Xcalibur			Sciex Multiquant		
LC-parameters						
Injection volume (µL)	2			1		
Auto-sampler temperature (°C)	4					
Flowrate (mL/min)	0.6			0.5		
Guard-column	Aquity UPLC® BEH C18 VanGuard (5 × 2.1 mm, dp = 1.7 µm)					
LC-column	Aquity UPLC® BEH C18 (30 × 2.1 mm, dp = 1.7 µm)					
Column temperature (°C)	40					
Solvent composition	%A (v/v)	%B (v/v)	%C (v/v)		%A (v/v)	%B (v/v)
Time (min)	(Buffer)	(ACN)	(MeOH)	Time (min)	(Buffer)	(ACN/MeOH)
0.0	65.0	17.5	17.5	0.0	75.0	25.0
1.5	55.0	22.5	22.5			
3.0	40.0	30.0	30.0	3.5	40.0	60.0
3.0	0.0	100.0	0.0	3.5	0.0	100.0
3.4	0.0	100.0	0.0	3.9	0.0	100.0
3.4	65.0	17.5	17.5	3.9	75.0	25.0
4.0	65.0	17.5	17.5	4.5	75.0	25.0
Ion-source and MS/MS parameters						
Skimmer Off-set (V)		0			–	
Entrance potential (V)		–			10	
Spray voltage (V)		3000			4500	
Capillary temperature (°C)		213			–	
Vaporizer temperature (°C)		395			700	
Ion-sweep / Curtain gas (psi)		2*			40	
Sheath gas / GS1 (psi)		60*			4	
Auxiliary gas / GS2 (psi)		35*			60	
Collision gas		1.5 mTorr Argon			'Medium' Nitrogen	
Resolution		0.7**			0.7**	
Dwell times (ms)		30			5 (erlotinib: 2)	

* Arbitrary units.
** Full-width-half maximum (unit resolution).

Table 3
Tuned MS/MS parameters for all quantified components on system 1 (Thermo TSQ Quantum Ultra) [1].

	erlotinib	OSI-413/-420	$^{13}C_6$–erlotinib	$^{13}C_6$–OSI-420
Precursor ion (*m/z*)	394.1	380.1	400.1	386.1
Product ions (*m/z*)	278.1	278.1; 322.1 *	284.1	284.1
Collision energies (V)	32	31; 23	32	31
Tube lens off-set	120	128	120	128

* Signals of multiple product ions of both desmethyl-erlotinib metabolites were added up for quantification.

optimized structures of both isomers that were generated using a systematic rotor conformer search (MMFF94 force field). Next, the structures were further optimized, first using the B3LYP density functional theory and the 3-21G basis set, and then with energy minimized using the 6-31G+(d) and 6-31G++(d,p) basis sets. Partial atomic charges were then determined using Mulliken, electrostatic surface potential (pop = mk,dipole) and natural population analysis (pop = npa) outputs. Vibrational frequencies and zero-point energies were calculated using the 6-31G++(d,p) basis set and finally, theoretical CCS values were calculated using the MOBCAL algorithm, with nitrogen as the buffer gas.

2.7. Sample preparation

To a 50 µl plasma sample, pipetted into a polypropylene micro-tube (1.5 mL), 75 µl of acetonitrile was added containing both internal standards (100 ng/mL $^{13}C_6$-erlotinib and 80 ng/mL $^{13}C_6$-OSI-420). After vortex mixing vigorously for a few seconds and centrifuging at 10,000 × *g* for 2 min at ambient temperature, 50 µl of the clear supernatant were transferred to a polypropylene conical 96-well plate and diluted with 50 µl water.

2.8. Cross validationCross validation

A laboratory scheme based on international guidelines, published by the EMA and FDA was used for the cross-validation procedure [20,21]. Cross-validation with spiked matrix standards and subject samples should be analyzed for each method to establish inter-system reliability [21]. For the cross validation, the same set of QC samples was analyzed by both the reference and comparator methods. The obtained mean accuracy by the different methods should be within 15%, and for the study samples, the difference between the two values obtained should be within 20% of the mean for at least 67% of the repeats.[20] The cross validation will determine whether the obtained data are reliable and whether they perform similarly.

2.8.1. Calibration

Stock solutions at 0.5 mg/mL of each individual target compound were prepared in DMSO, $^{13}C_6$-erlotinib was prepared at 0.5 mg/mL and $^{13}C_6$-OSI-420 was prepared at 0.2 mg/mL in methanol. Stock solutions were stored in 1.5-mL polypropylene tubes at −30 °C. One set of analyte stock solutions was combined and diluted in human lithium heparin plasma to obtain the highest calibration levels at 5000 ng/mL erlotinib and 1000 ng/mL OSI-420. This calibration sample was also stored in polypropylene micro-tubes at −30 °C and used for further dilution in human plasma. Additional calibration samples were then prepared daily at 1000, 250, 50, 12.5, and 5 ng/mL for erlotinib and 200, 50, 10, 2.5 and 1 ng/mL for OSI-420. The calibration ranges were expanded with 0.5 and 0.1 ng/mL for system 2 for quantitation of the low-abundant

Table 4
Tuned MS/MS parameters for all quantified components on system 2 (Sciex Qtrap5500) and their retention times. The entrance potential was set at 10 V for all compounds, dwell-time was 2 ms for erlotinib and 5 ms for all other compounds.

Compound	t_R (min)	Q1 (*m/z*)	Q3* (*m/z*)	DP (V)	CE (V)	CXP (V)	Compound	t_R (min)	Q1 (*m/z*)	Q3* (*m/z*)	DP (V)	CE (V)	CXP (V)
Erlotinib	3.14	394.2	278.1	31	38	12	M9	1.26	490.2	352.1	45	33	12
			336.1	31	34	14				410.1	45	29	12
erlotinib (^{13}C)	3.14	395.1	279.1	31	41	12	M11	1.34	394.2	276.1	45	38	12
			337.1	31	33	14				336.1	45	34	12
OSI-420/-413	2.55/2.64	380.1	278.0	61	41	12	M12	2.09	366.2	278.1	45	38	12
			322.1	61	31	14				322.1	45	35	12
M2	1.32	414.2	312.1	45	36	12	M16	1.62	410.2	294.1	45	37	12
			370.1	45	32	12				352.1	45	33	12
M3/M20	1.44/n.a.	586.2	352.1	45	33	12	M17/M21	1.59/1.35	396.2	294.1	45	37	12
			410.1	45	29	12				338.1	45	34	12
M5	1.42	336.2	278.1	45	38	12	M18	1.15	512.2	336.1	45	34	12
										278.1	45	38	12
M6	1.56	428.2	312.1	45	36	12	M19	1.86	428.2	312.1	45	36	12
			370.1	45	32	12				370.1	45	32	12
M7	1.48	430.2	314.1	45	36	12	$^{13}C_6$-erlotinib	3.14	400.1	284.1	31	41	12
			372.1	45	32	12				342.1	31	33	14
M8/10	1.17;1.34	556.2	278.1	45	38	12	$^{13}C_6$-OSI-420	2.55	386.1	284.1	61	41	12
			380.1	45	31	12				328.0	61	31	14

* Signals of multiple product ions of one compound were added up for quantification.

metabolites. All calibration samples were processed in duplicate for each daily calibration accompanied by duplicate blank samples. Least-squares regression was employed to define the calibration model using the ratios of the peak area of the analyte (calculated by adding up the signals of both available product ions of each compound) and the IS used for the calibration samples. The reversed square of the concentration ($1/x^2$) served as the weighting factor for both systems. For erlotinib and OSI-420 their $^{13}C_6$-labeled compounds were used as ISs. For OSI-413, M2, M3, M5-12, M16-21, $^{13}C_6$-OSI-420 was used as the IS as well. On system 1 a second-degree polynomial (quadratic regression) function was used for calibration of OSI-420 and OSI-413. On system 2, hill-regression was used for erlotinib. OSI-420′s linear calibration was used for OSI-420 itself, and the semi-quantitative determination of OSI-413, M2, M3, M5-12, M16-21.

2.8.2. Precision and accuracy

A second set of stock solutions was used to prepare quality control (QC) samples at four levels in human lithium heparin plasma. QC-high contained 4000 ng/mL erlotinib and 800 ng/mL OSI-420. Other samples contained 200 and 40 ng/mL (QC-med), 10 and 2 ng/mL (QC-low), 5 and 1 ng/mL (QC-LLOQ), and 0.5 and 0.1 ng/mL (QC-LLOQ2) of these compounds in plasma. Precisions and accuracies were determined in 6-fold in one analytical run for all QCs on both systems. Relative standard deviations were calculated for the within-run precisions.

2.8.3. Selectivity and matrix effect

Six individual human plasma samples were processed to test the selectivity of the assay. Samples were processed without target compounds and without ISs (double blank), and with erlotinib, OSI-420, and M16 at 1 ng/mL, supplemented with the ISs.

2.8.4. Matrix effect

The matrix effects were analyzed at high, medium, and low QC levels (n = 6) by comparing relative peak-areas of erlotinib, OSI-420, and M16 spiked into blank plasma extracts with the relative peak area of each compound in matrix-free solvent.

2.8.5. Stability

The stability of the compounds was investigated trough incurred sample reanalysis of patient samples stored in polypropylene tubes after storage at −30 °C for 1 month. Stability of processed samples, together with freshly prepared calibration samples, was tested after additional storage of the diluted extracts at −30 °C for 200 days.

Table 5
Retention times of OSI-420 and OSI-413, determined by LC-MS/MS analysis on system 2 (n = 3).

Supplier	Compound stated on label	t_r (min)
Supplier 2	OSI-420	2.55 ± 0.01
	[$^{13}C_6$]-OSI-420	2.54 ± 0.00
Supplier 3	OSI-420	2.54 ± 0.01
Supplier 4	OSI-413 ‘batch 1’	2.55 ± 0.01
	OSI-413 ‘batch 2’	2.65 ± 0.01
HLM-generated	OSI-420	2.54 ± 0.00
	OSI-413	2.64 ± 0.01

2.8.6. Analysis of erlotinib, OSI-420, and the semi-quantitation of OSI-413 and 16 other metabolites in human plasma

The assay was used to monitor plasma levels of drug and metabolites in patients treated with erlotinib. Drug analysis was part of a therapeutic drug monitoring service and inter-laboratory quality control. A random selection of 20 samples from patients treated with erlotinib was taken from a larger cohort of 277 patients. The samples were analyzed for erlotinib and OSI-420. OSI-413 and the other metabolites were quantified semi-quantitatively using OSI-420 as the calibrant. The result for both desmethyl metabolites and M2, M3, M5-12, M16-19, and M21 were verified by (visually) examining the metabolite *vs* erlotinib ratios for the larger cohort by means of Passing-Bablok regression, as interfering peaks would not be correlated to the plasma concentration of erlotinib. Afterwards, the ionization efficiency of M16 was compared to that of OSI-420 for verification by comparing the target to IS-ratio of both compounds over the calibration range of OSI-420 for a set of 28 spiked plasma samples.

3. Results and discussion

3.1. Confirmation of OSI-413 and OSI-420 structures

The isomers of O-demethylated erlotinib are hard to distinguish due to the two 2-methoxy-ethoxy groups in erlotinib. Frequently used techniques like high resolution MS, even combined with fragmentation, or NMR experiments are not capable to distinguish the two isomers. Therefore, confusion is a high risk, for instance, a journal that published a bioanalytical paper with the structure of OSI-413, indexed the compound as OSI-420 [10]. Therefore, the purchased metabolite standards were all subjected to LC-separation followed by MS/MS detection to

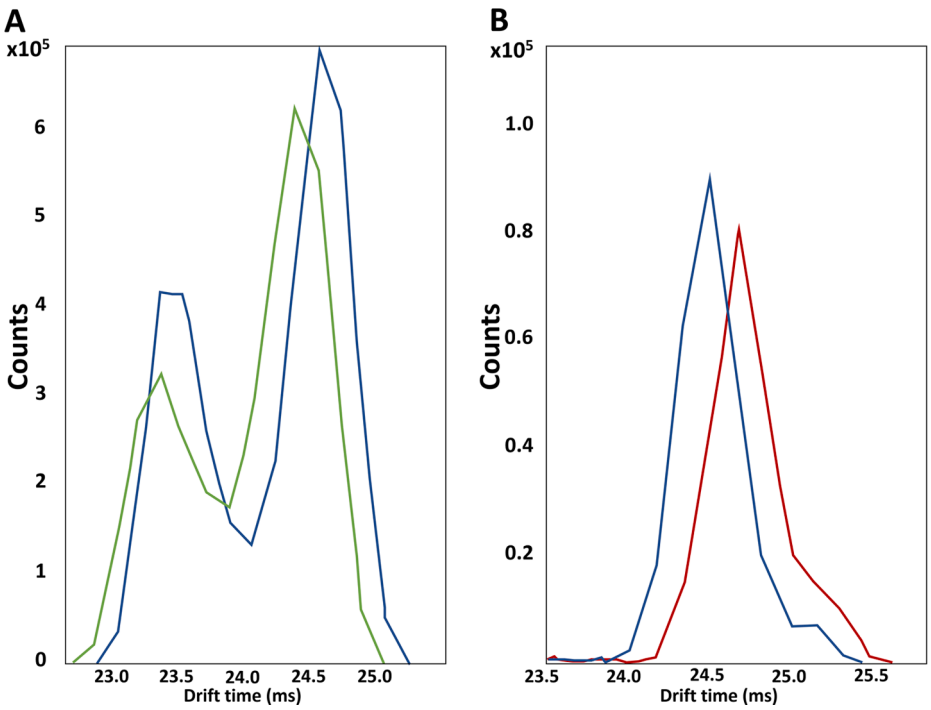

Fig. 2. DTIM-MS drift-times of OSI-420 and OSI-413 in positive ionization, (A green: OSI420, blue: OSI-413) and negative ionization, (B, blue: OSI-420, red: OSI-413). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 6
Experimental collisional cross sections of OSI-420 and OSI-413, determined by Drift Tube Ion Mobility - Mass Spectrometry in positive and negative ionization (n = 6, p-value < 0.0001 for all conformers).

Conformer	Ionization	OSI-420		OSI-413		Difference
	charge	CCS (Å^2)	RSD (%)	CCS (Å^2)	RSD (%)	(Å^2)
1	+	200.15 ± 0.08	0.04%	201.10 ± 0.15	0.08%	−0.95
2	+	191.82 ± 0.12	0.06%	192.32 ± 0.13	0.07%	−0.50
3	–	198.62 ± 0.13	0.07%	200.02 ± 0.15	0.07%	−1.40

confirm the retention times and thus whether they are OSI-413 or OSI-420. Results are depicted in Table 5. The first batch of OSI-413 that was acquired from supplier 4 eluted at the retention time of OSI-420 from suppliers 2 and 3. Supplier 4 was asked for an explanation for this and reported that there had been an error in the synthesis. A newly synthetized batch was supplied, which conformed with OSI-413.

But as also the vendors may not be able to distinguish the compounds, and possible switching could have occurred, we searched for an identification method for the isomers, leading us to DTIM-MS. Using DTIM-MS, two charged species of both desmethyl metabolites could be detected and distinguished in positive ionization, one was found in negative ionization (Fig. 2). The difference in CCS values for conformer 1 (Table 6) of both isomers might be caused by the difference in interaction of the hydroxy-tail of OSI-420 and the methoxy-tail of OSI-413 with a charge on the secondary amine. The second conformer is possibly due to a charge on either one of the nitrogen atoms in the quinazoline, leading to a less pronounced difference. DTIM-MS could be used as a method to further distinguish the two metabolites, even between labs. While interlaboratory deviations for single field measurements are generally within 0.30%, creating some possible overlap, the relative CCS difference should not change between laboratories [13]. The differences were statistically significant for all comparisons (p-value < 0.0001 for all conformers). Calculated CCS values show that OSI-420 should have a smaller CCS compared to OSI-413 (183.3 ± 5.6 *vs* 197.7 ± 4.6 Å^2), but the theoretical values differ a lot from the actual measurements. DTIM-MS spectra are shown in Fig. 2, the most efficient separation can be seen in negative ionization mode (Fig. 2B, Table 6) and expected identities of all desmethyl metabolite batches could be confirmed now.

Table 7
Accuracies and precisions for erlotinib and OSI-420 on both systems (n = 6).

Compound	QC-level (ng/mL)	System 1 (ref.)		System 2	
		Accuracy	Precision	Accuracy	Precision
Erlotinib	High (4000)	101.9%	3.7%	98.7%	6.0%
	Medium (200)	98.9%	3.6%	102.1%	4.9%
	Low (10)	105.4%	5.6%	104.0%	9.2%
	LLOQ (5)	106.2%	8.7%	103.0%	13.2%
	LLOQ2 (0.5)	109.5%	60.3%	92.0%	18.1%
OSI-420	High (800)	99.4%	4.1%	99.8%	10.0%
	Medium (40)	99.3%	2.8%	100.5%	4.6%
	Low (2)	99.2%	11.9%	100.2%	8.3%
	LLOQ (1)	99.1%	13.4%	100.3%	8.7%
	LLOQ2 (0.1)	97.8%	35.1%	100.9%	15.7%

3.2. Metabolite identification

The SRM transitions for all metabolites (Table 4) were adopted from previous papers on the bioanalysis of erlotinib and its metabolites [2–4,18], and were optimized using human liver microsomes generated metabolites, as well as pooled human plasma samples of patients that received erlotinib. The optimized SRM-settings are shown in Table 4 with all retention times. The M16-glucuronide conjugate M20 was not found in any sample using the transitions provided by Svedberg et al. [3] All other conjugates were found in the plasma pool by using the identifying fragments from Ling et al. [11] and Svedberg et al.[3] from which for each metabolite the sum of the two fragments with the highest response were used. In addition to OSI-420 and OSI-413, erlotinib has

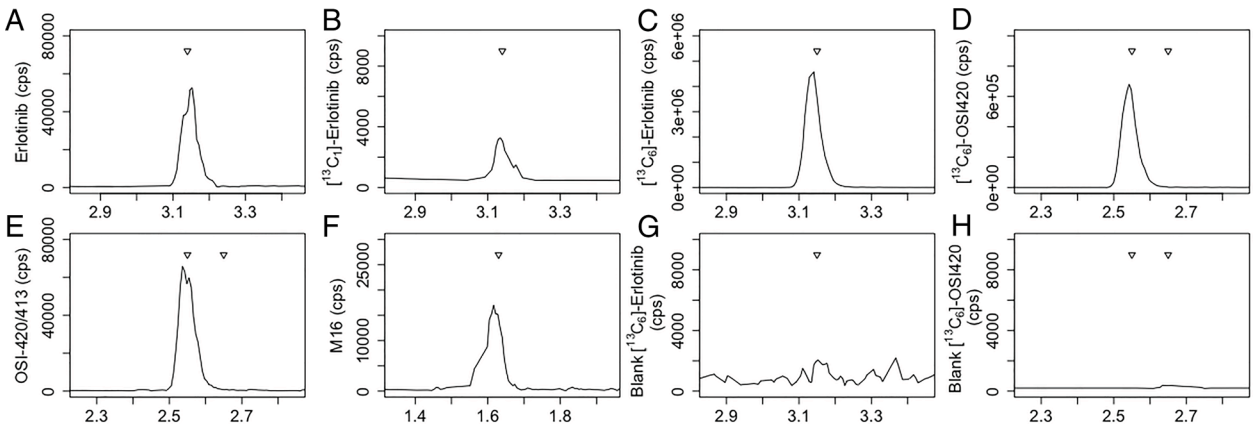

Fig. 3. Chromatograms of a (blank) plasma sample spiked at 1 ng/mL for erlotinib, OSI-420, and M16, with internal standards on system 2. The view is focused on the region of 0.3 min around the retention time of the compounds A) Erlotinib (Q1: *m/z* 394.1) B) ^{13}C-Erlotinib (Q1: *m/z* 395.1), C) $^{13}C_6$-erlotinib, D) $^{13}C_6$-OSI-420, E) OSI-420/-413, F) M16, G) $^{13}C_6$-erlotinib (blank), H) $^{13}C_6$-OSI-420 (blank). Compound names are also listed along the Y-axis, time (min) is plotted on the X-axis. The triangles indicate the expected retention times.

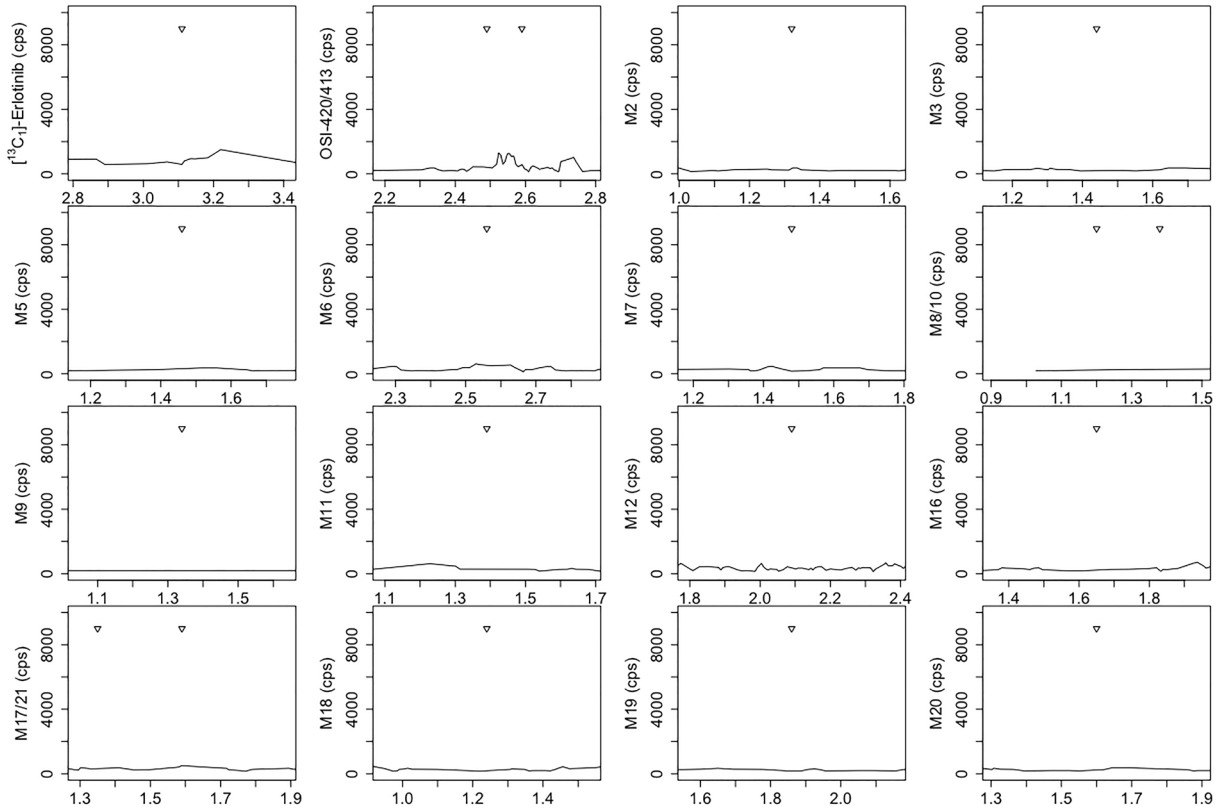

Fig. 4. Chromatograms of a blank plasma sample on system 2. The view is focused on the region of 0.3 min around the retention time of the compound. Compound names are listed along the Y-axis, time (min) is plotted on the X-axis. The triangles indicate the expected retention-times.

several other (potential) isobaric and isomeric metabolites as reported in Table 1. Except for both M16-glucuronides (M3 and M20) all these (potential) isomeric pairs origin from the two 2-methoxy-ethoxy groups in erlotinib [3,11,22].

3.3. Cross validation

3.3.1. Precision and accuracy

Assay performance data of the validation samples at four concentrations are reported in Table 7. Only within run variation was established, as the comparison of both systems gave good results. Accuracies and precisions met the required ±15% variation (±20% for the LLOQ) for both systems, and inter-method variation was within 15%, as provided by the EMA [20]. For system 2, a lower LLOQ (LLOQ2) was also successfully evaluated as it met the ±20% variation [20,21]. This effort was taken as the system was also used to quantify low abundant metabolites using the OSI-420 calibration. The QCs showed excellent agreement between both systems as shown in Table 7. While in the original range systems 1 and 2 performed comparably, system 2 was able to accurately and precisely analyze 10-fold lower levels (LLOQ2).

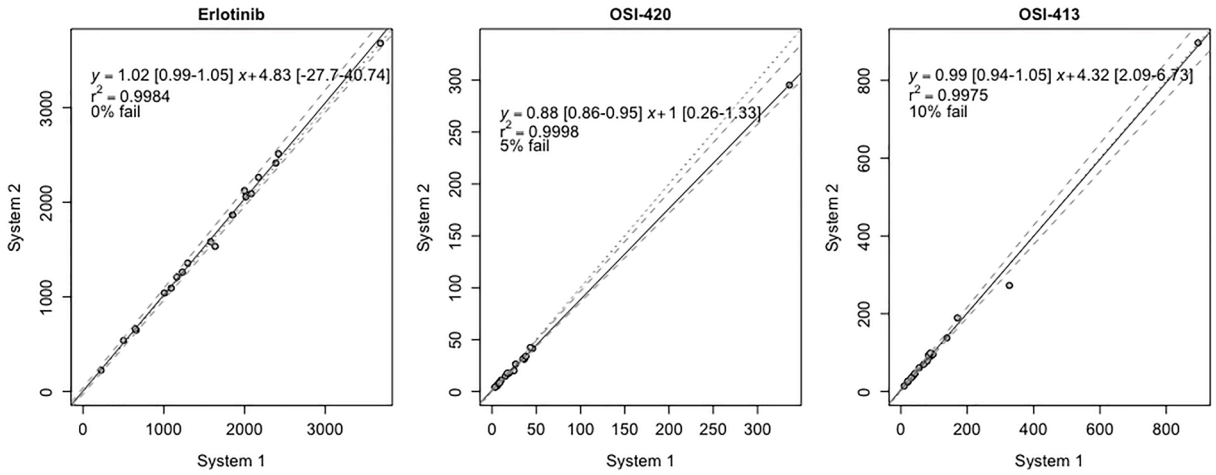

Fig. 5. Erlotinib, OSI-420, and OSI-413 concentrations as measured by system 2 plotted against concentrations measured on system 1 (reference). Solid line: regression line; dashed line: 95% CI; dotted line: line of equality (x= y).

3.3.2. *Selectivity and matrix-effects*

No significant interfering peaks were found in the all SRM traces for six blank human plasma lots. Blank responses were all <5% of the LLOQ response of the available target compounds, as is similar to the full validation of system 1 [2], and also met the required 20% [20,21]. For system 2, the IS responses in the traces of the target compounds were 0.024 ± 0.005% for $^{13}C_6$-erlotinib and 0.018 ± 0.006% for $^{13}C_6$-OSI-420, so isotopic interference is negligible. The accuracies and precisions at these levels, as well as the area of the blank compared to the LLOQ levels (n = 6) are reported in Supplemental Table 1, and the chromatogram with erlotinib, OSI-420 and M16 traces of selectivity sample 1 is shown in Fig. 3 and as well both IS traces with and without ISTDs present. Blank traces for all compounds are shown in Fig. 4, and were also taken from injection of inter-lot sample 1.

3.3.3. *Matrix effect and recovery*

Matrix effect coefficients of variation were within 15% for all levels (Supplemental Table 2), as per EMA requirements [20]. Since there was no significant change of the sample preparation or change of ISTD [2], recovery assessment was not additionally performed.

3.3.4. *Stability*

Stability of the plasma extracts at −30 °C was evaluated after 200 days for 177 samples. Excellent results were obtained for erlotinib (+10 ± 9%), OSI-420 (−5 ± 7%) and OSI-413 (−1 ± 12%) with only 12.6%, 5.6%, and 4.0% of the samples showing a difference greater than 20% for either. Stock and sample stability were not further investigated, as this was already successfully tested in our existing assay [2] as well as in several other studies [3–5,8,23–27].

3.3.5. *Analysis of erlotinib, OSI-420 and OSI-413, and 16 other metabolites in human plasma*

The concordance and variation between a previously published LC-MS/MS method for the simultaneous measurement erlotinib, OSI-420,

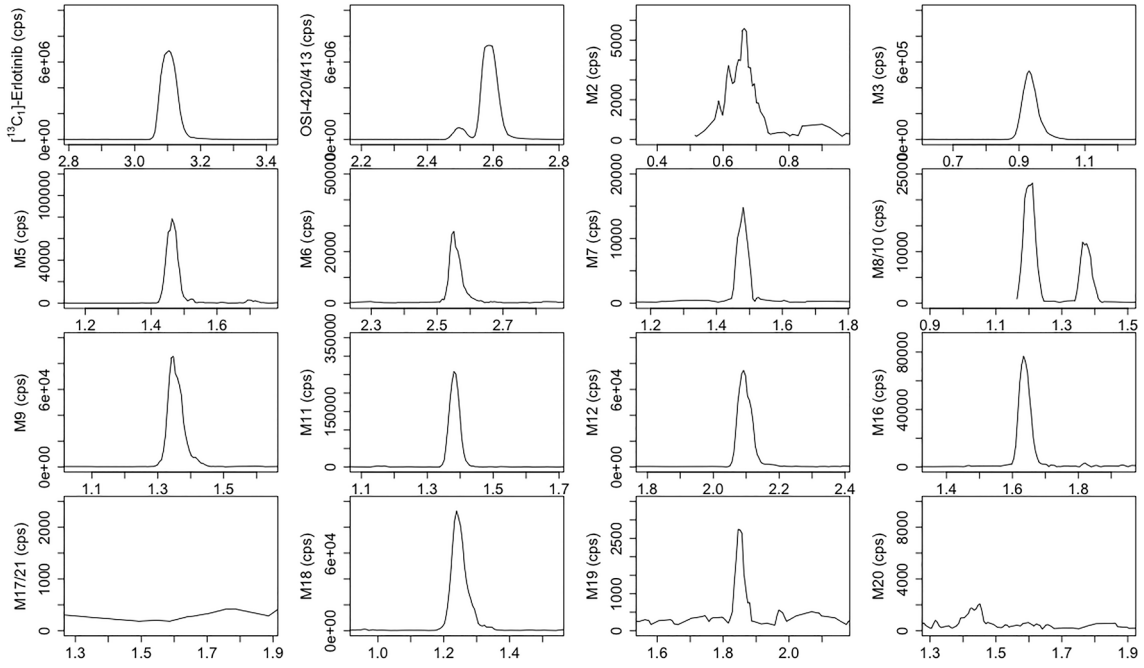

Fig. 6. Chromatogram of a patient sample on system 2. The view is focused on the region of 0.3 min around the retention time of the compound. Compound names are listed along the Y-axis, time (min) is plotted on the X-axis.

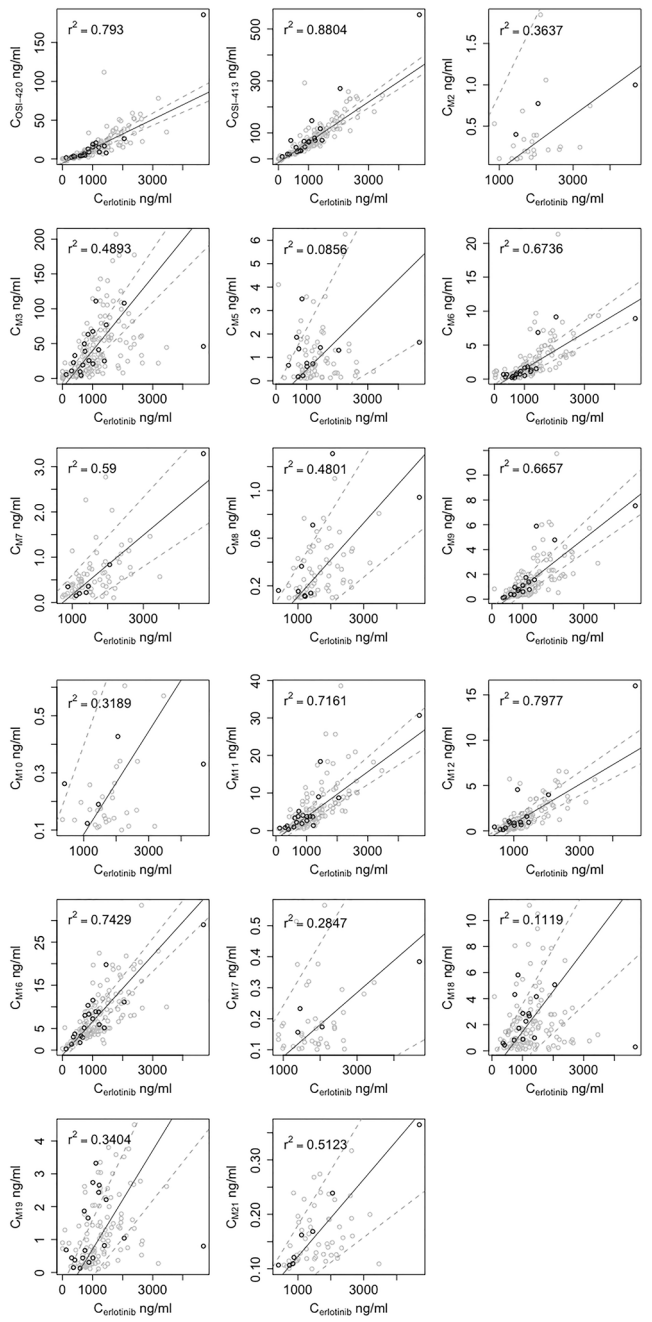

Fig. 7. Visual analysis of drug metabolites of erlotinib. Solid line: linear regression line (Passing-Bablok regression); dashed line: 95% CI.

and OSI-413 [2] and the present assay was investigated for 20 patient samples. For erlotinib (1.02, 95% CI: 0.99–1.05) and OSI-413 (0.99, 95% CI: 0.94–1.05), the concordance of the results for the LC-MS/MS methods investigated was high. The assays agreed well over their entire concentration ranges. For OSI-420 a slight proportional difference was found for the agreement among both investigated systems, with a slope of the regression line of 0.88 (95% CI: 0.86–0.95, n = 20, Fig. 5). Only 3 out of all the measurements failed to report within a 20% level of agreement for all three compounds together. While for OSI-420 a slight bias might be present, this is still within 15% deviation overall. For both, OSI-420 and OSI-413 a small systematic difference of 1.00 ng/mL for OSI-420, and 4.32 ng/mL for OSI-413 appears to be present. The full details are shown in Supplemental Table 3, a typical chromatogram is shown in Fig. 6.

Detailed TDM results for erlotinib and all metabolites in 20 patients are shown in Supplemental Table 4. For most metabolites, the method showed comparable levels to the results presented by Svedberg *et al.* [3] We did however observe higher maximum values. This was attributable to one patient sample (no. 15), and the limited sample size of the study by Svedberg et al. (n = 4). Patient 15 received a once weekly dose of erlotinib due to leptomeningeal metastases from EGFR mutant lung cancer. The visual examination of all metabolites led us to believe that all quantified compounds were indeed metabolites related to erlotinib (Fig. 7). This was performed for the larger data-set of 177 patient samples using Passing-Bablok regression, which assumes outliers on both axes. Metabolites M2 and M20 did not meet the criteria and should be reported as 'not detected' or '<LLOQ'. M20 was not included in the regression-analysis, as it was not detected in any of the 20 samples, as well as the 177 patient samples. For M16, the ionization efficiency was determined afterwards based on the target to ISTD-ratio, which appeared to be 22.3 ± 1.9% (n = 28) relative to OSI-420. The concentrations of M16 were corrected for this. When looking at the data of the other metabolites, the possibility of this effect has to be considered too. For OSI-413, the effect of using the calibration of OSI-420 was also assessed. The difference was less than 5% (n = 28) when the slopes of samples at the calibration levels were assessed, showing the validity of using OSI-420 calibration for its isomer. For erlotinib, OSI-420, M6, and M11 mass-balance reference values are available from a single-dose ^{14}C-labeled erlotinib study [11]. This study showed that OSI-420 and OSI-413 were separable by LC in feces, but they did not show OSI-413 in plasma. In their study OSI-420 encompassed 5.4% of the total plasma radioactivity at 2 h. In our study, overall, OSI-420 accounted for 1.5% of all related compounds, and OSI-413 for 7.4% (together they accounted for 8.9%). For M6 and M11 substantial lower levels were found, which might also be due to the semi-quantitative approach. Also, these results were gathered during steady-state, while in that case it was a single dose study.

4. Conclusions

Recently, the separation and quantitation of erlotinib desmethyl isomers OSI-420 and OSI-413 gathered interest. These metabolites were quantified as a combined fraction in earlier assays, named mostly as OSI-420. However, OSI-413 appeared to be the most abundant metabolite, but the structure could not be confirmed after synthesis so far, and obtaining the wrong isomer was therefore a significant risk. The method of DTIM-MS along with molecular dynamic simulation now gave additional assurance to obtain maximal certainty of the identity of both major isomeric metabolites. Further, we successfully transferred our existing method for the quantitation of erlotinib, OSI-420, and OSI-413 to a more sensitive system, demonstrating the robustness of the original method. Along with that, 16 metabolites were additionally included for the semi-quantitative determination of these metabolites. Among them, 15 were detected in human samples of patients treated with erlotinib, and 14 could be confirmed as actual metabolites. With a simple crash, dilute, and shoot sample preparation and a 4.5 min run time the method outperformed most other published LC-MS/MS methods for erlotinib and OSI-420 in plasma in terms of sensitivity and speed [3,4,6,8]. The new assay is suitable for TDM, as well as further enhancement of the understanding of the kinetics of erlotinib and its metabolites, also in relation to its pharmacological properties.

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CRediT authorship contribution statement

Johannes J.M. Rood: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Visualization, Writing - original

draft, Project administration. **Javier Sastre Toraño:** Visualization, Resources. **Victor J. Somovilla:** Resources. **Jos H. Beijnen:** Resources, Supervision, Writing - review & editing. **Rolf W. Sparidans:** Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2021.122554.

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