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Liquid chromatography–tandem mass spectrometric assay for therapeutic drug monitoring of the B-Raf inhibitor encorafenib, the EGFR inhibitors afatinib, erlotinib and gefitinib and the *O*–desmethyl metabolites of erlotinib and gefitinib in human plasma

Rolf W. Sparidans[a,b,*], Hilde Rosing[c], Johannes J.M. Rood[a], Jan H.M. Schellens[a,d], Jos H. Beijnen[a,c,d]

[a] *Utrecht University, Faculty of Science, Department of Pharmaceutical Sciences, Division of Pharmacoepidemiology & Clinical Pharmacology, Universiteitsweg 99, 3584 CG Utrecht, The Netherlands*
[b] *Utrecht University, Faculty of Science, Department of Pharmaceutical Sciences, Division of Chemical Biology & Drug Development, Universiteitsweg 99, 3584 CG Utrecht, The Netherlands*
[c] *MC Slotervaart, Department of Pharmacy & Pharmacology, Louwesweg 6, 1066 EC Amsterdam, The Netherlands*
[d] *The Netherlands Cancer Institute, Department of Clinical Pharmacology, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands*

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1. Introduction

Encorafenib (LGX818, Fig. 1A) is new generation B-Raf inhibitor investigated in the treatment of melanoma and other solid tumors harboring a *BRAF* mutation. A mechanism of its anti-tumor activity in B-Raf cell lines containing the *BRAF* V600E mutation was recently unraveled [1]. In the treatment with targeted therapies the focus is more and more shifting to the combination of drugs targeting different stages of the signaling pathways to overcome drug resistance. For encorafenib in *BRAF* mutated advanced melanoma, the combination with the MEK inhibitor binimetinib (MEK162) has been the most successful treatment thus far, compared to the B-Raf/MEK inhibition combinations vemurafenib/cobimetinib and dabrafenib/trametinib [2,3]. Alternatively, the combination of a B-Raf inhibitor with an epidermal growth factor receptor (EGFR) inhibitor can also show a strong synergistic effect as initially reported by Prahallad et al. [4] for the combination of vemurafenib with erlotinib (Fig. 1B), gefitinib (Fig. 1C) or cetuximab. Such a combination was clinically investigated later for vemurafenib with panitumumab in *BRAF*-mutant metastatic colorectal cancer [5] and might be a new step in the treatment of this aggressive and chemo-resistant cancer type.

For encorafenib, investigations have even evolved to triple targeted therapies. Promising results were recently obtained in humanized mouse models for the encorafenib/binimetinib combination with the pan-PI3K inhibitor buparlisib (BKM120) and also with the c-Met inhibitor capamatinib (INC280, INCB28060)

Abbreviations: EGFR, epidermal growth factor receptor; IS, internal standard; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LLOQ, lower limit of quantification; SRM, selected reaction monitoring; QC, quality control.

* Corresponding author at: Utrecht University, Faculty of Science, Department of Pharmaceutical Sciences, Division of Pharmacoepidemiology & Clinical Pharmacology, Universiteitsweg 99, 3584 CG Utrecht, The Netherlands.
E-mail addresses: R.W.Sparidans@uu.nl (R.W. Sparidans), H.Rosing@nki.nl (H. Rosing), J.J.M.Rood@uu.nl (J.J.M. Rood), j.schellens@nki.nl (J.H.M. Schellens), jos.beijnen@slz.nl (J.H. Beijnen).

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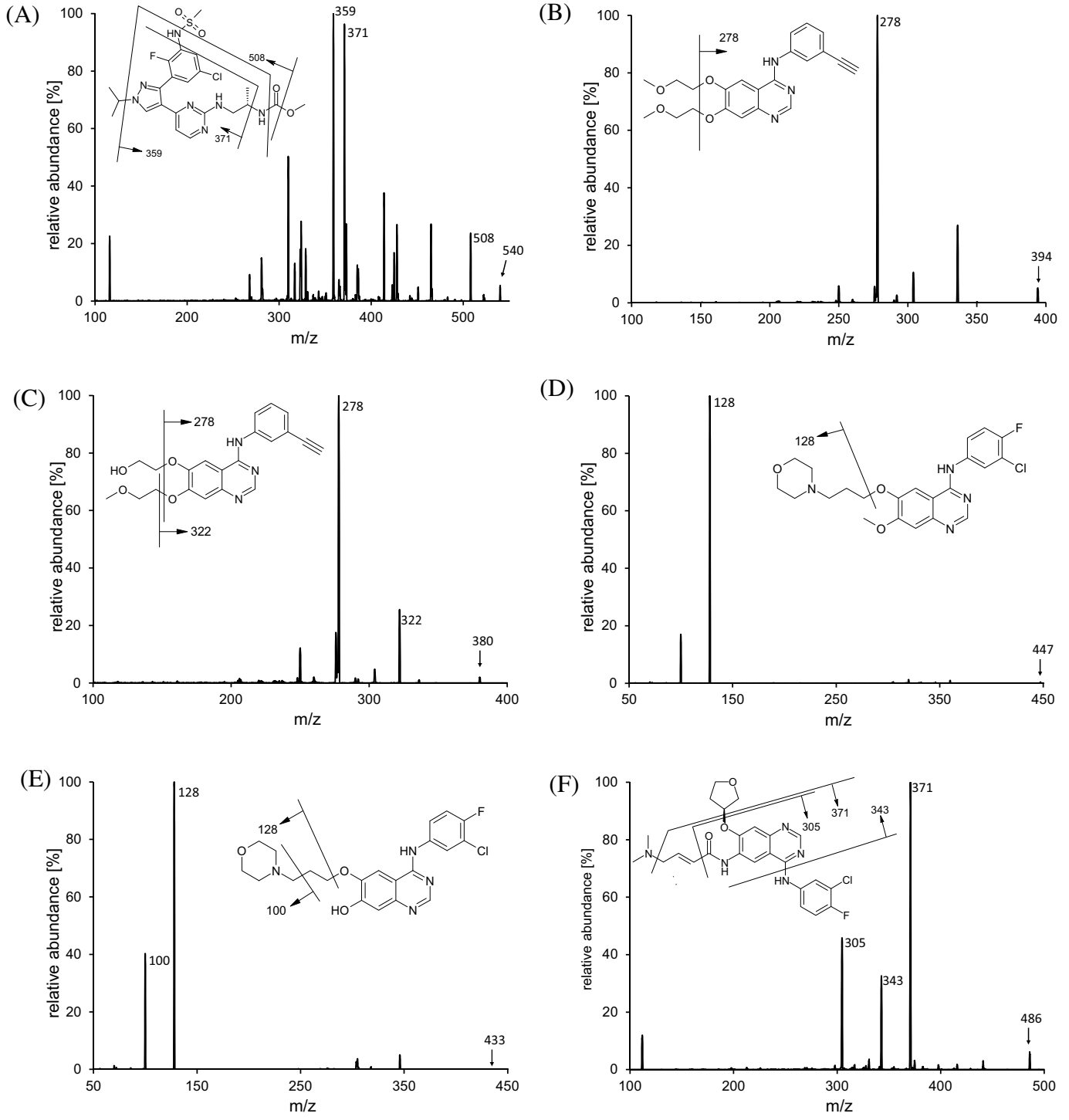

Fig. 1. Chemical structures and product ion spectra, formed by collision-induced dissociation of the protonated molecules of (A) encorafenib; *m/z* 540.15@–41 V, (B) erlotinib; *m/z* 394.1@−30 V, (C) OSI-420; *m/z* 380.1@−31 V, (D) gefitinib; *m/z* 447.1@–30 V, (E) *O*−desmethyl-gefitinib; *m/z* 433.1@−35 V and (F) afatinib; *m/z* 486.1@−30 V. Dissociation pathways of the monitored transitions have been proposed.

[6]. Finally, encorafenib is being investigated in combination with cetuximab (EGFR inhibitor) with and without alpelisib (BYL719) in advanced *BRAF*-mutant colorectal cancer as well with binimetinib and several third agents (buparlisib, capamatinib, ribociclib [LEE011; cyclin-dependent kinase inhibitor], infigratinib [BJG398; fibroblast growth factor receptor inhibitor]) in advanced B-Raf melanoma [7].

In order to support clinical studies with encorafenib the availability of a bioanalytical assay is pivotal. A validated method for this drug is, however, not yet been published. The potential of combined targeted therapies with encorafenib decided us to develop an assay for encorafenib in combination with three EGFR inhibitors: erlotinib, gefitinib and afatinib (Fig. 1F). Active *O*−desmethyl metabolites of erlotinib and gefitinib (Fig. 1E) were also included in the assay. Bioanalytical methods for erlotinib and gefitinib, sometimes including their metabolites, have been frequently reported, mainly using liquid chromatography–tandem mass spectrometry (LC–MS/MS) [8–11]. OSI-413 was recently indi-

Table 1
Compound dependent parameters of SRM detection.

	encorafenib	*O*–desmethyl-gefitinib	*O*–desmethyl-erlotinib[a]	$^{13}C_6$-OSI–420[b]	erlotinib	$^{13}C_6$-erlotinib	gefitinib	gefitinib-d_8	afatinib	$^{13}C_6$-afatinib
Precursor ion (*m/z*)	540.15	433.1	380.1	386.1	394.1	400.1	447.1	455.1	486.15	492.1
Product ions[c] (*m/z*)	359.1 371.1 508.15	100.05 128.05	278.1 322.1	284.1	278.1	284.1	128.05	136.05	305.1 343.1 371.1	377.1
Collision energies (V)	−36 −38 −25	−41 −23	−31 −23	−31	−32	−32	−24	−24	−35 −37 −26	−29
Tube lens off-set	134	123	128	128	120	120	128	128	133	134
Skimmer Off-set (V)	0	0	0	0	0	0	0	0	−6	−6
Dwell times (ms)	30	30	30	30	30	30	30	30	50	50
Time range (min)	0–1.8	0–1.8	0–2.5	0–2.5	0–3.5	0–3.5	1.8–3.5	1.8–3.5	2.5–3.5	2.5–3.5

[a] Parameters used for both isomers: OSI-413 and OSI-420.
[b] Also used as internal standard for encorafenib and *O*–desmethyl-gefitinib.
[c] Signals of multiple product ions of one compound were added up for quantification.

cated as the main circulating metabolite of erlotinib [12,13] and not the OSI-420 isomer (Fig. 1C). OSI-420 was originally indicated as the main circulating erlotinib metabolite [14] and has frequently been included in previous assays as *O*–desmethyl metabolite without separation of both isomers [9]. The first validated assay for afatinib was reported recently for human plasma, with simultaneous determination of erlotinib and gefitinib, using liquid–liquid extraction [8] and for mouse plasma using salt-assisted liquid–liquid extraction [15], both using LC–MS/MS. Here, we report a new validated quantitative LC–MS/MS assay for encorafenib, erlotinib, OSI-420, gefitinib, *O*–desmethyl-gefitinib and afatinib in human plasma from low ng/ml concentrations up to the clinical levels using a simple sample pre-treatment procedure and LC–MS/MS. OSI-413, separated from its isomer, could be quantified semi-quantitatively.

2. Experimental

2.1. Chemicals

Erlotinib (as hydrochloric acid) and encorafenib were supplied by Sequoia Research Products (Pangbourne, UK). Afatinib, $^{13}C_6$-afatinib, $^{13}C_6$-erlotinib (as hydrochloric acid), OSI-420 (as hydrochloric acid, 95.9%), $^{13}C_6$-OSI-420 (as hydrochloric acid, 98.0%), gefitinib, gefitinib-d_8 and *O*–desmethyl-gefitinib were obtained from Alsachim (Strasbourg, France). Non-defined purities were all >99%.

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).

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). 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 (2 μl) 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 and 0.01% (v/v) formic acid in water (pH 9.8), freshly prepared daily, solvent B being acetonitrile and solvent C being methanol. After injection, the percentages of solvent B and C were both increased linearly from 17.5 to 22.5% during 1.5 min. From 1.5 to 3 min percentages of B and C were increased further linearly to 30%. Next, the column was flushed with 100% (v/v) B for 0.4 min and finally, the column was reconditioned at the starting conditions (65% (v/v) A, 17.5% (v/v) B and 17.5% (v/v) C) for 0.6 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 3.4 min after injection. The electrospray was tuned in the positive ionization mode by

Table 2
Mean calibration parameters (±SD) and the assay performance for 6 calibrations ($y = A + B \cdot x + C \cdot x^2$).

Compound	A (intercept)	B (slope)	$C \cdot 10^8$	R^2	Range of precisions (%)	Range of accuracies (%)
encorafenib	0.0034 ± 0.0042	0.0039 ± 0.0005	−11.0 ± 2.1	0.991 ± 0.008	4.5–11.7	96.6–101.8
O−desmethyl-gefitinib	0.00089 ± 0.00045	0.0073 ± 0.0008	−105 ± 32	0.996 ± 0.003	2.5–7.4	98.2–101.5
OSI-420	0.0018 ± 0.0010	0.0143 ± 0.0003	0	0.9967 ± 0.0015	3.3–6.3	98.4–103.3
erlotinib	0.0036 ± 0.0024	0.00674 ± 0.00012	0	0.9980 ± 0.0009	2.1–3.7	95.5–102.5
gefitinib	0.0034 ± 0.0011	0.0093 ± 0.0002	0	0.9930 ± 0.0014	3.5–9.1	91.9–104.8
afatinib	0.00089 ± 0.00085	0.0231 ± 0.0006	0	0.9950 ± 0.0019	2.3–9.8	97.4–101.5

introducing 0.6 ml/min of a solvent mixture containing 50% (v/v) of 0.1% (v/v) formic acid and 50% (v/v) methanol and 5 μl/min of 10 μg/ml of each respective target compound. A 3000 V spray voltage, 213 °C capillary and 395 °C vaporizer temperatures and nitrogen sheath, ion sweep and auxiliary gasses set at 60, 2 and 35 arbitrary units showed to be optimal for *O*−desmethyl-gefitinib and was selected for the positive electrospray ionization. The selected reaction monitoring mode (SRM) 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 both separating quadrupoles. Compound dependent parameters are listed in Table 1.

2.4. Sample pre-treatment

To a 50 μl plasma sample, pipetted into a polypropylene micro-tube (1.5 ml), 75 μl of acetonitrile was added containing all 4 internal standards (100 ng/ml $^{13}C_6$-erlotinib, 80 ng/ml of $^{13}C_6$-OSI-420, gefitinib-d_8 and $^{13}C_6$-afatinib). After vortex mixing vigorously for a few seconds and centrifuging at 10,000 × *g* for 2 min at ambient temperature, 100 μl of the clear supernatant were transferred into a glass injection vial and diluted with 100 μl water.

2.5. Validation

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

2.5.1. Calibration

Stock solutions at 0.5, 1 or 2 mg/ml of each individual target compound were prepared in methanol, $^{13}C_6$-erlotinib was prepared at 0.5 mg/ml and other internal standards (ISs) were 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 10,000 ng/ml encorafenib, 5000 ng/ml erlotinib, 2000 ng/ml gefitinib and 1000 ng/ml of the other compounds (afatinib, OSI-420 and *O*−desmethyl-gefitinib). 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 2000/1000/400/200 (encorafenib/erlotinib/gefitinib/afatinib, OSI-420 and *O*−desmethyl-gefitinib), 500/250/100/50, 100/50/20/10, 25/12.5/5/2.5 and 10/5/2/1 ng/ml. 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 all 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. When available, corresponding labeled compounds were used as IS to obtain linear calibrations. For both, encorafenib and *O*−desmethyl-gefitinib, $^{13}C_6$−OSI-420 was used as IS with a second degree polynomial (quadratic regression) function used for calibration.

2.5.2. Accuracy and precision

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 8000 ng/ml encorafenib, 4000 ng/ml erlotinib, 1600 ng/ml gefitinib and 800 ng/ml of the other compounds. Other samples contained 400/200/80/40 ng/ml (QC-med), 20/10/4/2 ng/ml (QC-low) and 10/5/2/1 ng/ml (QC-LLOQ) of these compounds in plasma. Samples were stored in polypropylene tubes at −30 °C. Precisions and accuracies were determined in 6-fold 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 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 all six compounds at the lower limit of quantification (LLOQ) levels, supplemented with the four ISs.

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 solutions in blank pooled plasma extract with all target compounds added at the same levels.

The matrix effect was assessed using post-column infusion experiments. A mixture containing all target compounds (8000 ng/ml encorafenib, 4000 ng/ml erlotinib, 1600 ng/ml gefitinib and 800 ng/ml of the other compounds in 50% (v/v) methanol) and a mixture of both, target compounds and ISs (4000 ng/ml encorafenib, 2000 ng/ml erlotinib, 800 ng/ml gefitinib, 400 ng/ml of the other target compounds, 50 ng/ml $^{13}C_6$−erlotinib, 40 ng/ml of $^{13}C_6$−OSI-420, gefitinib-d_8 and $^{13}C_6$-afatinib in methanol/water/acetonitrile 25/25/50 (v/v/v)) were tested. A mixture was mixed post-column at 3 μl/min with the eluent while blank extracted samples (inter-lot ($n = 6$)) were injected without using the divert valve for each mixture. Responses for each compound 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 and −high levels by processing each sample and by comparing the relative peak area of each compound added to blank extract with the relative peak area of each compound in matrix-free solvent.

2.5.5. Stability

The stability of target compounds 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 8 h, three additional freeze-thaw cycles (thawing at 20 °C during *ca.* 1 h and freezing again at −30 °C for at least one day), and storage at −30 °C for 2 months, respectively. Furthermore, a complete series of QC samples ($n = 24$) was re-injected, together with freshly prepared calibration samples, after additional storage of the diluted extracts

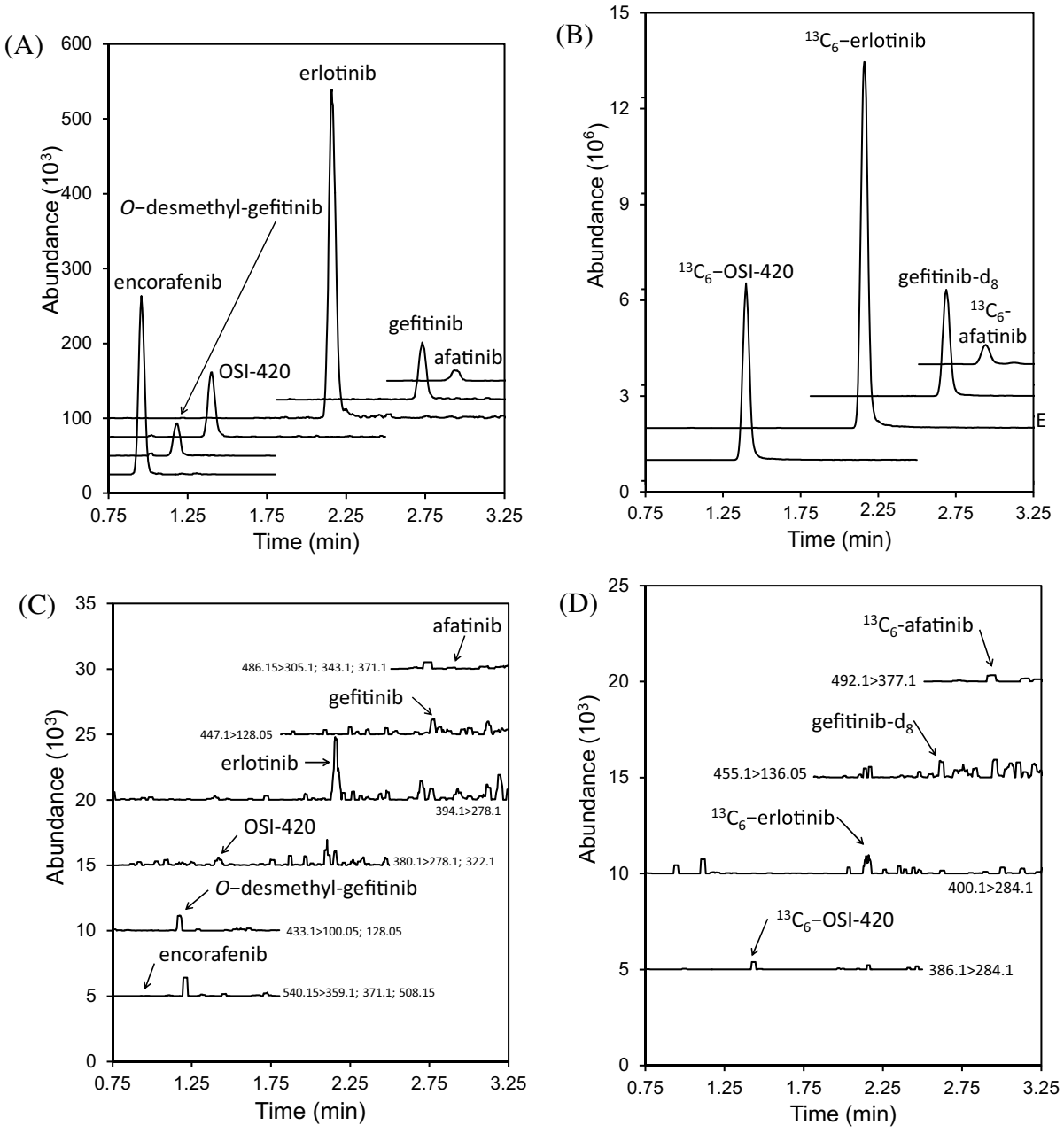

Fig. 2. SRM chromatograms of all compounds: (A) Target compounds at LLOQ levels (10 ng/ml encorafenib, 5 ng/ml erlotinib, 2 ng/ml gefitinib, 1 ng/ml of afatinib, OSI-420 and *O*–desmethyl-gefitinib) with (B) corresponding internal standards; (C) blank responses of target compounds with (D) corresponding blank responses of internal standards.

at 4 °C for five nights, to test the stability in the final extract in the auto-injector.

Finally, the responses of all target compounds (except afatinib) from the stock solutions in methanol after 6 h at 20 °C ($n = 2$) and after 2 months at −30 °C ($n = 2$) were compared to fresh stock solutions with LC–MS/MS after appropriate dilution of the samples and adding the ISs.

2.6. Therapeutic drug monitoring

The assay was used to monitor plasma levels of drugs and metabolites in treated patients. Drug analysis was part of a therapeutic drug monitoring service and inter-laboratory quality control. Plasma samples of 5 patients treated with erlotinib, 5 with gefitinib and 3 (from 2 different patients) with afatinib were assayed. OSI-413 was quantified semi-quantitatively using the OSI-420 calibration standards. For encorafenib only a whole blood sample was available, this sample was measured after 5-fold dilution with blank plasma. Therefore, an additional QC-sample in whole blood was assayed analogously at 1000 ng/ml ($n = 6$) using the same dilution with plasma. In order to test incurred sample reanalysis all 14 samples were analyzed a second time in a separate run.

3. Results and discussion

3.1. Method development

Electrospray ionization-MS/MS settings were optimized for all target compounds to obtain maximal sensitivity; product spectra are presented in Fig. 1 with the proposed dissociation pathways. Based on chromatographic peak areas, methanol showed higher

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

Compound	Nominal concentration (ng/ml)	Within-day precision (%)	Between-day precision (%)	Accuracy (%)
encorafenib	8000	8.6	10.4	93.5
	400	7.7	10.3	94.8
	20	6.8	9.3	96.7
	10	8.4	12.3	98.9
O–desmethyl-gefitinib	800	8.4	8.7	95.1
	40	7.9	8.8	93.7
	2	8.0	8.1	97.7
	1	7.9	10.2	102.4
OSI-420	800	2.4	3.7	103.0
	40	2.7	3.0	105.2
	2	5.3	6.8	105.8
	1	6.8	9.1	100.0
erlotinib	4000	2.7	2.8	99.9
	200	1.5	2.2	101.7
	10	1.9	5.2	102.8
	5	3.9	9.2	102.5
gefitinib	1600	3.0	3.8	97.7
	80	3.6	4.3	104.7
	4	4.2	6.3	106.4
	2	6.6	10.5	107.2
afatinib	800	3.9	4.0	94.9
	40	2.8	3.3	98.9
	2	11.2	11.2	101.5
	1	11.7	11.9	101.7

responses than acetonitrile. In addition, ammonium hydroxide in the eluent showed higher responses than formic acid. Both, signal and noise improved using a relatively low ammonium hydroxide concentration. Afatinib initially showed almost equal responses for the single and double charged protonated ions under acidic conditions. In addition to the use of ammonium hydroxide the use of the skimmer off-set also favored the formation of the single charged ion.

Chromatographic conditions were derived from our earlier reported assay for afatinib in mouse plasma [15]. However, because acetonitrile showed narrower peaks than methanol, a mixture of both was applied as a compromise between optimal detector response and peak shape. Gradient profile and column temperature were optimized to obtain maximal resolution between both *O*–desmethyl-erlotinib isomers (OSI-413 as well as OSI-420 were initially obtained using a liver microsomal incubation of erlotinib), sufficient retention for the first eluting compound and a run time as short as possible. During initial experiments encorafenib and *O*–desmethyl-gefitinib showed a day-to-day increase of the retention and it was shown that the pH of 0.1% ammonium hydroxide (10.7 when freshly prepared) was lower in older solutions, typically about one unit after two weeks in used solvent remainders. This pH shift can probably be explained by the absorption of carbon dioxide from air. The retention shifts can be explained by the pK_a values of the sulfonamide group in encorafenib and the phenolic acid group in *O*–desmethyl-gefitinib near this pH, resulting in a loss of charge at such a negative pH shift. Therefore, ammonium hydroxide solutions were prepared freshly daily. Further, the beneficial increase in retention of encorafenib and *O*–desmethyl-gefitinib at lower pH, eluting closer to the other compounds (and the ISs) then, resulted in the decision to add a small amount of formic acid to the ammonium hydroxide solution to obtain pH 9.8.

The use of the selective MS/MS detection and sufficient chromatographic retention facilitated the use of a simple pre-treatment procedure. Protein precipitation using acetonitrile or another organic solvent is a simple procedure used frequently for the bioanalysis of kinase inhibitors [9] including for example erlotinib and gefitinib included in the present assay. In addition, the development of a more efficient extraction procedure may be a tedious task for four drugs and the metabolites, all possessing different chemical properties. Strongly retained constituents like phospholipids, remaining from the plasma matrix 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.

Stable isotopically labeled ISs were available for four target compounds: erlotinib, OSI-420, gefitinib and afatinib. No detectable (all $\leq$0.01%) cross-analyte mass spectrometric interferences were observed for target compounds and the labeled analogues. For encorafenib and *O*–desmethyl-gefitinib, being the two fastest eluting compounds, the first eluting IS, $^{13}C_6$–OSI-420, showed the best performance in terms of accuracy and precision. For OSI-413, the most prominent metabolite of erlotinib [12,13] and being the isomer of iso420 with the other *O*–methyl group desmethylated, reference compounds were extremely expensive and relatively impure at the start of the project. Therefore, this compound was not included in the validation of the assay but semi-quantitative analysis was performed using OSI-420 calibration standards. The OSI-413 peak was assigned in clinical samples based on the transitions similar to OSI-420 and because of the same elution order and similar OSI-413/OSI-420 ratios compared to previous studies [12,13] and the microsomal product used during method development.

3.2. Validation

Different calibration ranges were used for different compounds because higher clinical levels of erlotinib and encorafenib compared to the other compounds [9,10,12,13] could be expected. SRM chromatograms of blank and LLOQ spiked samples are depicted in Fig. 2.

3.2.1. Calibration

The relative responses of compounds with a labeled IS showed linearity in the whole investigated range. For encorafenib and *O*–desmethyl-gefitinib a second degree polynomial function was the most suiting alternative. 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 8.1% were observed for all compounds (Table 2), indicating a good suitability of the linear and second degree polynomial regression

Table 4
Results of therapeutic drug monitoring in plasma during once daily oral dosing.

sample number	Dose (mg) Encorafenib	Age (y)	Sex	Time point (h)[b]	Levels (ng/ml) Encorafenib		Ratios (%)		
1	200 Erlotinib	77	M	1.9	1527[c] erlotinib	OSI-420	OSI-413	OSI-413/420	OSI-413/erlotinib
2	150	70	F	5.5	1067	16.3	55.5	342	5.2
3	150	83	F	3.2	1681	27.5	65.6	239	3.9
4	100	70	F	22.0	1263	26.3	56	213	4.4
5	75	80	M	2.4	1397	21.5	45.5	211	3.3
6	150 Gefitinb	63	F	2.8	1831 gefitinib	32.8 O−desmethyl-gefintinib	67.2	205 O−desmethyl-gefintinib/gefitinib	3.7
7	250	42	M	15.7	339	121	33		
8	250	60	F	25.4	155	187	120		
9	250	50	F	20.3	737	381	52		
10	250	49	F	17.8	172	77	45		
11	250 Afatinib	42	M	13.8	955 afatinib	203	21		
12	40	51	M	2.5	15.3				
13	30	67	F	4.3	42.6				
14[a]	30	67	F	7.3	31.1				

[a] From same patient as sample 13.
[b] Time after the last dose.
[c] Whole blood instead of plasma.

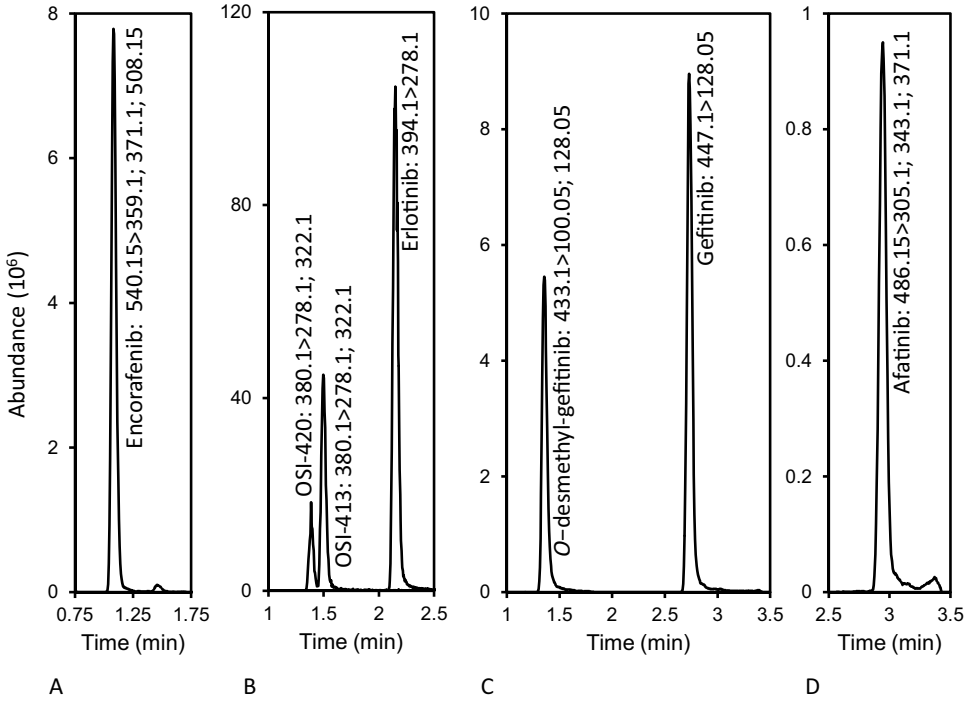

Fig. 3. SRM chromatograms from samples of patients receiving (A) encorafenib; sample 1, (B) erlotinib; sample 2, (C) gefitinib; sample 7 and (D) afatinib; sample 12. Quantitative sample results and patient characteristics are reported in Table 4. The signal of the of *O*−desmethyl-erlotinib mass transition (OSI-420 and OSI-413) has been multiplied a factor 10.

models [16,17]. Further, variations of the slope and regression coefficient were relatively small when a labeled IS was used and variability of the erlotinib calibrations was superior compared to the other compounds.

3.2.2. Accuracy and precision

Assay performance data of the validation samples at four concentrations are reported in Table 3. Within-run and between-run variations ≤12.3% were observed with lower values for higher concentrations and for the compounds with a labeled IS available. Accuracies ranged from 93.5-107.2%. 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 human plasma samples showed no significant interfering peaks in the SRM traces for all compounds. Blank responses were all ≤6% of the LLOQ response of the target compounds, meeting the required 20% [17], and ≤0.12% of the IS responses. The accuracies and precisions at the LLOQ levels ($n = 6$) are reported in Supplemental Table 1, these data demonstrate the applicability of the investigated LLOQ levels as well as

the absence of endogenous compounds interfering with the quantification of the analytes [16].

3.2.4. Recovery and matrix effect

Extraction recoveries showed not more than minor losses with average recoveries ranging from 79.9-102.8% (data not shown) in plasma. Using the post-column infusion experiment, matrix effects ($n = 6$, inter-lot) were only observed in irrelevant time ranges (suppression from 0.14-0.8 min for all compounds and some enhancement at 1.2 min in the transitions of erlotinib, $^{13}C_6$−erlotinib and $^{13}C_6$−OSI-420). The inter-lot ($n = 6$) average relative matrix factors at the QC-low levels ranged from 91.4-101.1% at the QC-low levels and from 88.2–103.0% at the QC-high levels (Supplemental Table 2). Overall, the small extraction losses and the absence of relevant matrix effects contributed to a successful validation of the assay.

3.2.5. Stability

The stability of six target compounds in human lithium heparin plasma after different storage procedures is presented in Supplemental Table 3. All drugs and metabolites showed sufficient stability (levels within ±15% [17]) under all conditions. Extracted QC samples were re-analyzed after additional storage at 4 °C for 5 days, and all analytes were stable under these conditions. The maximum number of QC failures out of 24 was two for all compounds, thus QC failures remained far below a 33% frequency as required in an analytical run [16,17] during five days for all compounds.

Recoveries in methanolic stock solutions after additional storage were all within ±5% for encorafenib, erlotinib, OSI-420, gefitinib and, *O*−desmethyl-gefitinib. Recoveries ranged from 96.4 to 103.4% after 6 h at 20 °C ($n = 2$) and from 97.0 to 103.9% after 2 months at −30 °C ($n = 2$), respectively. For afatinib, sufficient stability under these conditions was reported previously [15].

3.3. Therapeutic drug monitoring

After a successful validation procedure, the new assay was used to monitor of drug levels and metabolites in 14 samples of 13 patients. Results are shown in Table 4 and for each drug a chromatogram including metabolites is shown in Fig. 3. All levels were within the validated ranges. For encorafenib in whole blood (1000 ng/ml, diluted 5–fold with plasma) the precision was 13.1% and the accuracy 95.5%, the results of this experiment were also successful for all other compounds (data not shown).

Erlotinib and its metabolites levels show relatively small variations and drug levels are well within known therapeutic ranges [18]. Further, ratios of both desmethyl metabolites confirm the recent new insights about OSI-413 being the main metabolite [12,13]. Gefitinib, administered at a fixed daily dosage to each patient, and its metabolite levels show relative large variations. This may be an indication for the benefit of therapeutic drug monitoring in treatment with this drug. However, in a very recent paper by Kobayashi et al. [19] variations in gefitinib an *O*−desmethyl-gefitinib exposure, resulting from polymorphisms in the Cytochrome P450 2D6 enzyme and the ABCB1 and ABCG2 drug transporters, had no relation with any of the investigated side effects in 36 Japanese patients with NSCLC.

Reanalysis of all 14 samples resulted only for one (semi-quantitative) measurement of OSI-413 in a difference exceeding the ±20% limit of the guidelines [17] for incurred sample reproducibility. All other 28 levels measured were within this limit that should be met by at least 67% of the samples.

4. Conclusions

The first fully validated assay for the quantification of encorafenib in plasma has been reported now. The LC–MS/MS assay includes a fast and simple sample pre-treatment procedure, and can also monitor three EGFR inhibitors and some of their metabolites. Results showed values of accuracy, precision, recovery and stability compliant to international guidelines [16,17]. The new assay was successfully used to the monitor the individual drugs and metabolites in patients and can therefore also be a useful tool when encorafenib will be combined with erlotinib, gefitinib or afatinib.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2016.09.012.

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