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Bioanalysis of EGFRm inhibitor osimertinib, and its glutathione cycle- and desmethyl metabolites by liquid chromatography-tandem mass spectrometry

J.J.M. Rood [a], M.J. van Haren [b], J.H. Beijnen [a,c], R.W. Sparidans [a,b,*]

[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] The Netherlands Cancer Institute, Department of Pharmacy & Pharmacology, Louwesweg 6, 1066 EC Amsterdam, the Netherlands

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ABSTRACT

Osimertinib is a "third-generation" oral, irreversible, tyrosine kinase inhibitor. It is used in the treatment of non-small cellular lung carcinoma and spares wild-type EGFR. Due to its reactive nature, osimertinib is, in addition to oxidative routes, metabolized through GSH coupling and subsequent further metabolism of these conjugates. The extent of the non-oxidative metabolism of osimertinib is unknown, and methods to quantify this metabolic route have not been reported yet. To gain insight into this metabolic route, a sensitive bioanalytical assay was developed for osimertinib, the active desmethyl metabolite AZ5104, and the thio-metabolites osimertinibs glutathione, cysteinylglycine, and cysteine conjugates was developed. The ease of synthesis of these metabolites was a key-part in the development of this assay. This was done through simple one-step synthesis and subsequent LC-purification. The compounds were characterized by NMR and high-resolution mass spectrometry. Sample preparation was done by a simple protein crash with acetonitrile containing the stable isotopically labeled internal standards for osimertinib and the thio-metabolites, partial evaporation of solvents, and reconstitution in eluent, followed by UHPLC–MS/MS quantification. The assay was successfully validated in a 2–2000 nM calibration range for all compounds except the glutathione metabolite, where the LLOQ was set at 6 nM due to low accuracy at 2 nM. Limited stability was observed for osimertinib, AZ5104, and the glutathione metabolite. The clinical applicability of the assay was demonstrated in samples of patients treated with 80 mg osimertinib once daily, containing all investigated compounds at detectable and quantifiable levels.

1. Introduction

Osimertinib (Fig. 1) is a "third-generation" tyrosine kinase inhibitor (TKI), that targets sensitized mutant epidermal growth factor receptor (EGFRm) and TKI-resistant mutant T790 M non-small cellular lung carcinoma (NSCLC). It is an oral, irreversible, selective inhibitor that spares wild-type EGFR [1]. When in a favorable position in the target protein, osimertinib covalently binds to the cysteine 797 residue within the ATP binding pocket of the EGFRm, and irreversibly inhibits the target. Two circulating metabolites of osimertinib are the active desmethyl metabolites, encoded earlier as AZ5104 and AZ7550 (Fig. 1), at roughly 10% of total osimertinib related material [2]. The reactive nature of osimertinib gives way for a metabolic pathway that – for most therapeutics – rarely plays a role. Coupling of osimertinib to glutathione (GSH), and subsequent downstream metabolism to other small molecule thiol (SMTs) conjugates has been previously described by Dickinson et al., who suggested that GSH conjugates are formed chemically and not catalyzed by glutathione-S-transferase, but the extent had not been quantified [2]. For three different TKIs, Shibata and Chiba reported the correlation between the predicted clearance from hepatocyte incubations and total body clearance [3]. A mismatch between *in-vivo* and *in-vitro* clearance however was found, most likely caused by extrahepatic conjugation to glutathione, a route that is less prominently present in the investigated hepatocyte systems. The lack of quantitative insight in

* Corresponding author at: Utrecht University, Faculty of Science, Department of Pharmaceutical Sciences, Division of Pharmacology, Universiteitsweg 99, 3584 CG Utrecht, The Netherlands.
E-mail address: r.w.sparidans@uu.nl (R.W. Sparidans).

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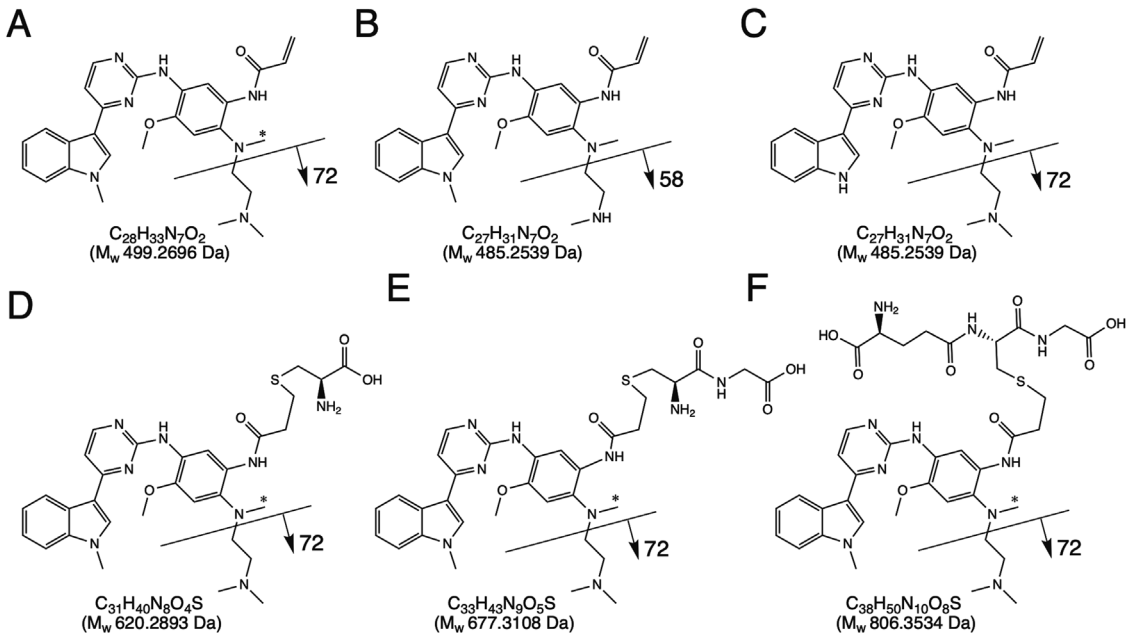

Fig. 1. Structures, molecular formula, calculated monoisotopic molecular weight, and proposed fragmentation for A) osimertinib, B) AZ7550, C) AZ5104, D) OCYS, E) OCGS, and F) OGSH. The locations of the $^{13}C^2H_3$-labels are denoted with an asterisk (*).

this route of metabolism might cause hepatocyte and (recombinant) cytochrome P450 studies to overestimate the role of oxidative metabolism. GSH-conjugates are quickly metabolized to a cysteinyl-glycine (CGS) conjugate by γ-glutamyl transferase (γ-GT), and then further metabolized to a cysteine (CYS) conjugate by dipeptidases. The final step before elimination via either the urine or – as in this case – feces is acetylation of the cysteine residue to form a mercapturate [2]. While earlier assays were published on the bioanalysis of osimertinib in human [4,5] and rat plasma [6], none have been developed for AZ7550 or any of the osimertinib-SMTs (OSMTs) so far. The reactivity of osimertinib allows for relatively simple synthesis of osimertinib-GHS (OGSH, M10), osimertinib-CGS (OCGS, M8) and osimertinib-CYS (OCYS, M21). This approach has been used before by us for ibrutinib [7]. In this study we describe the synthesis of the three OSMTs, and the development and validation of a simple, sensitive and specific assay for osimertinib, AZ5104, OGSH, OCGS, and OCYS in human plasma, and the semi-quantitative determination of AZ7550. The clinical applicability of the developed assay was demonstrated by the analysis of plasma samples of NSCLC patients treated with osimertinib.

2. Materials and methods

2.1. Chemicals and reagents

Osimertinib (>99.9%, M_w:499.61 g/mol) was obtained from Sequoia Research Products (Pangbourne, UK) and the metabolite AZ5104 (>99%, M_w:485.59 g/mol) from Selleckchem (Houston, TX, USA). [$^{13}C^2H_3$]-labeled osimertinib (>98%, 99.4% ^{13}C, 99.8% $^2H_3$, M_w:503.63 g/mol) was obtained from Alsachim (Illkirch Graffenstaden, France). Acetonitrile of HPLC-S gradient grade quality was from Biosolve (Valkenswaard, The Netherlands). Water for all purposes was purified in-house by a Synergy UV apparatus (Merck Millipore, Darmstadt, Germany). Dimethyl sulfoxide (DMSO, analytical grade) was supplied by VWR (Fontenais-sous-Bois, France) and deuterated DMSO (DMSO-d_6) by Buchem (Apeldoorn, The Netherlands). Cysteinylglycine was acquired from Bachem (≥95%, Bubendorf, Switzerland), and formic acid (≥98%), cysteine−HCl (≥98%), reduced glutathione (≥98%), and all other chemicals from Merck (Darmstadt, Germany). Human sodium-EDTA plasma was obtained from Sera Laboratories (Haywards Heath, West Sussex, UK). For the evaluation of selectivity and matrix effect, human sodium-EDTA plasma of six individual donors were obtained from Innovative Research (Novi, MI, USA).

2.2. Synthesis of small molecular thiol-conjugates

For the synthesis of the osimertinib thio-metabolites, 2 mL of 2 mM osimertinib in ethanol was added to 2 mL of a 50 mM thiol-solution in water in a glass vial. The pH of each combined solution was adjusted to 10 with 25% (w/v) NH_4OH, and the mixture was allowed to react overnight at room temperature (18 h). The amount of residual osimertinib was quantified by LC–MS/MS to check whether the reaction was complete. After completion of the reaction, the end-products were purified using semi-preparative HPLC. After filtration of insoluble products, the whole mixture was purified using a ReproSil-pur 120 C18-AQ column (250 × 10 mm, 10 μm particle size, Dr. Maisch, Ammerbusch, Germany) and a chromatographic system that consisted of two LC10 pumps and an SPD10Av UV/VIS detector (Shimadzu, 's-Hertogenbosch, The Netherlands) coupled to a Gilson 215 fraction collector (Gilson, Middleton, WI). Mobile phase A was 0.1% (v/v) formic acid in water, and mobile phase B 0.1% (v/v) formic acid in acetonitrile, with a flow rate of 6 mL/min and with gradient elution described as percentage B. A gradient program with linear steps was used (0 min. 2%, 5 min. 2%, 22 min. 25%, 23 min. 80%, 24 min. 80%, 25 min. 2%, 30 min. 2%). The fractions were collected based on their UV absorption (214 and 256 nm) and were combined after confirmation with LC–MS data for $[M+H]^+$ at *m/z* 621.3 (OCYS), 678.3 (OCGS), and 807.2 (OGSH). After pre-concentration by rotary evaporation (Buchi R100, Flawil, Switzerland) and dilution in *tert*-butanol/water (1:1, v:v), the compounds were freeze-dried using a Christ Alpha 2–4 LSC lyophylizer (Osterode am Harz, Germany). The synthesized compounds were stored in sealed vials under nitrogen with a desiccant at −30 °C. For the SIL internal standards of the OSMTs, 10 μl of a 10-fold molar concentration of each thiol in water was added to 10 μL of the 2 mM [$^{13}C^2H_3$]-osimertinib, and NH_4OH (25%(w/v) was added to adjust to a pH to above approximately 9. After 18 h, the amount of resid-

ual [$^{13}C^2H_3$]-osimertinib was quantified by LC–MS/MS, using the non-labeled osimertinib as internal standard.

2.3. Chromatography and MS/MS detection

A binary ultra-high-performance liquid chromatography (UHPLC) system, with two LC-30AD pumps, a SIL30-ACmp autosampler, a CTO-20AC column oven, and a DGU-20A5R degasser, all from Shimadzu was used for chromatography. Gradient elution was done on a Waters Acquity CSH-C18 column (2.1 × 100 mm, 1.7 μm particle size, Waters, Milford, USA), using water containing 0.1% (v/v) formic acid (A) and acetonitrile containing 0.1% (v/v) formic acid (B), with a run-time of 10 min. The solvent composition was held at 10% B for 1 min, and was then increased linearly to 20% at 7 min, to 80% from 7 till 7.5 min, and to 10% at 7.5 min till the end of the run at 10 min. For detection a Sciex QTRAP® 5500 triple quadrupole mass spectrometer, with Analyst 1.6.2, and MultiQuant 3.0.1 software (Sciex, Ontario, Canada) was used. Settings used for the ionization source were curtain gas, 30 psi; collision gas, 'medium'; ionspray voltage, 4500 V; temperature, 500 °C; ion source gas 1, 50 psi; ion source gas 2, 50 psi. Dwell times were 10 ms, and the entrance potential was set to 10 V; compound specific parameters can be found in Table 1. The Q1 *m/z* values for osimertinib, AZ5104 and AZ7550, and the osimertinib-SIL were shifted +1 to the ^{13}C isotope (*m/z* 501.2, 487.2, and 505.2) to avoid saturation of the continuous electron multiplier, and thus improve linearity. [$^{13}C^2H_3$]-osimertinib was used as an ISTD for osimertinib, AZ5104, and AZ7550. The [$^{13}C^2H_3$]-OSMTs were used as ISTDs for and the OSMTs. For the OSMTs, both transitions were summed to improve sensitivity.

2.4. H- nuclear magnetic resonance spectroscopy

The samples were dissolved in DMSO-d_6. The synthesized OCYS, OCGS and OGSH compounds were characterized by ^1H nuclear magnetic resonance (NMR). Spectra were recorded at 500 MHz on a Varian INOVA-500 (Palo Alto, USA), with chemical shifts reported in parts per million (ppm) downfield relative to DMSO (δ 2.50). All samples were shimmed by automated gradient shimming and automatically tuned. Data are reported in the order of multiplicity (singlet (s), doublet (d), triplet (t), quartet (q) and multiplet (m)), coupling constant (*J*) in hertz (Hz) and the number of protons. In case of a broad signal, the multiplicity was preceded by 'br'.

2.5. Purity and HRMS

The purity of the compounds was analyzed by LC-UV on an Agilent 1260 Infinity II system with gradient elution (Agilent Technologies, Santa Clara, Ca, USA) on a Waters Acquity CSH-C18 column (2.1 × 100 mm), using water containing 0.1% (v/v) formic acid (A) and acetonitrile containing 0.1% (v/v) formic acid (B), with a run-time of 60 min and a flow of 0.25 ml/min. The run started at 1% B, and was increased linearly to 100% B from 0.5 till 55 min, after which the column was re-equilibrated at 1% B for 5 min. The purity was evaluated by peak area of absorption at 214 nm. For high-resolution mass spectrometry (HRMS) analysis the eluent was transferred to an Agilent 6560 Ion Mobility Q-TOF instrument.

2.6. Data processing

Sciex Analyst 1.6.2, and MultiQuant 3.0.1 software (Sciex, Ontario, Canada) were used to process quantitative MS data. For further processing of the data Microsoft Excel 2016 for MacOS (version 16.10) was used. NMR spectra were processed using MestReNova 10.0.2-15465 (Mestrelab Research, Santiago de Compostela, Spain).

2.7. Standard solutions and quality controls

Stocks of 1 mM osimertinib and AZ5104 were prepared by dissolving the compounds in an appropriate amount of DMSO. The 2 mM OGSH, OCGS and OCYS stocks were prepared in DMSO-d_6, so that they were suitable for NMR analysis. Separate combined working solutions of 200 nM in DMSO were made from these stocks and were used to obtain the calibration standards and QC-samples in human plasma. For the calibration, standards of 2000, 1000, 500, 200, 100, 50, 20, 10, 5 and 2 nM were made by serial dilution for each daily calibration. High, medium, and low QC-samples of 1600, 400, and 6 nM and LLOQ samples of 2 nM were prepared from stocks and working solutions. Aliquots of QCs were stored at −30 °C. The internal standard solution to be used in sample preparation consisted of [$^{13}C^2H_3$]-osimertinib (100 nM) and each of the [$^{13}C^2H_3$]-osimertinib-SMTs (50 nM) dissolved in 0.2% (v/v) formic acid in acetonitrile and stored at −30 °C. The formic acid was added to improve stability by lowering the reactivity of osimertinib towards any thiol.

2.8. Sample preparation

All calibration, QC, and plasma samples were kept on ice during thawing and treatment to prevent degradation. Sample preparation was performed by addition of 100 μl acetonitrile containing the four ISTDs to 50 μL plasma in a 96-deep well polypropylene plate. The plate was closed and shaken for 5 min at 1000 RPM, and then centrifuged for 5 min at 3500 × *g*. Fifty μL of the supernatant was transferred to a microtiter plate, and the solvent was evaporated to approximately 10–15 μL under a gentle stream of nitrogen for 10 min (Porvair MiniVap, 40 L/min, 40 °C). The samples were diluted with 40 μL water containing 0.1% (v/v) formic acid to ensure compatibility with the chromatographic system and mixed for 5 min at 1000 RPM before LC–MS/MS analysis.

2.9. Analytical method validation

A laboratory scheme based on international guidelines, published by the EMA and FDA was used for the validation procedures [8,9].

2.9.1. Calibration

For each daily calibration, calibration standards were prepared as described above. The calibration samples were processed in duplicate with duplicate accompanying blanks. For all conjugates least-squares $1/x^2$ weighted linear regression of the analyte/ISTD peak ratios was used to define the calibration curves, where x is the concentration of the analyte. For osimertinib and AZ5104 Hill regression was used alternatively. AZ7550 was quantified in a semi-quantitative manner using the calibration of AZ5104 and the SIL-osimertinib as an ISTD.

2.9.2. Precision and accuracy

The accuracies and precisions were determined by analyzing the three QC-levels and LLOQ samples in six-fold, on three separate days, with a total of 18 samples per QC-level. For both the within- and between-day precisions relative standard deviations were calculated.

2.9.3. Selectivity

Selectivity was assessed by analyzing six separate human plasma samples spiked with each compound at its LLOQ level sup-

Table 1
Tuned MS/MS parameters for all quantified components and ISTDs. The entrance potential was set at 10 v for all compounds, dwell-time was 10 msec.

Compound	Q1 (m/z)	Q3 (m/z)	z	DP	CE	CXP
Osimertinib (^{13}C)	501.2	72.1	1	151	75	10
AZ-5104 (^{13}C)	487.2	72.1	1	156	65	10
AZ-7550 (^{13}C)	487.2	58.0	1	150	75	10
OCYS	621.3	72.1	1	160	100	34
	311.2	72.1	2	75	20	34
OCGS	678.3	72.1	1	155	110	33
	339.6	72.1	2	70	20	33
OGSH	807.2	72.1	1	160	125	31
	404.2	72.1	2	62	20	31
Osimertinib-SIL ($^{13}C_2$)	505.2	72.1	1	151	75	10
OCYS-SIL	625.3	72.1	1	160	100	34
	313.2	72.1	2	75	20	34
OCGS-SIL	682.3	72.1	1	155	110	33
	341.6	72.1	2	70	20	33
OGSH-SIL	811.2	72.1	1	160	125	31
	406.2	72.1	2	62	20	31

Q1: quadrupole 1, Q3: quadrupole 3, z: charge, DP: declustering potential, CE: collision energy, CXP: collision cell exit potential. (^{13}C) indicates that the Q1 *m/z* was shifted + 1 from the most abundant *m/z*.

plemented with the ISTDs. Samples were also processed without analytes and ISTDs.

2.9.4. Stability

Stability of osimertinib was tested previously [4]. The stability of AZ5104, OGSH, OCGS and OCYS was tested in separate samples at the high (1600 nM), medium (400 nM) and low (6 nM) QC-levels. For long term stability, four samples of each level were prepared, and stored at −30 °C for 90 days. For freeze-thaw stability, samples underwent three cycles of thawing on wet ice for 1 h and freezing at −30 °C for 12–24 h, and thereafter they were analyzed. An analytical run was re-injected after additional storage of the extracts at 4 °C for 48 h to test the stability of extracted samples. The stability of stock solutions was tested by comparing the responses of osimertinib, AZ5104, OGSH, OCGS, and OCYS from the stock solutions in DMSO after 2 weeks at room temperature ($n = 2$) and after 2 months at −30 °C ($n = 2$) to fresh stock solutions with LC–MS/MS after appropriate dilution of the samples with 10% (v/v) acetonitrile and adding ISTDs.

2.10. Analysis of osimertinib and its metabolites in human plasma

For 5 NSCLC patients receiving oral osimertinib therapy, 80 mg once daily, osimertinib and metabolite blood-plasma levels were analyzed. The analysis was performed on samples taken as part of a therapeutic drug monitoring service to support therapy. The samples were analyzed in two separate runs to evaluate incurred sample reanalysis.

3. Results and discussion

3.1. Synthesis of small molecular thiol-conjugates

After overnight synthesis, non-soluble reaction by-products (only for OCYS, where most likely the insoluble cystine is formed) were removed by centrifugation. The reaction mixtures were purified by LC on a semi-preparative scale, and the fractions were lyophilized to remove the solvents, producing off-white to yellow powders. All three compounds had NMR spectra and mass spectra consistent with the assigned structures, as shown below. The [$^{13}C^2H_3$]-OSMTs were not further purified after synthesis, as there was not enough starting material available for synthesis and subsequent purification of these compounds, and the exact quantities and yields were not relevant for their use as ISTDs. It was confirmed that less than 1% of the [$^{13}C^2H_3$]-osimertinib starting material was present.

3.1.1. Purity and HRMS

The purities of the compounds were calculated at 94.0% for OCYS, 98.9% for OCGS, and 95.6% for OGSH from the total peak area at 214 nm absorption, without any detectable amounts of osimertinib or the other compounds. For HRMS the calculated *m/z* values for OCYS ($C_{31}H_{40}N_8O_4S$) were 621.2966 for $[M+H]^+$, and 311.1520 for the $[M+2H]^{2+}$. The *m/z* values were found at 621.2983 (Δm/z: $1.7 \cdot 10^{-3}$), and 311.1531 (Δm/z: $1.1 \cdot 10^{-3}$). For OCGS ($C_{33}H_{43}N_9O_5S$) the calculated *m/z* were [M+H]+ 678.3186 and [M + 2H]2 + 339.6632, and the *m/z* values 678.3200 (Δm/z: $1.4 \cdot 10^{-3}$) and 339.6638 (Δm/z: $0.6 \cdot 10^{-3}$) were found. Calculated *m/z* values for OGSH ($C_{38}H_{50}N_{10}OS$) were $[M+H]^+$ 807.3612 and $[M+2H]^{2+}$ 404.1845. The found *m/z* values were 807.3626 (Δm/z: $1.4 \cdot 10^{-3}$) and 404.1852 (Δm/z: $0.7 \cdot 10^{-3}$).

3.1.2. H NMR-data

The recorded proton-NMR spectra were referenced on the DMSO solvent peak, and the peaks were noted and compared to assigned structures. OCYS ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.77 (s, 1H), 8.98 (s, 1H), 8.62 (s, 1H), 8.34 – 8.27 (m, 3H), 8.25 (d, J = 8.1 Hz, 1H), 7.89 (s, 1H), 7.53 (d, J = 8.2 Hz, 1H), 7.25 (t, J = 7.7 Hz, 1H), 7.21 (d, J = 5.3 Hz, 2H), 7.16 (t, J = 7.5 Hz, 1H), 6.99 (s, 1H), 3.91 (s, 3H), 3.84 (s, 3H), 3.43 – 3.36 (m, 1H), 3.02 – 2.98 (m, 3H), 2.93 – 2.59 (m, 7H), 2.69 (s, 3H), 2.34 (s, 6H).

OCGS ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.63 (s, 1H), 9.00 (s, 1H), 8.64 (s, 1H), 8.31 (d, J = 5.1 Hz, 1H), 8.24 (d, J = 8.1 Hz, 1H), 8.12 (br s, 1H), 7.88 (s, 1H), 7.53 (d, J = 8.3 Hz, 1H), 7.25 (t, J = 7.6 Hz, 2H), 7.22 (d, J = 5.3 Hz, 1H), 7.16 (t, J = 7.5 Hz, 1H), 6.98 (s, 1H), 3.92 (s, 3H), 3.85 (s, 3H), 3.62 (s, 2H), 3.07 – 3.03 (m, 3H), 2.72 – 2.62 (m, 7H), 2.41 (s, 6H).

OGSH ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.61 (s, 1H), 9.03 (s, 1H), 8.65 (s, 1H), 8.43 (d, J = 8.1 Hz, 1H), 8.31 (d, J = 5.3 Hz, 1H), 8.29 (br s, 1H), 8.24 (s, 3H), 7.89 (s, 1H), 7.55 – 7.51 (m, 1H), 7.25 (t, J = 7.6 Hz, 1H), 7.22 (d, J = 5.4 Hz, 1H), 7.16 (t, J = 7.6 Hz, 1H), 6.99 (s, 1H), 4.48 (q, J = 8.3 Hz, 1H), 3.92 (s, 3H), 3.85 (s, 3 H), 3.68-3.51 (m, 2H), 3.42 – 3.36 (m, 1H), 3.09 – 3.01 (m, 3H), 2.97 (dd, J = 13.9, 4.5 Hz, 2H), 2.86 – 2.78 (m, 3H), 2.75 – 2.62 (m, 6H), 266 (s, 3H), 2.43 (s, 6H), 2.34 – 2.28 (m, 2H), 2.01 – 1.91 (m, 1H), 1.91 – 1.80 (m, 1H).

3.2. Method development

Method development was aimed at using minimal steps for sample preparation in a 96-well format. For this, protein pre-

Table 2
Accuracies and precisions for osimertinib, AZ5104, OCYS, OCGS, and OGSH (n: 3 × 6 = 18 per level).

Compound	Nominal concentration (nM)	Within day precision (%)	Between day precision (%)	Accuracy (%)
Osimertinib	1600	4.3%	3.5%	97.0%
	400	2.7%	4.0%	106.1%
	6	5.9%	7.3%	107.9%
	2	8.7%	11.1%	109.8%
AZ5104	1600	4.6%	4.5%	101.7%
	400	4.6%	4.0%	101.6%
	6	6.4%	5.6%	103.3%
	2	6.1%	5.0%	107.4%
OCYS	1600	3.3%	5.4%	95.3%
	400	6.8%	8.1%	105.7%
	6	13.2%	13.4%	108.7%
	2	17.0%	19.7%	114.0%
OCGS	1600	4.9%	4.8%	96.3%
	400	7.7%	6.7%	105.9%
	6	10.1%	11.3%	110.8%
	2	9.3%	13.7%	99.3%
OGSH	1600	6.0%	4.9%	101.0%
	400	8.1%	8.4%	105.7%
	6	12.6%	10.3%	113.7%
	2	17.6%	16.2%	125.5%[a]

[a] For OGSH, the LLOQ was not successfully validated at the 2 nM level and the 6 nM level was defined as the LLOQ.

cipitation using acetonitrile was selected. A previous assay for only osimertinib used salt assisted liquid-liquid extraction because matrix effect could be detrimental to the method, as no stable isotope was available at that time [4]. The new method used SIL-ISTDs for osimertinib and the OSMT-metabolites, which corrects for most effects. The ion-source parameters were chosen for the optimal response of the OSMTs in SRM-mode. The proposed fragmentation schemes of osimertinib and the metabolites are shown in Fig. 1. Chromatography was performed using a low concentration formic acid (0.1% (v/v)) in water and acetonitrile. These conditions gave sufficient separation of the OSMT-metabolites, avoiding the interference of *'ghost-traces'* in the signals of the OSMT-metabolites due to in-source fragmentation of the respective upstream metabolites.

For osimertinib, AZ5104, and AZ7550 the *m/z* values for the first quadrupole (Q1) were shifted to their mono-^{13}C-isotopes. This approach has been used before in similar cases [7,10]. The method was optimized for the sensitivity of the OSMTs in terms of ion-source parameters and injection volume. This was pivotal because the OSMT-compounds have a significantly less intense signal in the mass spectrometer compared to osimertinib and the desmethyl-metabolites. Lowering of the injection volume or further dilution would also cause the signals of the OSMTs to reduce, decreasing the sensitivity for these compounds. The areas of summed transitions of OGSH were 1.3% of that of osimertinib, and 4.3% of mono-^{13}C-osimertinibs area. The area of mono-^{13}C-osimertinib was 29.7% of the osimertinib response.

3.3. Analytical method validation

A range of 2–2000 nM was chosen for osimertinib, as maximum drug levels were about 1600 nM [4]. The analytical limits of the OSMTs was mainly limited at the LLOQ by the sensitivity for these compounds with relatively poor ionization. A range of three decades was deemed appropriate, allowing for inhibition studies were much higher OSMT metabolites could be expected. Chromatograms can be seen in Fig. 2, showing chromatograms of a blank sample; Fig. 3, a sample spiked at the LLOQ; and Fig. 4, a representative patient sample.

3.3.1. Calibration

Both osimertinib and AZ5104 showed strong non-linearity at the desired range due to the limited linear range of the detector and the relatively high signals of both compounds compared to the conjugates. For this reason, the *m/z* of the parent ions of osimertinib and AZ5104 were shifted + 1 to their ^{13}C isotopes. Still non-linearity occurred, so alternative regression models were tested. For both, osimertinib and AZ5104 Hill (sigmoidal) regression gave the most appropriate fit. Typical regression curves can be seen in Fig. 5, which shows the need for an alternative regression model for osimertinib and AZ5104.

Table 3
Stability of all compounds at 4 h on wet ice, after three freeze thaw cycles, and −30°C for 90 days (mean ± SD, n = 4, osimertinib at −30°C for 75 days [4]).

Long term stability (%)			
Level (nM)	High (1600)	Medium (400)	Low (6)
Osimertinib[a]	92.5 ± 4.7	97.7 ± 9.8	89.1 ± 2.5
AZ5104	87.7 ± 6.3	91.1 ± 2.9	103.3 ± 5.6
OCYS	96.3 ± 4.7	114.3 ± 10.3	110 ± 11.8
OCGS	99.7 ± 6.4	101.9 ± 5	107 ± 9.5
OGSH	74.5 ± 31.6	76.8 ± 24.4	69.5 ± 30.3
Freeze-thaw stability (%, thawing on wet ice)			
Level (nM)	High (1600)	Medium (400)	Low (6)
Osimertinib[a]	95.7 ± 2.4	91.8 ± 1.8	89.1 ± 10.0
AZ5104	92.0 ± 2.2	94.1 ± 8.9	90.7 ± 5.3
OCYS	97.6 ± 5.7	98.2 ± 3.3	103.6 ± 5.6
OCGS	99.6 ± 3.0	96.9 ± 6.8	100.5 ± 10.5
OGSH	90.1 ± 8.4	91.2 ± 3.0	92.2 ± 7.8
Short term stability (%, benchtop on wet ice)			
Level (nM)	High (1600)	Medium (400)	Low (6)
Osimertinib	98.6 ± 3.6	97.6 ± 2.8	95.6 ± 4.2
AZ5104	96.0 ± 6.8	97.1 ± 3.8	96.1 ± 3.6
OCYS	102.7 ± 5.0	99.5 ± 5.8	101.7 ± 4.7
OCGS	96.7 ± 7.1	103.1 ± 3.3	99.9 ± 8.7
OGSH	90.5 ± 7.6	88.0 ± 6.4	91.9 ± 10.7

[a] data from [4], thawing at ambient temperature.

3.3.2. Precision and accuracy

The performance of the assay was evaluated by determining the within- and between-day precisions and the accuracy for three QC-levels and at LLOQ-level in three separate runs. The results are shown Table 2. Except for OGSH, all compounds were successfully evaluated for accuracies and precisions. For OGSH, the accuracy at the lowest level of 2 nM was not within ± 20% of the nominal value (+25.5%). The compound had the lowest intensity, and also the low-

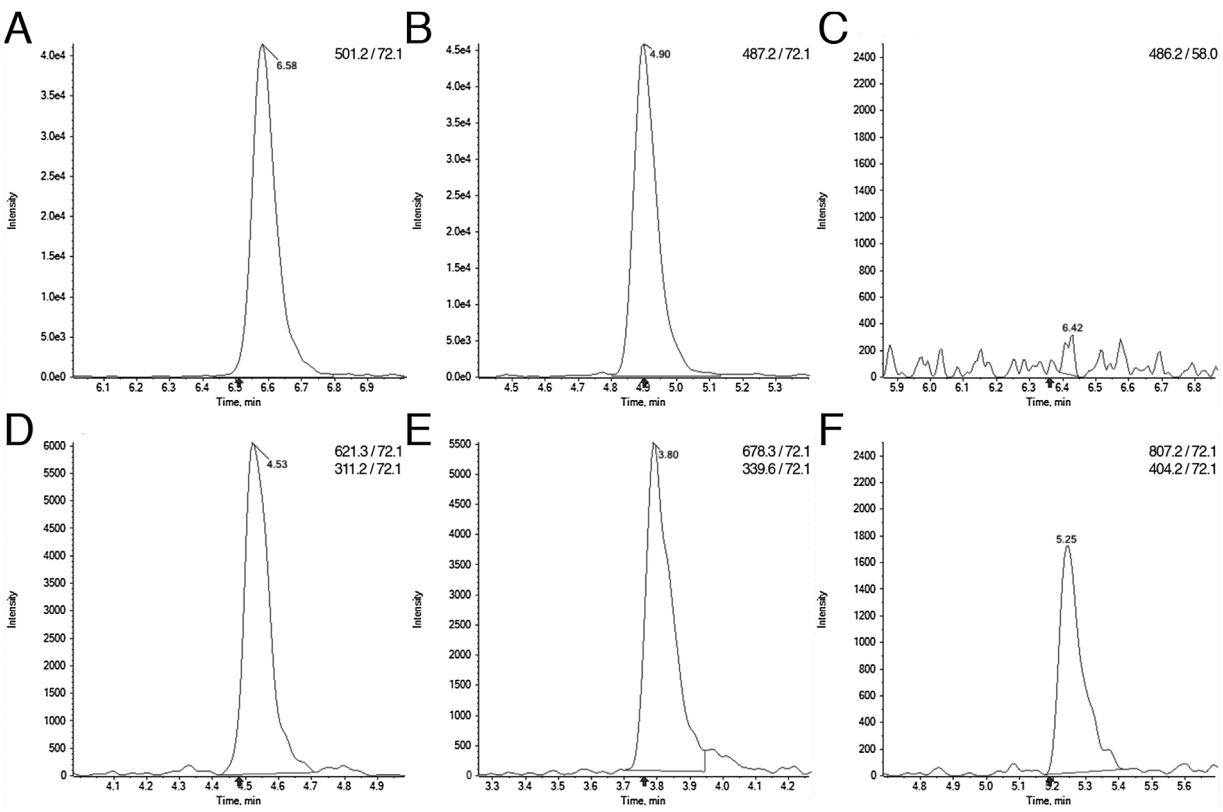

Fig. 2. Chromatogram of A) osimertinib, B) AZ5104, C) AZ7550, D) OCYS, E) OCGS, and F) OGSH for a blank plasma sample, spiked at LLOQ-level (2 nM), except AZ7550. The arrow at the x-axis denotes the expected retention times.

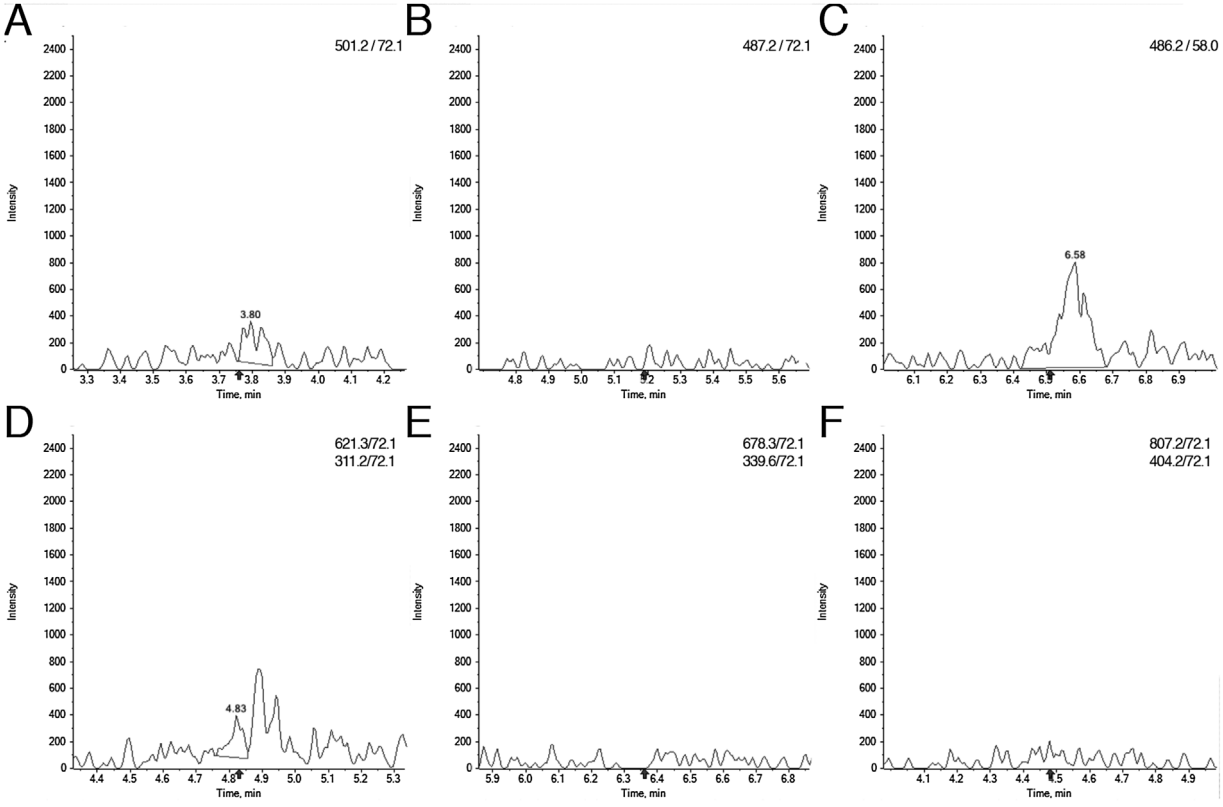

Fig. 3. Chromatogram of A) osimertinib, B) AZ5104, C) AZ7550, D) OCYS, E) OCGS, and F) OGSH for a blank plasma sample. The arrow at the x-axis denotes the expected retention times.

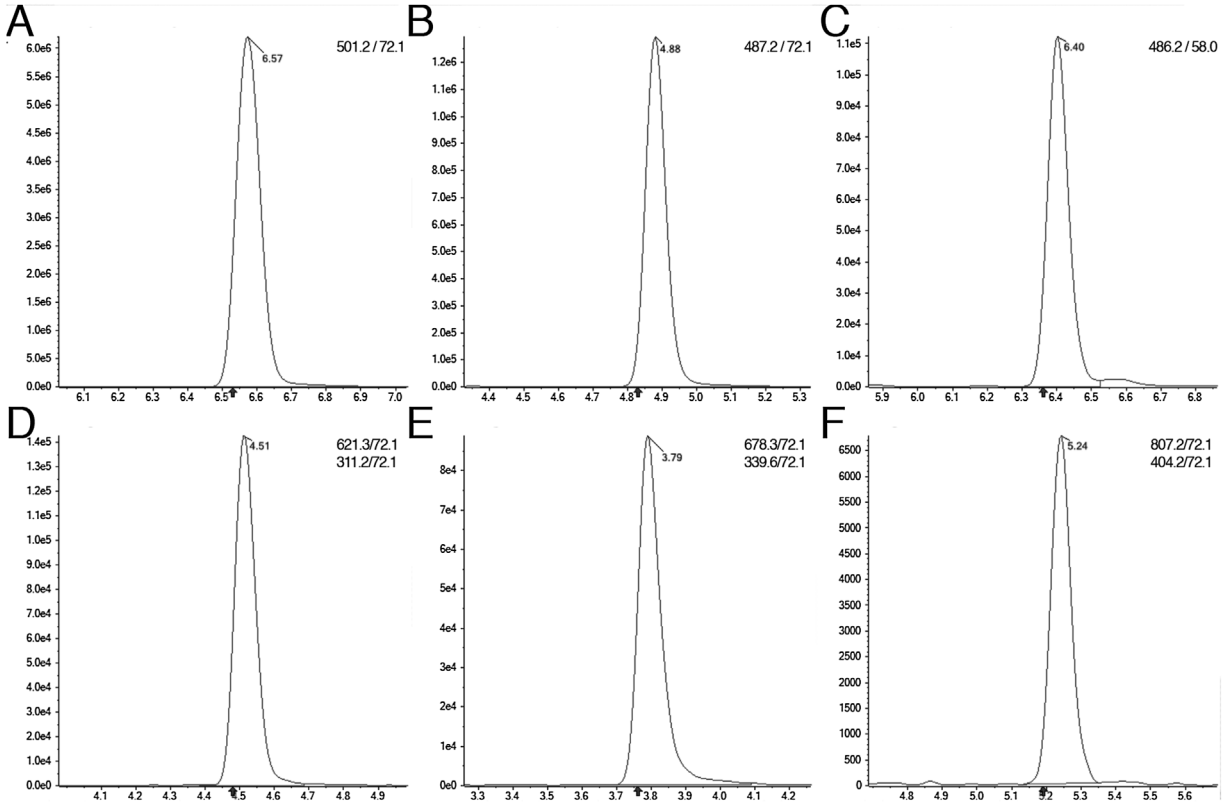

Fig. 4. Chromatogram of A) osimertinib, B) AZ5104, C) AZ7550, D) OCYS, E) OCGS, and F) OGSH a sample from an 88 yr patient receiving 80 mg osimertinib once daily (y-axes are scaled to 100%). The arrow at the x-axis denotes the expected retention times.

est extraction efficiency. These findings combined led to setting of the LLOQ for OGSH at 6 nM.

3.3.3. Selectivity

For six independent blank plasma samples no interfering peaks were shown in the SRM traces for all compounds, and responses of the blanks were all below 20% of the LLOQ response as required by the EMA [8]. Blank ISTD responses were below 0.5% of the normal response (Fig. 3). The signals at the LLOQ level were all distinguishable from blank responses with the signal of the blank ≤ 20% than that of the LLOQ, demonstrating the applicability of the investigated LLOQ level. The *'ghost-traces'* mentioned under method development did not pose a problem. The thiol compounds were separated chromatographically without any overlap (t_Rs: OCYS, 4.53; OCGS, 3.83; OGSH, 5.25 min), and this phenomenon due to in-source fragmentation did not occur.

3.3.4. Stability

For osimertinib, the stability in human plasma is limited at room temperature, with a loss of >10% after 4 h [4,5]. Stability data of osimertinib and the quantified metabolites are shown in Table 3. Among the OSMT-metabolites, only the glutathione-conjugate was unstable in human plasma due to cleavage by γ-GT. For this reason, all samples were prepared and processed on ice, and stored at −30 °C. At −30 °C for 90 days, OGSH is the most unstable compound through conversion to OCGS by plasma γ-GT, and thus still accounts for the amount of osimertinib that is metabolized through this route. OCYS and OCGS were proven stable under the same conditions. AZ5104 shows comparable results to osimertinib itself, with 88–103% recovery (osimertinib: 89.1–97.7% after 75 days) [4]. Stability was sufficient for 4 h on wet ice for all compounds for all compounds. Osimertinib itself shows a degradation of 7.4–11.4% after 4 h in human plasma, as described in our previous publication [4]. After phlebotomy, the blood is handled on ice, and blood cells and plasma are separated at the collection facility. For this reason, only plasma stability experiments were performed. Veerman et al. showed better stability for osimertinib in whole blood compared to plasma [5]. Injection of a validation run, showed that the extracts were stable under the tested conditions in the autosampler (48 h at 4 °C). No QCs exceeded ± 15%, or ± 20% for the LLOQ. Absolute responses were compared and were generally within ± 20% for the overall measurements. Incurred reanalysis of 50 plasma samples also showed comparable results for all samples, no difference between the initial concentration and the repeat analysis was greater than 20%. Stocks were stable for multiple freeze-thaw cycles over a course of two months, and after 2 weeks at room temperature (<±5% in both occasions).

3.3.5. Recovery and matrix effect

The analytes showed good recoveries, with low variation for osimertinib and AZ5104, but for the OSMT-compounds the recoveries were lower, around 50–65% (Table 4). The variation was largely compensated by the ISTDs. Matrix effects (Table 5) varied between compounds, but the ISTDs corrected for the matrix effects of each compound. Since no SIL ISTD was available for AZ5104, SIL-osimertinib was used as an internal standard, potentially leading to a quantitative bias when there is suppression or enhancement of either AZ5104 or SIL-osimertinib [11]. A possible mitigation is the generation of a metabolite-SIL trough in-vitro bio-synthesis using (human) liver microsomes or recombinant enzymes [12].

There seemed to be a concentration dependent matrix effect for the OSMTs, which was corrected for by the internal standards, proving the need for SIL-ISTDs in this method. The RSDs of the relative matrix effect of OGSH were greater than 15%, not satisfying the EMA-guidelines [8]. This was due to a large variation in the response of matrix-free solutions of OGSH that was not present in the spiked blank matrix extracts, possibly due to unspecific binding

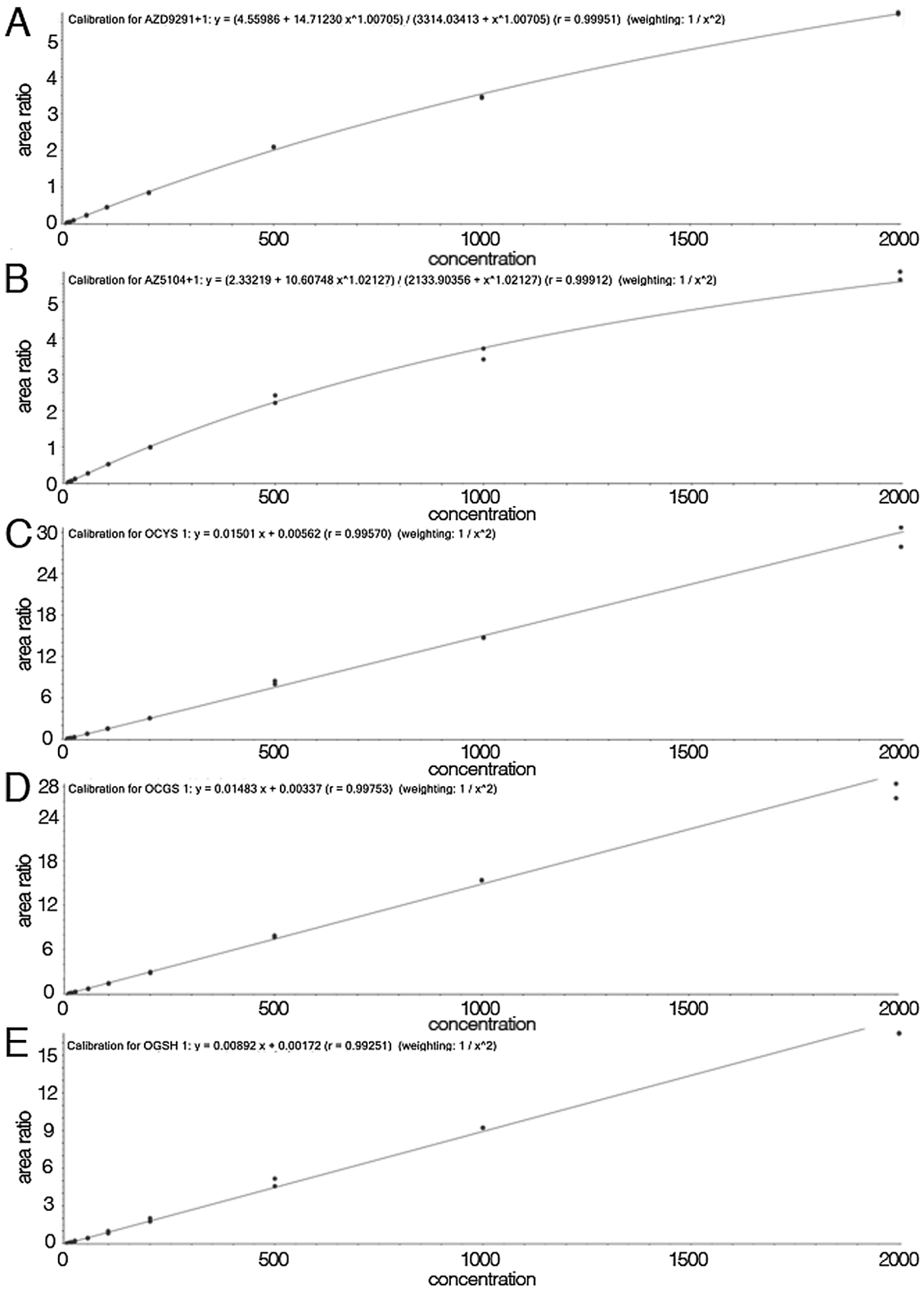

Fig. 5. Typical calibration curves for A) osimertinib, B) AZ5104, C) AZ7550, D) OCYS, E) OCGS, and F) OGSH. Concentration in [nM].

Table 4
Extraction recoveries from plasma for all compounds (mean ± sd, n = 6).

Level (nM)	Osimertinib	AZ5104	OCYS	OCGS	OGSH
High (1600)	95.6 ± 3.5	94.3 ± 2.8	58.4 ± 5.6	55.1 ± 4.5	55.6 ± 4.5
Medium (400)	104.0 ± 5.0	93.6 ± 4.8	58.8 ± 9.4	50.3 ± 8.5	53.6 ± 6.2
Low (6)	94.6 ± 9.3	104.7 ± 10.2	65.3 ± 20.5	62.7 ± 13.9	62.0 ± 35.3

Table 5
Relative and absolute matrix effects for all compounds (mean ± sd, n = 6 per level).

	Level (nM)	Osimertinib	AZ5104	OCYS	OCGS	OGSH
Absolute	High (1600)	96.4 ± 4.7	104.8 ± 2.7	94.8 ± 6.1	85.8 ± 3.8	93.3 ± 4.0
	Medium (400)	83.9 ± 8.5	103.0 ± 5.1	73.6 ± 4.5	68.0 ± 9.4	68.7 ± 37.6
	Low (6)	97.8 ± 15.3	108.4 ± 9.7	61 ± 21.4	66.9 ± 18.5	50.8 ± 38.7
Relative	High (1600)	98.4 ± 3.6	105.9 ± 5.7	97.9 ± 4.6	102.5 ± 3.5	104.1 ± 3.9
	Medium (400)	97.8 ± 5.4	118.1 ± 6.7	88.8 ± 8.0	97.0 ± 7.0	104.7 ± 18.1
	Low (6)	89.3 ± 13.2	113.9 ± 8.9	85.2 ± 13.6	91.5 ± 13.5	94.3 ± 38.1

Table 6
Plasma measurements for all compounds in five patient samples at steady state after once-daily dosing of 80 mg.

	Age (yr.)	t (h)	Osimertinib (nM)	AZ5104 (nM)	AZ7550 (nM)	OGSH (nM)	OCGS (nM)	OCYS (nM)
1	64	1.8	154	39.1	2.77	6.61	10.6	14.3
2	66	22.7	461	75.0	4.87	10.5	37.4	91.9
3	45	4.0	414	42.1	4.19	7.56	18.3	62.6
4	88	3.3	458	79.4	5.28	9.53	18.1	42.2
5	79	4.7	559	67.6	2.44	12.8	36.8	58.4
Mean ± SD	68.4 ± 16.3	7.3 ± 8.7	409 ± 152	60.7 ± 18.8	3.91 ± 1.26	9.39 ± 2.44	24.2 ± 12.1	53.9 ± 28.5

or solubility issues. The precision and accuracy performed well in plasma at this limit.

3.4. Analysis of human plasma samples

Plasma levels were analyzed for 5 NSCLC patients (68 ± 16 y) receiving oral osimertinib therapy (80 mg once daily). The average plasma-levels are depicted in Table 6 with none of the samples showing a concentration outside the assay range of 2–2000 nM (or 6–2000 nM for OGSH). Along with the incurred sample reanalysis of these initial samples, it was shown that the method can reliably report the subject sample analyte, however sample stability between sampling and analysis is a critical point. A typical chromatogram is shown in Fig. 4. Dickinson et al. suggested that AZ5104 and AZ7550 are found in roughly equal concentrations, with C_{max} values of 1.4 and 0.9 nM after a single dose of 20 mg [2]. Semi-quantitative determination of both compounds shows that AZ7550 levels are roughly 5–7 times lower than AZ5104 levels so caution is warranted in interpreting the results of AZ7550.

4. Conclusions

Thioether metabolites of osimertinib were successfully synthesized and characterized. All synthesized compounds had NMR spectra and mass spectra consistent with the assigned structures. HRMS-data was almost identical to the calculated theoretical *m/z*. An LC–ESI–MS/MS method has been developed and validated for quantitation of osimertinib, AZ5104, OGSH, OCGS, and OCYS in human plasma. The method used was successfully validated for the analysis of osimertinib and several metabolites in samples from patients with a T790 EFGR mutated form of NSCLC and is the first published validated assay for these drug metabolites. Accuracy and precision met the limits as they are described in international guidelines, except for OGSH the sensitivity was somewhat lower and the LLOQ was set at 6 nM (low QC). Long-term, bench-top, freeze-thaw and auto-sampler stability of the samples met the required criteria as well, with the exception of OGSH, which is converted to OCGS in plasma. In the analysis of osimertinib in human plasma all samples were within the validated range, demonstrating the applicability of the method for the detection of osimertinib metabolites in patient-derived samples.

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