



Recent developments in the chromatographic bioanalysis of approved kinase inhibitor drugs in oncology



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ABSTRACT

In recent years (2010–present) there has been an increase in the number of publications reporting the development, validation and use of bioanalytical methods in the rapidly expanding drug class of small molecule protein kinase inhibitors. Most reports describe the technological set-up of the methods that have allowed for drug concentration measurements from various sample types. This includes plasma, dried blood-spot, and tissue-analysis. Also method development, exploration of various techniques, as well as measurement and identification of metabolites were addressed. For the bioanalysis, a variety of sample-pretreatment methods like protein-precipitation, liquid-liquid extraction, and solid-phase extraction have been employed, all varying in complexity, cleanliness and time-consumption. Chromatographic separation, nowadays, is more focused on separating components from ion-suppressive effects, since for MS/MS detection, various components do not have to be baseline separated. For detection multiple types of detectors were used, ranging from state-of-the-art high resolution, and tandem mass spectrometry with low picogram per milliliter detection limits to the classical UV-detector with several nanograms per milliliter limits. As new bioanalytical methods have arisen that do rely on chromatographic separation, for example for high-throughput analysis, these are addressed in this review as well.

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1. Introduction

The small molecule signal transduction inhibitor class tyrosine and serine/threonine kinase inhibitors (TKIs) are a relatively novel group of medicinal drugs. Their main targets, as the name indicates, are cellular kinases. These signal transduction proteins are responsible for regulation of numerous cellular pathways. Their mechanism of action has been first described around 28 years ago, in 1988 [1]. In this article by Yaish et al., the inhibition of the catalytic properties of epidermal growth factor receptor (EGFR) by what was then called ‘thyrophostins’ (tyrosine phosphorylation inhibitors) was described. This receptor is still a popular drug target for TKIs, as shown by the rapid increase in registered TKIs targeting these receptors. Although kinases have been recognized as potential drug targets for decades, wide acceptance was only relatively recent due to the idea that potency against, and selectivity between

the 518 human kinases would not be possible when targeting the ATP binding pocket of these kinases [2]. Since the registration of the first selective TKI imatinib [3], 25 other oncological TKIs have entered the market, and many more are under development, or never made it through initial (clinical) trials [4]. The bioanalysis of these drugs is also gaining interest. Initially, only pharmaceutical companies, contract research organizations (CROs) or large universities and academic centers performed bioanalytical assays on these drugs. Their uses were mainly for drug-development or research into, amongst others, drug-drug interactions, CSF- or target tissue penetration, and transporter elucidation. Later, with most notably triple quadrupole mass spectrometers (MS) becoming more accessible, also smaller (non-academic) facilities started to use these highly sensitive detectors, since information about the necessity for therapeutic drug monitoring (TDM) became progressively more available [5–10]. A step back from the mass spectrometer detection is the use of UV or fluorescence detection (FLD). These relatively low cost detectors are still widely spread across the world, and although they are not as sensitive and selective as MS or tandem MS (MS/MS) detection, the techniques are still valid in bioanalysis. For this rea-

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son UV(-VIS) and FLD are included in this review, especially now that the first TKI patents start to expire and these expensive drugs become more and more available to developing countries [11].

Nowadays TKIs are mostly used in a 'one-dose-fits-all' manner, with little inter-individual tailoring. While this is usually practical for both patient and caregiver, it might not be optimal [5,12]. Also, for TKIs the dose is set at the maximum tolerated dose, at which not only effect is maximized, but also the toxicity. Furthermore, although TKIs are better tolerated than traditional cytotoxic drugs, they still possess significant toxicity. This can be explained by inhibition of intended targets or off-target toxicity by the TKI or by formed metabolites [2]. TKIs are widely metabolized by CYP enzymes in both the liver and the intestine, especially but not exclusively by CYP3A4/5 [9], as well as transported back to the intestine by efflux transporters like P-glycoprotein (P-GP; ABCB1) and breast cancer resistance protein (BCRP; ABCG2), and thus varying oral bioavailability [13]. These enzymes (like CYP2D6) are not only subject to a high inter-individual variation in gene expression due to genetic polymorphism [14], but also to metabolism related pharmacokinetic drug-drug interactions due to inhibition or induction of these enzymes [9,13,15]. Individual dosing requires TDM, which in its way creates the need for additional interpretation and action. A recent paper reviewed the exposure-effect relationships for the registered TKIs and concluded that for 9 out of 18 TKI's registered in the Netherlands at the time, a relationship between exposure and effect could be found, and for 16 of those TKI's there is a relationship between exposure and (often) reversible toxicity [5]. Although recommended therapeutic targets for efficacy are already available for crizotinib, erlotinib, gefitinib, imatinib, nilotinib, pazopanib, and sunitinib [12] they are still not validated due to the lack of prospective studies [5,9,12,16].

Although the majority of the TKIs that are now on the market focus on oncology, signal transduction therapy can, in principle, also apply for non-cancer proliferative diseases and for inflammatory conditions. Tofacitinib, a Janus kinase inhibitor, has been registered for the treatment of rheumatoid arthritis, and more research about the use of kinase inhibition in these fields is being

performed [17]. Since it is not used in oncology, the review does not focus on it (Figs. 1 and 2).

2. Methods

The FDA and EMA websites were searched for registered TKIs (noted in Table 1). The relevant scientific databases were then searched for bioanalytical papers regarding these kinase inhibitors and using some form of chromatography. Only original research articles, published between January 2010 and March 2016, that focus on the bioanalysis of currently registered TKIs (EMA or FDA) using validated assays were admitted to this review. In all articles, method validation was performed in accordance with the FDA guidelines for bioanalytical method validation ([18]). In most cases the EMA guidelines ([19]) were withheld as well. cursory information of TKI bioanalysis, indicating if metabolite quantification is published, and what matrices, preparative methods, detection methods and limits were used is noted in Table 2. Everolimus, sirolimus, and temsirolimus were excluded from this review as they are indirect kinase inhibitors of mTOR-kinase via a complex formed with FK506-binding protein 12 [2]. Also idealisib is excluded, as it is an inhibitor of phosphoinositide 3-kinase [20].

From the initial search ([“name of TKI”] + [chromatography] OR [HPLC]), 241 articles were included. From these we excluded all articles that did not meet our inclusion criteria (validated, bioanalytical papers, 2010-now, current registered TKIs). From these 241 articles, 106 articles remained.

3. Sample types

3.1. Species

Validated assays were found using samples obtained from one of the following species: human (n = 82, 77.4%), rat (n = 19, 17.9%), mouse (n = 14, 14.2%), dog (n = 1, 0.9%), and non-human primates (n = 2, 1.9%).

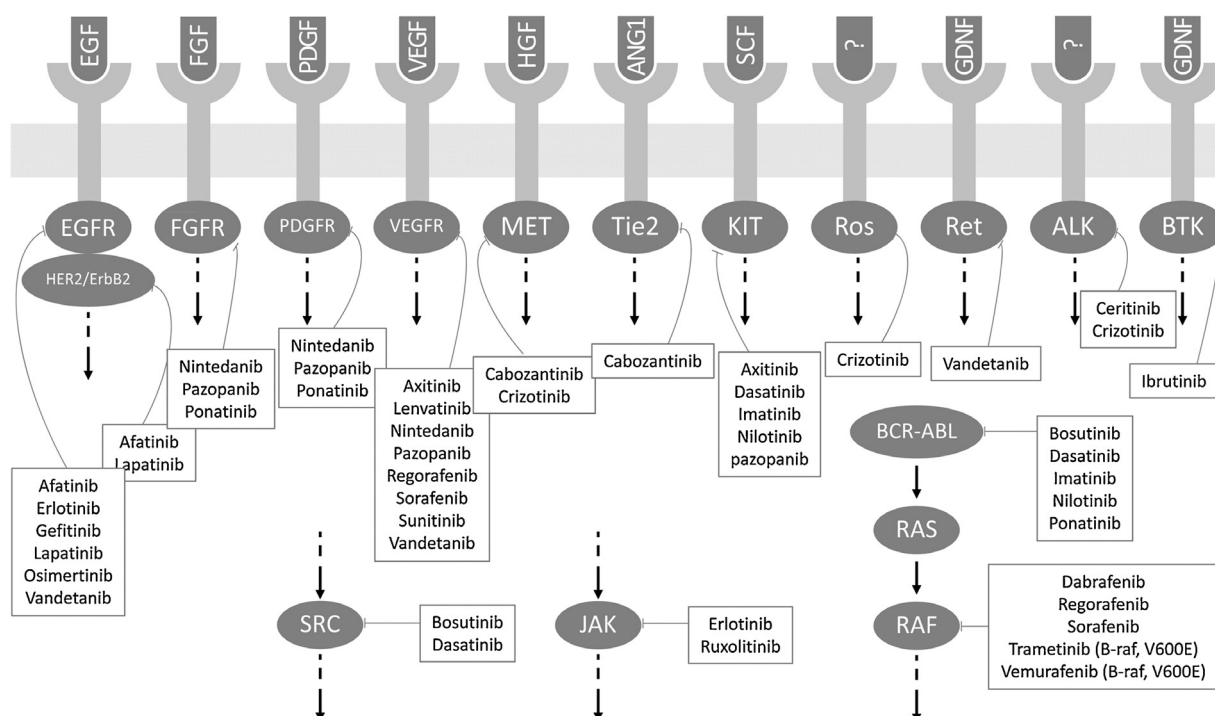


Fig. 1. Target-map of tyrosine kinase inhibitors currently approved by the USFDA and/or EMA.

Table 1
Tyrosine kinase inhibitors currently approved by the USFDA and/or EMA.

Drug	Brand-name	Research code	Disease	Main target	Covalent inhibition	Source
afatinib	Gilotrif, Giotrif	BIBW2992	NSCLC	HER2/EGFR	Yes, Cys797	[79,83,98,145,146]
axitinib	Inlyta	AG013736	Renal cell carcinoma	VEGFR1-3, c-KIT, PDGFR	No	[15,101,127]
bosutinib	Bosulif	SKI-606	Ph + CML (Bcr-Abl)	BCR-ABL, Src, Lyn, Hck	No	[127]
cabozantinib	Cabometyx, Cometriq	XL184	Medullary thyroid cancer	c-Met, VEGFR2	No	[64,147]
ceritinib	Zykadia	LDK378	ALK+ NSCLC	ALK	No	[22,117]
crizotinib	Xalkori	PF02341066	ALK-fusion NSCLC	ALK, ROS1, c-Met	No	[32,92,133]
dabrafenib	Tafinlar	GSK2118436	B-raf V600E mut. melanoma	B-Raf-V600E	No	[148]
dasatinib	Sprycel	BMS354825	Ph + CML (Bcr-Abl)	BCR-ABL, PDGFR, Src, c-Kit, . . .	No	[7,15,23,31,47,62,83,95,99,114,119,124,126,130,149]
erlotinib	Tarceva	OSI774	NSCLC	EGFR, JAK2V617F	No	[15,23,51,61,67,84,91,105,107,114,118,124,125,128,129,135,150,151]
gefitinib	Iressa	ZD1839	NSCLC	EGFR	No	[15,52,53,107,114,124,125,152]
ibrutinib	Imbruvica	PCI32765	MCL, CLL, WM	Brutons TK (BTK)	Yes, Cys481	[72,76,98,113,145]
imatinib	Gleevec, Glivec	ST1571	Ph + CML (Bcr-Abl), GIST	BCR-ABL, PDGFR, SCF, c-KIT	No	[7,8,10,15,23,26,27,31,33,36,47,60,94,97,99,100,102,105,108,114,115,119,121,124,129,130,139,150,153,154]
lapatinib	Tykerb, Tyverb	GSK572016	HER2+ breast cancer	EGFR, HER2	No	[15,23,25,105,106,114,121,124,129,155]
lenvatinib	Lenvima	E7080	Thyroid cancer	VEGFR2,3 [156]	No	[103,138]
nilotinib	Tasigna	AMN107	Ph + CML (Bcr-Abl)	BCR-ABL, PDGFR, c-KIT	No	[15,23,31,47,93,99,100,114,119,121,124,129,130,157,158]
nintedanib	Ofev, Vargatef	BIBF1120	NSCLC	VEGFR, FGFR, PDGFR	No	[159]
osimertinib	Tagrisso	AZD9291	NSCLC, T790 M mut.	EGFR [160]	Yes, Cys797	[87]
pazopanib	Votrient	GW786034	Renal/Soft Cell Carcinoma	c-KIT, FGFR, PDGFR, VEGFR	No	[6,83,101,112,130,155,161]
ponatinib	Iclusig	AP24534	Ph + CML (Bcr-Abl), T3151 mut.	Bcr-Abl, FGFR	No	[21]
regorafenib	Stivarga	BAY73–4506	MCC, advanced GIST	VEGFR, PDGFR, B-Raf	No	[130,162,163]
ruxolitinib	Jakafi, Jakavi	INCB18424	Myelofibrosis	JAK1, JAK2	No	[157]
sorafenib	Nexavar	BAY439006	Adv. RCC, HCC, RI resist. ATC	VEGFR, PDGFR, B/C-Raf	No	[6,15,23,54,96,105,114,119,124,125,129,164–166]
sunitinib	Sutent	SU11248	RCC, GIST	PDGFR, VEGFR, c-KIT	No	[6,15,23,24,35,57,65,69,101,104,105,114,119,124,125,129,130,134,150,167–169]
trametinib	Mekinist	GSK1120212	B-raf V600E mut. melanoma	B-Raf-V600E/K	No	[170]
vandetanib	Caprelsa, Zactima ^a	ZD6474	Medullary thyroid cancer	VEGFR, EGFR, RET	No	[23,55,83,171]
vemurafenib	Zelboraf	PLX4032	B-raf V600E mut. melanoma	B-Raf-V600E	No	[38,80,111,128,172–175]

Diseases: ALK: Anaplastic Lymphoma Kinase, ATC: Advanced Thyroid Cancer, CLL: Chronic Lymphocytic Leukemia, GIST: Gastro-Intestinal Stromal Tumor, HCC Hepatocellular Carcinoma, IPF: Idiopathic Pulmonary Fibrosis, MCC: Metastatic Colorectal Cancer, MCL: Mast Cell Leukemia, NSCLC: Non-Small Cellular Lung Carcinoma, Ph + CML: Philadelphia Chromosome Positive Chronic Myeloid Leukemia, RCC: renal cell carcinoma, RI: Radioactive Iodine, WM: Waldenströms Macroglobulinemia. Targets: ALK: Anaplastic lymphoma kinase, c-KIT: Mast/stem cell growth factor receptor, c-Met: hepatocyte growth factor receptor, EGFR: Epidermal Growth Factor Receptor (HER1), FGFR: Fibroblast growth factor receptor, HER2: Human Epidermal growth factor Receptor 2, JAK: Janus Kinase, Raf: RAF proto-oncogene, RET: RET proto-oncogene, ROS1: ROS proto-oncogene 1 receptor tyrosine kinase, SCF: Stem Cell Factor, SRC: Proto-oncogene tyrosine-protein kinase Src, TK: Tyrosine Kinase, VEGFR: vascular endothelial growth factor receptor. ^a: withdrawn application.

Table 2

Cursory information of TKI bioanalysis; indicating if metabolite quantification is published, and what matrices, preparative methods, and detection modes were used.

Analyte	Metabolite	Matrix	Preparation	Molecular formula	Mw(g/mol)	LLOQ	ULOQ	Therapeutic range (ng/ml)	Sample volume (μl)	Detection mode	Ionization method	Reference
afatinib	No	PI	PP, SPE, SALLE	C ₂₄ H ₂₅ ClFN ₅ O ₃	485.938	0.5 ^Q 700 ^U	500 ^Q 7,000 ^U	C _t : 10–55 [176]	10 ^Q 100–1000 ^U	QqQ, DAD	ESI	[79,83,98,145,177]
axitinib	No	PI, Ser	SPE, LLE	C ₂₂ H ₁₈ N ₄ O ₅	386.470	0.1 ^Q 10 ^U	200 ^Q 250 ^U	C _{pl} ^a : 45–56 [12] C _{pl} : 10–100 [178]	300 ^Q n.a. ^U	QqQ, DAD	ESI	[15,101,127]
bosutinib	No	PI	PP, LLE	C ₂₆ H ₂₉ Cl ₂ N ₅ O ₃	530.446	0.1 ^Q 20 ^U	500 ^Q 10,000 ^U	C _t : 75–215 [5]	100 ^Q 300 ^U	QqQ, UV	ESI	[123,127]
cabozantinib	No	PI, He, Li, Sp, Lu, Ki	PP, LLE	C ₂₈ H ₂₄ FN ₃ O ₅	501.506	0.5 ^Q	5000 ^Q	C _t : ~750–2,250 [179]	100 ^Q	QqQ	ESI	[64,147]
ceritinib	No	PI	PP, SALLE	C ₂₈ H ₃₆ ClN ₅ O ₃ S	558.135	1 ^Q	1,000 ^Q	C _t : ~450–1,300 [180]	100 ^Q	QqQ	ESI, APCI	[22,117]
crizotinib	Semi	PI, DBS	PP, SPE	C ₂₁ H ₂ Cl ₂ FN ₅ O	450.337	1 ^Q	10,000 ^Q	C _t : >200 [178]	5–50 ^Q	QqQ	ESI	[32,92,133]
dabrafenib	No	PP	PP	C ₂₃ H ₂₀ F ₃ N ₅ O ₂ S ₂	519.562	2 ^Q	2,000 ^Q	C _t : 8–90 [5]	200 ^Q	QqQ	ESI	[148]
dasatinib	Yes	PI, Ser, Br, PBMC	PP, LLE, SPE	C ₂₂ H ₂₆ ClN ₇ O ₂ S	488.006	0.96 ^Q 20 ^U	2,500 ^Q 10,000 ^U	C _t : >1.5 [12], 1.4–3.4 [5]	10–300 ^Q 200–1000 ^U	QqQ, Q, UV, OT	ESI, APCI	[7,15,23,31,47,62,83,95,99,114,119,124,126,130,149]
erlotinib	Yes	PI, Ser, CSF, WhB, Tu	PP, SPE, LLE, SLE, UF	C ₂₂ H ₂₃ N ₃ O ₄	393.436	0.25 ^Q 12.5 ^U	10,000 ^Q 5,000 ^U	C _t : Ct: >500 [178], 700–1,700	20–300 ^Q 200–400 ^U	QqQ, UV	ESI, APCI	[15,23,51,67,84,91,105,107,114,118,124,125,129,135,150,181,182]
gefitinib	Yes	PI, Ser, CSF, WhB	PP, SPE	C ₂₂ H ₂₄ ClFN ₄ O ₃	446.902	0.25 ^Q 11 ^U	10,000 ^Q 1,000 ^U	C _t : >200 [178], 265–814 [5]	20–500 ^Q 250–400 ^U	QqQ, DAD, UV	ESI, APCI	[15,52,53,107,114,124,125,152,182]
ibrutinib	Yes	PI	PP, LLE, SPE	C ₂₅ H ₂₄ N ₆ O ₂	440.497	0.5 ^Q 5 ^U	5,000 ^Q 400 ^U	C _{max} : ~170 [183]	10–50 ^Q 100 ^U	QqQ, DAD	ESI	[72,76,98,113,145]
imatinib	Yes	PI, Ser, DBS, CSF, He, Li, PBMC	PP, LLE, SPE, UF	C ₂₉ H ₃₁ N ₇ O	493.603	8.4 ^Q 10 ^U 296 ^I	50,000 ^Q 12,000 ^U 9,000 ^I	C _t : >1,000 [5,178]	10–300 ^Q 100–500 ^U	QqQ, Q, LIT, UV, OT, DAD	ESI, APCI	[7,8,10,15,23,26,27,31,33,36,47,60,94,97,99,100,102,105,108,114,115,119,121,124,129,130,139,150,153,154]
lapatinib	No	PI	PP, LLE, SPE, UF	C ₂₉ H ₂₆ ClFN ₄ O ₄ S	581.058	10 ^Q 50 ^U	10,000 ^{Q,U}	C _t : >500 [178], 780 [12], 50–500 [5]	50–300 ^Q 200 ^U	QqQ, UV	ESI, APCI	[15,23,25,105,106,114,121,124,129,155]
lenvatinib	Yes	PI, ser, WhB, Ur, Fe	PP, LLE, UF	C ₂₁ H ₁₉ ClN ₄ O ₄	426.853	0.08 ^Q	400 ^Q	C _{max} : 518 ± 209 [184]	200–250 ^Q	QqQ	ESI	[103,138]
nilotinib	No	PI, Ser, DBS, PBMC	PP, SPE	C ₂₈ H ₂₂ F ₃ N ₇ O	529.516	0.86 ^Q 10 ^U	5,000 ^Q 12,000 ^U	C _t : >600 [178], 480–1,580 [5]	50–300 ^Q 100–500 ^U	QqQ, Q, UV, OT	ESI, APCI	[15,23,31,47,93,99,100,114,119,121,124,129,130,157,158]
nintedanib	Yes	PI	PP	C ₃₁ H ₃₃ N ₅ O ₄	539.625	1 ^Q	1000 ^Q	C _{max} : 44.9–67.6 [185]	100 ^Q	QqQ	ESI	[159]
osimertinib	No	PI	Salle	C ₂₈ H ₃₃ N ₇ O ₂	499.607	1 ^Q	1,000 ^Q	C _{max} : 80–1000 [186]	20 ^Q	QqQ	ESI	[87]
pazopanib	No	PI, Ser	PP, LLE, SPE	C ₂₁ H ₂₃ N ₇ O ₂ S	437.518	100 ^Q 20 ^U	100,000 ^Q 200,000 ^U	C _t : >20,000 [178]	50–100 ^Q 100–1000 ^U	QqQ, DAD, UV	ESI	[6,83,101,112,130,155,161]
ponatinib	No	PI	SPE	C ₂₉ H ₂₇ F ₃ N ₆ O	532.559	0.5 ^Q	200 ^Q	C _t : 12–73 [5]	150 ^Q	QqQ	ESI	[21,187]
regorafenib	Yes	PI	PP	C ₂₁ H ₁₅ ClF ₄ N ₄ O ₃	482.815	2 ^Q	25,000 ^Q	C _t : ~1800–2500 [188]	50–100 ^Q	QqQ	ESI	[130,162,163]
roxolitinib	No	PI	PP	C ₁₇ H ₁₈ N ₆	306.365	0.16 ^Q	248 ^Q	C _{max} : 1160 (25.6%) ^d [189]	50 ^Q	QqQ	ESI	[157]
sorafenib	Yes	PI, Ser, CSF, WhB	PP, LLE, SPE, UF	C ₂₁ H ₁₆ ClF ₃ N ₄ O ₃	464.825	5 ^Q 30 ^U	10,000 ^Q 30,000 ^U	C _t : >3,000 [178], 3,750–4,300 [5,12]	20–300 ^Q 100–250 ^U	QqQ, UV, OT	ESI, APCI	[6,15,23,54,96,105,114,119,124,125,129,164–166]
sunitinib	Yes	PI, Ser, WhB, Sweat, Br, Tu	PP, LLE, SPE, UF	C ₂₂ H ₂₇ FN ₄ O ₂	398.474	0.06 ^Q	500 ^Q	C _t : >50 ^d [178], 31–70 ^b [5]	10–500 ^Q n.a. ^U	QqQ, DAD, UV, OT	ESI, APCI	[6,15,23,24,35,57,65,69,101,104,105,114,119,124,125,129,130,134,150,167–169]
trametinib	No	PI	LLE	C ₂₆ H ₂₃ FIN ₅ O ₄	615.395	0.25 ^Q	250 ^Q	C _{max} : 2.5–25 [170]	50 ^Q	QqQ	ESI	[170]
vandetanib	No	PI, CSF	PP, LLE	C ₂₂ H ₂₄ BrFN ₄ O ₂	475.354	1 ^Q 80 ^U	3,500 ^Q 7,000 ^U	C _t : >400 [178], 400–2,000 [5]	50–100 ^Q 60–1000 ^U	QqQ, DAD, UV	ESI	[23,55,83,171]
vemurafenib	Semi	PI, CSF, DBS	PP, LLE	C ₂₃ H ₁₈ ClF ₂ N ₃ O ₃ S	489.922	100 ^Q 1250 ^U	100,000 ^{Q,U}	C _t : >40,000 [178], 34,100–93,500 [5]	10–50 ^Q 200 ^U	QqQ, UV	ESI	[38,80,111,128,132,172–175]

Matrix: Br: brain, Fe: feces, He: heart, Ki: kidney, Li: liver, Lu: lung, PBMC: peripheral blood mononuclear cells, PI: blood-plasma, Ser: blood-serum, Sp: spleen, Tu: tumor, Ur: Urine. Preparation: PP: protein precipitation, SPE: solid phase extraction, LLE: liquid-liquid extraction, SALLE: salt- or saline-assisted liquid-liquid extraction, UF: ultrafiltration. Detection: DAD: Diode Array Detector, FLD: Fluorescence Detector, OT: Orbitrap MS, QqQ: triple-quadrupole MS, Q: single-quadrupole detector, MS, UV: UV/VIS-detector. ^a: cycle 1, day 1, 1–2 h post-dose concentration, ^b: possibly body-size dependent ^c: depending on dose, ^d: geometric mean (percent coefficient of variation [CV%]), ^e: sunitinib + N-desethyl-sunitinib, ^Q: QqQ, ^U: UV-VIS/DAD, ^I: immunoassay, C_t: C_{trough}, C_{pl}: C_{plasma}, n.a.: not available.

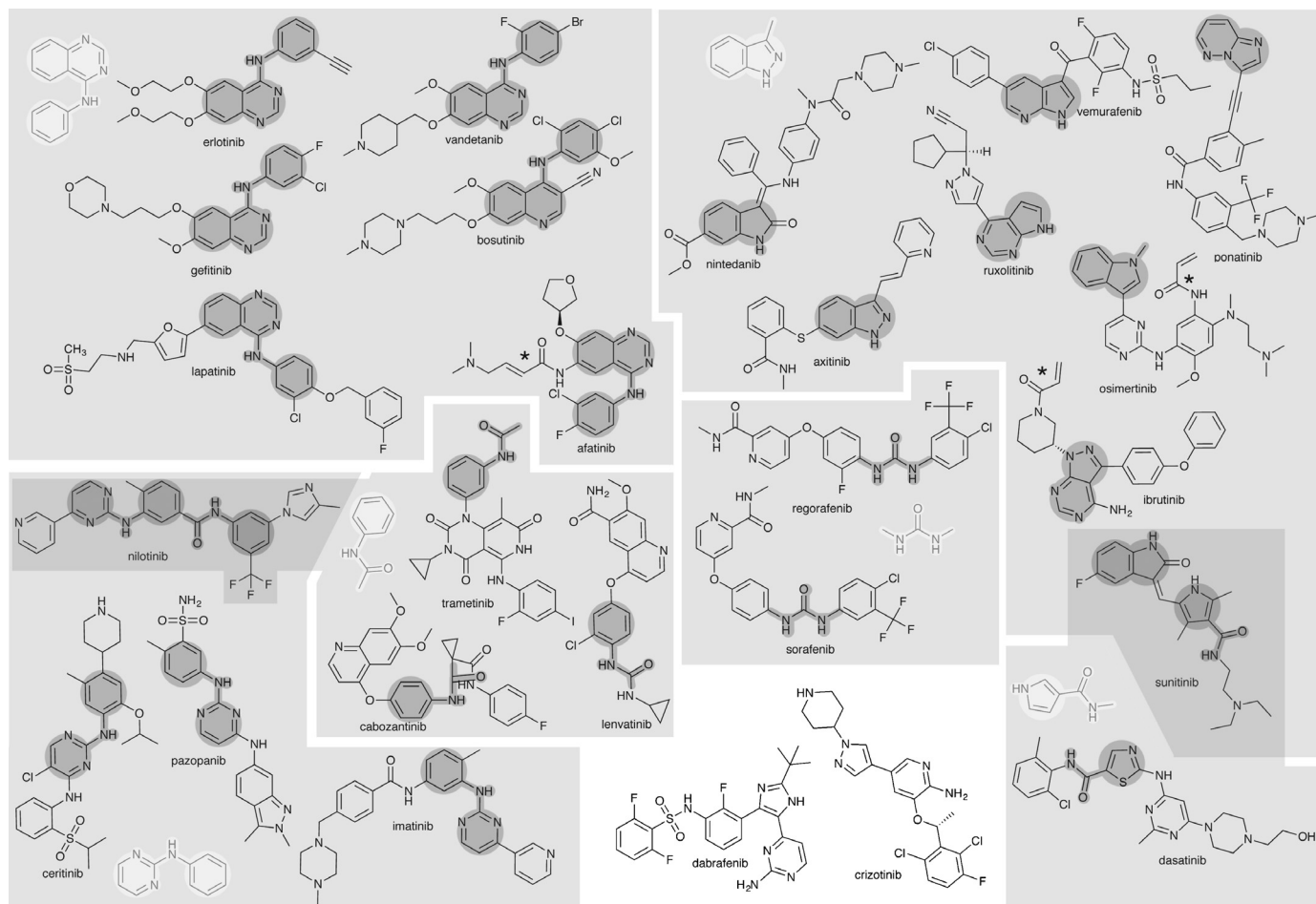


Fig. 2. Chemical structures of all included kinase inhibitors, sorted by similar chemical groups. * denotes a reactive group (Michael donor) capable of irreversibly inhibiting the target.

3.2. Blood and blood-components

3.2.1. Plasma and serum

Most assays focus on bioanalysis of the drugs in plasma. This is the most common matrix for drug level analysis, during the initial developmental testing of new compounds to evaluate the concentration time profile, starting most often in small rodents, such as rats or mice. Subsequently, the concentration time profiles in human subjects are determined. Drug levels are not only determined to assess initial pharmacokinetic (PK) parameters, but also interaction testing is performed, such as drug–drug [9] or food–drug interactions [21]. In a clinical setting, plasma is the most used matrix in which drugs, in general, are quantified, TKIs are no exception. Over 90 percent of the included matrices are either plasma or serum. A possible concern in plasma analysis of TKIs is hemolysis. Several articles investigated the interference of hemolysis on matrix effect (ceritinib [22], dasatinib, sunitinib, imatinib, nilotinib, erlotinib, lapatinib, sorafenib, vandetanib [23]), precision and accuracy (sunitinib/N-desmethyl-sunitinib [24], lapatinib [25]), cross-talk, carry-over, and interference (imatinib [26,27]). They only tested spiking of either drug to hemolyzed plasma, hemolyzed blood to drug-containing plasma, or hemolytic blanks. Potentially, TKIs bound to erythrocytes could be freed during hemolysis [22–27]. This is discussed in the next section.

3.2.2. Whole blood

A difference in concentration can be found when plasma concentrations were compared to whole blood. For dasatinib, a

plasma-whole blood ratio (PBR) of 0.7 was found, whereas for imatinib and desmethyl-imatinib the concentration was approximately 1.4 times higher in plasma [7]. Hemolysis of samples with high PBRs (≥ 2.0) could lead to an apparent increase in plasma concentration [28]. Another concern is the potential instability due to oxidative degradation, as the hemoglobin acts as an oxygen donor [29]. In pharmacokinetics, clearance is most commonly denoted as plasma clearance, as blood–plasma is the most commonly analyzed fluid. This PBR is important for PK-calculations. When whole blood concentrations rather than plasma concentrations are used, the PBR should be taken into account [30].

3.2.3. Dried blood spot

During recent years dried blood spot (DBS) methods gain interest [31–36]. DBS, especially in combination with LC–MS/MS has many diverse applications, including screens for inherited metabolic disorders in newborn, workplace drug testing, therapeutic drug monitoring, toxicokinetics, and PKs in drug development [32]. The main advantage lies mostly in the sample collection stage, as the matrix itself mostly adds complexity to the analysis. The variation in hematocrit between individuals and with disease state has an effect on the physical characteristics of DBS samples. The area of the spot of DBS samples decreases linearly with increasing hematocrit levels [37]. In a 2010 study in *Bioanalysis* a bias in the concentrations of acetaminophen and sitamaquine in DBS samples at different hematocrits was observed, which in some cases exceeded acceptable EMA or FDA values, particularly for hematocrits outside normal values [37]. This effect was also found by

Nijenhuis et al. They found that the measured concentration of spiked samples increases with the hematocrit [38]. The effects of hematocrit on DBS bioanalysis has extensively been reviewed by Denniff and Spooner [37], and was investigated for ten compounds by de Vries et al. [39]. Furthermore, the applied drop-size possibly adds additional variation, as described for vemurafenib. On average 10–20 μl blood is applied by the patient. Application of a larger amount increased the inaccuracy of the assay [38]. These effects can be ruled out by aliquoting a fixed sample amount onto the DBS card and using the entire spot [32]. This method may prove useful in a laboratory setting, but this eliminates the ease of use for outpatient TDM. The volume problem can be tackled by using a volumetric absorptive microsampling (VAMS) tool with a simple technique for collection and quantitative analysis of dried blood samples, such as the Mitra device. An accurate blood volume (10 μl) is collected into the absorbent tips, regardless of blood hematocrit [40]. Other advantages of DBS samples are the absence of water after drying of the sample collection card, possibly providing more stability at ambient temperatures [41,42], and the limited biohazard risks making transport and handling easier. When analyzing DBS samples, the results are reported as whole blood concentration rather than plasma concentration. For this reason, DBS methods always have to be investigated along with plasma [33]. DBS requires a different approach for the validation of sample pretreatment. The issues concerning validation are reviewed elsewhere [43]. With DBS, the type of material could introduce major variation in extraction efficiency. Not all reports about TKI bioanalysis mentioned the investigation of multiple paper-substrates [31,33], except for Rahavendran et al., reporting there was no effect between two different substrates [32], and Nijenhuis et al. reported ionization enhancement for FTA DMPK-A, but not 903 protein saver cards. The FTA DMPK-A cards consist of cellulose, SDS and trometamol (TRIS). SDS and TRIS are both surfactants and denaturants of proteins and may influence spot size, as well as ionization in the source of the MS (in this case for vemurafenib 60% enhancement of ionization was shown with ESI) [38].

For the development of a DBS-method, various card types should be tested for spot homogeneity, extraction recovery, ionization effects, and accuracy and precision at different hematocrits, and selecting the card type that shows most consistent. Additional validation-steps are advisable as well, the accuracy and precision of the method at different hematocrit values over the intended hematocrit range to show the combined influence of spot size, spot homogeneity and absolute recovery at different hematocrits on accuracy and precision. Long-term stability at different hematocrits should be tested as absolute recovery can decrease in aged DBS in a hematocrit-dependent way [38,39].

3.2.4. Erythrocytes

Erythrocytes have shown to be the most important drug transporting cells, they have the largest volume and surface of all blood derived cells. In classic anti-cancer drugs, erythrocytes have been shown to carry high amounts of these drugs, among which the nitrogen-mustard species such as cyclophosphamide and ifosfamide, as well as other anticancer agents [44,45]. For TKIs the analysis of this matrix does not seem to provide added benefit. This is also reflected in the plasma-whole blood ratio around 1 (0.7 for dasatinib, and 1.4 for imatinib) [7].

3.2.5. Peripheral blood mononuclear cells

Only the drug-fraction reaching the intracellular compartment is expected to exert action. In the case of imatinib, dasatinib and nilotinib, specific bone-marrow derived circulating cancer cells are a reflection of the active compartments. These cells contain the BCR-ABL reciprocal translocation defect, found in 95% of the CML patients [46]. One article describes the development and success-

ful validation of such an assay [47]. PBMCs were isolated from the blood of patients by density gradient centrifugation, and after lysing of the PBMCs by sonication, quantify imatinib, dasatinib and nilotinib were quantified in an absolute manner with a limit of quantification of 0.25 ng/million cells. This method is an alternative for measuring receptor occupancy in PBMCs by a specially designed fluorescent affinity probe, as was done for ibrutinib [48]. The clinical relevance of determining PBMC drug concentrations in BCR-ABL targeted therapy is differentiating between patient groups, as Bouchet et al. showed by the PBMC-/plasma-concentration ratio. Patients with known response at 12 months following treatment initiation with imatinib showed a considerably higher ratio. The determination of intracellular concentrations could be beneficial in patients who are suboptimal or non-responding despite adequate plasma concentrations [49].

3.2.6. Plasma and DBS sample stability

The most frequent used matrix for drug-analysis in TDM and clinical trials is blood-plasma. Although in most articles the true stability is not evaluated, a minimal stable period can be derived from the provided data. Most TKIs are relatively stable in plasma, as noted in Table 3. With the tested stability ranging from several hours to a number of months even at room temperature Lapatinib was tested unstable in human plasma when stored at room-temperature for over 6 h [23], and axitinib for 24 h [15]. At sub-zero temperatures ($<-20^{\circ}\text{C}$), almost all TKIs are stable in human plasma for more than 1 month, and the others have not been tested. As part of the validation procedures, the stability during three freeze-thaw cycles and auto sampler stability should be investigated. All TKIs passed these criteria. Since DBS is gaining interest, DBS stability data have been included in Table 3. DBS use provides drug stability over a wide temperature range, allowing home-sampling of blood-samples, and mailing in the cards. DBS samples are often tested at elevated temperatures, as the temperature during shipping is uncontrolled. For crizotinib, dasatinib, imatinib, nilotinib, and vemurafenib DBS stability data were known. All samples were stable at temperatures ranging from -20 to 43°C [31–33,35,38], except dasatinib, which was unstable on DBS-cards at 40°C ($>15\%$ loss) [31].

3.3. Cerebrospinal fluid

The incidence of brain metastases from melanoma, breast cancer, and non-small cell lung cancer (NSCLC) is apparently on the rise in cancer patients, and possibly threatens the gains acquired by introduction of TKIs. To assess the blood-brain barrier (BBB) permeability of TKIs, cerebrospinal fluid (CSF) concentrations have been compared to blood-concentrations [50]. A number of TKI assays for the analysis and subsequent calculation of the CSF-penetration rate have been published for erlotinib, gefitinib, sorafenib and vandetanib [8,51–55]. CSF is a relatively clean ultrafiltrate, with a low protein level of approximately 0.15–0.60 g/L (blood-plasma contains ca. 70 g/L) [56]. One of the problems that arises when validating an assay for TKIs in CSF is acquiring drug-free CSF. A way to overcome this is to use artificial CSF [53], if justifiable [18,19]. To incorporate CSF bioanalysis in plasma-assays, CSF is sometimes diluted with drug-free plasma. The herein reviewed articles did not make use of this. Although this may ease the development of the assay, it also lowers the detection limit by the dilution.

3.4. Sweat

Lankheet et al. developed a method for the quantitative analysis of sunitinib and *N*-desethyl-sunitinib in the sweat of patients. This was done to investigate the function of sweat as a carrier of the drug and metabolite to the skin surface. Sweat containing the drug may

Table 3TKI stability in plasma and DBS, indicating the time the compound is minimally stable^a (unless stated otherwise). In the case that human data were unavailable, other sources are mentioned.

Analyte	Species	Matrix	+20–25 °C	+2–8 °C	–20–30	–60–80	Other	FT (cycles)	Extract/AS	Source
afatinib	Human	Plasma	8h	24h	30d	–	–	3	–	[177]
axitinib	Human	Plasma	1d (15–20%)	7d	30d	–	–	–	–	[15]
bosutinib	Human	Plasma	3h	–	180d	–	–	3	12 h (+4 °C)	[123,127]
cabozantinib	Rat	Plasma	2–16h	25h	32d	–	–	3	24 h (RT)	[64,147]
ceritinib	Human	Plasma	24h	–	–	630d ^b	–	3 (<–65 °C)	137 h (+10 °C)	[22,117]
crizotinib	Human/Mouse	Plasma/DBS	24 h 60d ^c	– –	60d –	– –	– –	3 –	4d (+4 °C) 4d (+15 °C)	[92,133] [32]
dabrafenib	Mouse	Plasma	24h	–	90d	–	–	3 (–30 °C)	4d (+4 °C)	[148]
dasatinib	Human	Plasma/DBS	90d 28d	90d –	270d 3d	180d –	–3d (40 °C, >15%)	6 –	168 h (RT) –	[7,15,23,95,114,130] [31]
erlotinib	Human	Plasma	3d	7d	365d ^b	227d	–	3	72 h (+4 °C)	[15,23,84,91,107,114]
gefitinib	Human	Plasma	48h	7d	150d	90d	–	6	24 h (+4 °C)	[107,114,124,125,152]
ibrutinib	Human	Plasma	6h	–	451	451d	16 h (0 °C) ^d	6 ^d	120 h (+4 °C)	[72,79,113]
imatinib	Human	Plasma/DBS	90d 36d	150d –	730d ^b 36d	180d –	– 36d (43 °C)	6 –	168 h (RT) 12h	[15,23,100,114,124,130][33]
lapatinib	Human	Plasma	6h	7d	45d	180d	–	3	12 h (+4 °C)	[15,23,114,124]
lenvatinib	Human	Plasma	24h	21d	30d	–	18 (37 °C)	3	96 h (+4 °C)	[103,138]
nilotinib	Human	Plasma/DBS	90d 28d	90d –	365d 3d	180d –	– 3d (40 °C)	6	168 h (RT) –	[15,23,100,114,124,130][31]
nintedanib	Mouse	Plasma	3h	48h	35d	–	–	3	48 h (+4 °C)	[159]
osimertinib	Human	Plasma	4h	–	75d	–	–	3	24 h (+4 °C)	[87]
pazopanib	Human	Plasma	90d	90d	270d	80d	–	6	168 h (RT)	[130,161]
ponatinib	Human	Plasma	6h	–	–	14d	–	3	–	[140]
regorafenib	Human	Plasma	90d	90d	90d	540d	–	6	168 h (RT)	[130,190]
ruxolitinib	Rat	Plasma	6h	–	–	30d	–	3	24 h (+10 °C)	[157]
sorafenib	Human	Plasma	2d	7d	45d	180d	–	3	12 h (+4 °C)	[15,23,124,164]
sunitinib ^e	Human	Plasma	2d ^f	7d	45d	180d	–	3	12 h (+10 °C)	[15,23,65,114,124,134]
trametinib	–	–	–	–	–	–	–	–	–	no data
vandetanib	Human	Plasma	3d	2d	–	180d	–	3	6 h (+4 °C)	[23,55]
vemurafenib	Human	Plasma/DBS	30d 163d	90d –	120d 120d	– –	– –	3 –	7d (+4 °C) 7d (+4 °C)	[111,174] [38]

When time is printed BOLD, the stability does not match the FDA/EMA stability criteria for the selected time-frame; ^a stability is described as less than 15% loss over the mentioned time-span; ^b: trough incurred sample analysis; ^c: under desiccator, in the dark; ^d: (thawing) on wet ice; ^e: sunitinib shows reversible isomeric photo-conversion, peaks of both isomers can be summed; ^f: one paper described 15% loss after two days.

penetrate into the stratum corneum and cause the dermal toxicity [57]. This technique has been previously used for drugs-of-abuse screening, however, using sweat drug-concentrations as a measure for plasma-levels may not be directly useful without further knowledge. Sweat is slightly acidic (pH around 6.3), thus for basic drugs concentrations might differ from blood, based on pH partition theory [58]. Also, since sweat is an ultrafiltrate, only free drugs are able to pass through.

3.5. Tissue

With regards to tissue samples, the samples have to be converted to a liquid format before further sample preparation. This is often done by rotor-stator [59–61], micro-pellet homogenizer [62,63], or acoustic cell disruption [64] with addition of water [59], saline [65], drug-free plasma [61], or a BSA-solution [66]. Quantitative analysis of TKIs in tissue is mainly done to evaluate target site drug-exposure in patients or for transporter elucidation in small animals.

3.6. Tumor tissue

Penetration of drugs into tumor tissue is affected by different factors including tumor vascularization, plasma protein binding, and drug efflux pumps in tumor cells. Intratumoral drug metabolism may also occur. Tumors may not optimally respond to systemic therapy if minimal effective therapeutic levels are not reached within the tumor [61,67]. Two reports on tumor tissue analysis were published, both using rotor-stator homogenization. For both erlotinib in lung tissue [61] and sunitinib [59] in mouse-brain and tissue no interference of endogenous compounds from the tissue could be detected with the analyte or the ISDT.

3.7. Other tissue

A number of murine models were used for elucidating which transporters are responsible for drug-transport. The model-organs are liver, brain, kidney, heart, spleen, lung, and testis, and are mainly studied in mice. These transporters, like P-glycoprotein (PGP; ABCB1) and breast cancer resistance protein (BCRP; ABCG2) are expressed on the apical membrane of epithelia in tissues involved in drug absorption and elimination, like liver, small intestine and kidney, but also on luminal membranes of barrier tissues like the blood-placenta, blood-testis and blood-brain barrier. At these sites PGP or BCRP substrates are immediately pumped out of the epithelial or endothelial cells back into the blood. As a consequence, only small amounts of drug can accumulate in, for instance, the brain to treat (micro)metastases that are present behind a functionally intact blood-brain barrier [66,68]. Bianchi et al. described an assay for imatinib in rat-heart and liver tissue. This was done to evaluate the effect of carvedilol on drug-distribution, in relation to cardiac damage [60]. The limited penetration of these drugs in these tissues poses an analytical challenge, as often tissue-levels can be (much) lower than plasma levels [69]. A difficulty in tissue-level analysis is the solubility of drugs in the homogenized matrix. For this reason, drug-free plasma [61], or a BSA-solution [66] is added for improved solubility due to protein binding [66], or direct protein precipitation with organic solvents like methanol and/or acetonitrile is performed [60,64]. An issue that may rise is the drug-stability in tissue samples, especially tissues involved in drug metabolism, like liver samples. For sunitinib and N-desethyl-sunitinib, the instability in liver (–15.7 to –30.6% loss) and spleen (–18.6 to –19.7% loss) samples, and not lung, kidney, and heart homogenates was shown after 6 hours at room-temperature. Therefore, tissue sta-

bility should not be assumed and stability experiments should be performed for tissue samples at appropriate time-points [65].

4. Sample preparation

Sample preparation plays a critical part in (high throughput) bioanalysis. Protein precipitation (PP), liquid/liquid extraction (LLE), and solid phase extraction (SPE) are the three most widely used sample preparation techniques [70]. Ideally, sample preparation methods do not chemically affect analytes of interest, and breakdown of other components (such as phase II-conjugated metabolites) into the analyte of interest should not occur. Also there should be no loss of analyte due to nonspecific adsorption or evaporation. Ideally, recovery for both the analyte and internal standard are adequate and at least reproducible. For mass spectrometry, matrix components that can cause significant matrix effects, such as phospholipids, should largely be removed or separated chromatographically. Ultimately, the method should have minimal human manual operation, few working steps and short extraction time with extracts that can be injected directly, or after a simple dilution, onto common LC or LC-MS(MS) so preferably without evaporation and reconstitution [71]. Classically for LC-UV or FLD, the preparative method had to eliminate any spectrometrically interfering substance. This frequently led to laborious time consuming methods, which coincidentally often used large amounts of organic solvents. With more selective analytical techniques, such as mass spectrometry, interference plays a substantially smaller role, although isobaric interferences can still occur [72]. In MS detection, the preparative technique still plays a crucial role, as the most frequent used ionization technique is ESI, which is sensitive for ion suppression. The preparative methods show varying levels of contaminant removal, the most prominent being protein removal. Most contaminants could contaminate the chromatographic system and mass spectrometer, and also these contaminants could decrease ionization efficiency (ion suppression). Frequently occurring ion suppressants are endogenous phospholipids, as they are highly abundant in biological matrices. Removal of these compounds can pose a challenge [73,74]. Matrix effects do not solely come from endogenous compounds, exogenous compound are also a source of interfering substances, such as polymers from plastic tubes [75]. Next to effects on ionization, isobaric interferences can prove problematic as well. Such an example can be taken from the analysis of ibrutinib, where in plasma samples from a clinical study in hepatically impaired subjects, taurocholic acid showed interference with the internal standard of the active metabolite dihydrodiol-ibrutinib [72].

Some TKIs require specific conditions when handling, such as sunitinib, where exposure of the samples to light causes isomerization, and all sample preparation must be done in light-protected conditions [69]. For ibrutinib, the samples are temperature sensitive in human plasma. Samples are best kept on ice during sample pretreatment [76]. In Table 2 the different pretreatment methods used in the analysis of each of the TKIs have been noted.

4.1. Micro-volume assays

An ongoing trend in bioanalysis is reducing sample volumes. Sample volumes from both clinical and nonclinical studies have been decreasing because more samples are collected from the same subject or animal to better define the PK parameters [70]. For analysis of TKIs in plasma, 20 µl is a manageable volume, but for this volume, approximately 50 µl blood has to be drawn. For larger animals or human subjects multiple time-points can be taken without any trouble. This is difficult for small rodents, as mice have a total blood volume of only 1.8 ml. Therefore, 5 time points add up

to roughly 15% of the total circulating volume [77,78]. Although micro-volumes like 10 μ l blood are not as manageable, this could provide more time-points for PK-analysis whilst increasing animal welfare by diminished blood-loss. Only one paper described the use of micro-sampling using a 5 μ l whole-blood method [32]. Ten microliter sample volumes have been reported for afatinib [79], dasatinib, and vemurafenib [80]. Another method is the use of DBS micro-sampling [32,40].

4.2. Protein precipitation

The simplest pretreatment for bioanalysis is protein precipitation (PP) with a water miscible organic solvent, such as ethanol, methanol or acetonitrile. This method has been around for decades, and the required volumes were extensively investigated, among others by Blanchard [81] and Polson et al. Organic solvent precipitation is based on the fact that the organic solvent displaces water from the protein surface. With smaller hydration layers, the proteins can aggregate by attractive electrostatic and dipole force, leading to a decrease in solubility of the protein [82]. Protein precipitation is a very crude technique, and still leaves a lot of contaminants in the sample. This makes the technique, without combination with a more selective preparation method, less suitable for unselective detection methods such as UV-VIS, DAD or FLD, although not always impossible [83]. This is discussed in more detail later on. In (TKI-) bioanalysis, mostly acetonitrile and methanol, or a mixture thereof is used as precipitating agent. Although more solvents can be used, these are not always compatible or less suitable for direct injection into the chromatographic system with most used separation techniques. One method to increase the efficiency of PP is the use of ice-cold PP reagent [26,84], or even cooling to -80°C on an ethanol/dry ice bath [26]. Cold-induced denaturation of proteins is a phenomenon that is caused by a temperature-dependent interaction of nonpolar groups within the protein with water. This results in unfolding at a sufficiently low temperature, exposing internal nonpolar groups to water [85]. As mentioned, PP produces less clean extracts that still contain a fairly large amount of endogenous components, interfering with the detection [26]. A way to overcome this limitation is lowering the volume that is injected onto the column, which is often not problematic given the high sensitivity of the present generation MS detectors. Clogging of the column is often proposed as an argument against PP [71], this is possibly caused by incomplete settling of the precipitate.

4.3. Liquid extraction

With liquid-liquid extraction clean extracts can be produced, and the methods are relatively simple. However, the development of such a method can prove to be difficult, especially when performing simultaneous analysis of multiple kinase inhibitors, as TKIs generally differ in pKa and do not share the same “non-charged” point.

4.3.1. Liquid-liquid extraction

The mixing of two immiscible liquids, known as liquid-liquid extraction (LLE) is one of the oldest sample preparation techniques [70]. As said, LLE provides clean extracts, but when complete recovery is desired optimization can prove troublesome and multiple extractions would be needed for optimal sample cleanliness. Most articles state that LLE provides clean extracts, but lipids tend to be co-extracted with the compound of interest. LLE sample preparation can also be time-consuming and hard to (fully-) automate, especially when compared to PP [86]. For LLE the solvents are mostly immiscible with water, requiring the removal of the solvents for compatibility with a reversed-phase chromatographic method.

This can be done by evaporation and reconstituting the sample with a compatible (aqueous) solvent or mobile phase [71]. The solvents normally used in LLE can be quite toxic for personnel and/or have a high environmental impact. Saline assisted liquid-liquid extraction (SALLE) exists for quite some time, although it seems to re-emerge for LC-MS bioanalysis. SALLE has the same advantages as regular LLE and simultaneously removes the complicating factor of evaporating and reconstituting the reversed phase LC-incompatible solvent, as well as often using the more common and less toxic acetonitrile. With SALLE, phase separation of a mixture of acetonitrile and water or a biological fluid is obtained by salting out the organic solvent. The upper layer consists of mostly organic solvent, with therein the analytes. For ceritinib, a LC-MS/MS assay has been published using acetonitrile and a saturated sodium chloride solution [22], for afatinib magnesium-chloride [79], and for osimertinib magnesium-sulfate was applied [87]. Other, non-TKI methods, make use of the salts ammonium-acetate, ammonium-formate, potassium-carbonate, and zinc-sulfate [71]. As solvent, most methods use acetonitrile. Theoretically, multiple water-miscible solvents can be used, such as acetone, dioxane, tetrahydrofuran, propanol, or isopropanol [88]. Salts like sodium chloride and magnesium sulfate are not ‘MS-compatible’, and could interfere with the ionization or even contaminate the ion-optics. The switching valve integrated in most mass spectrometers should be used to divert the flow of the LC to a waste container until at least the ‘dead-volume’ is eluted [79]. This way accumulation of non-retained, non-volatile, or non-ionizable contaminants like salts, and large molecules, such as proteins, phospholipids, and lipids do not reach the MS [26,89]. This is not only true for SALLE-based methods, but is applicable to most bioanalytical LC-MS methods. By using the diverter, maintenance required for the MS is reduced, and thus saves costs [90].

4.3.2. Solid supported liquid-liquid extraction

Solid-supported liquid-liquid extraction, or supported liquid extraction (SLE) has been around for a long time, but surprisingly it is seldom used to replace classical LLE. In SLE, the same aqueous phases used in LLE are coated onto an inert diatomaceous earth support, but instead of shaking the two immiscible phases together, the organic phase is passed through the column and a very efficiently extraction takes place. SLE offers many advantages over LLE, including equivalent or more efficient extraction, no emulsion formation, easy automation, less organic solvent use, less labor, and less glassware [86]. The introduction of a 96-well format with a robotic liquid handling system for the analysis of erlotinib significantly improved the throughput of SLE. The methyl-tert butyl ether extract could be directly injected into the HILIC-MS/MS system for analysis without the solvent evaporation and reconstitution steps [91].

4.4. Solid-phase extraction

For most compounds a SPE extraction method is possible, however, SPE suffers from having a complicated procedure, relatively poor reproducibility, and high cost [70]. The reproducibility is mostly affected by the repeated addition and elution of liquids, and can be corrected by the use of a preferably stable isotope labeled internal standard. While the most frequently stated reason for SPE in TKI bioanalysis is the reduced matrix effect compared to PP [15,92–94], most papers do not state which factors influenced their choice for preparative methods. SPE provides clean samples, and can relatively easily be automated to in-line systems, but in-line SPE could drastically reduce sample throughput of the equipment. For this reason, and the need of specialized equipment, SPE is often still performed off-line. Due to its complexity, SPE requires expertise to set up and requires relatively large amounts of solvents, with steps like conditioning of the SPE column, buffering and loading

sample, column washing and finally eluting the analyte of interest with organic solvents such as methanol or acetonitrile [15,94,95]. The advantage of these solvents over the ones usually used in LLE is that they are usually less toxic. When using other solvents that are not compatible with the further quantitative analysis, evaporation and reconstitution of eluted extracts is often needed [96]. With these steps, for normal size SPE columns, usually up to 1 ml per step is used. Micro-SPE column plates require less solvents, but still use 200 μ l for washing and elution [15,92]. In TKI bioanalysis, SPE is mostly used for less sensitive and specific detection like UV [93,96–101], or in multi-drug assays when short analytical run-times are desired [15], because there is not much space in the chromatography to accommodate ion-suppressive matrix components.

4.5. Ultrafiltration

Ultrafiltration (UF) is a sample-preparative method used for the determination of unbound drug fractions in plasma. Of the techniques to determine unbound drug, UF is the only one that also a preparative method, and is used in TKI bioanalysis [102]. Other methods to determine unbound drug are ultracentrifugation and equilibrium dialysis. The downside of UF and ultracentrifugation is the non-specific binding of drugs to membranes and devices, giving false-low results. Equilibrium dialysis is the most commonly used since non-specific adsorption of drugs to the device and membrane has less impact than other techniques, provided that an equilibrium is reached [103,104]. In TKI bioanalysis, ultracentrifugation is used for the determination of unbound imatinib [102] and erlotinib, imatinib, sunitinib, sorafenib, and lapatinib [105].

4.6. Novel developments

4.6.1. Dried blood spot

As mentioned earlier, DBS is a promising sampling method that is gaining popularity [31–33,35,38]. And although DBS has some potential issues in the blood drawing stadium, the sample preparation is relatively simple. The easiest method is punching out small discs and simply extract the drug with an organic solvent, such as methanol [32], acetonitrile or a mixture of both [38]. Extraction can be aided by sonication [32]. One group described an additional step, where they performed the extraction on an Agilent Captiva ND Lipids filter plate, removing potentially interfering substances such as phospholipids, previously precipitated proteins, surfactants, and other matrix interferences [31].

4.6.2. High throughput sample preparation

Sample preparation can be laborious and time-consuming, especially in the case of complex sample preparation methods such as LLE or SPE, or steps like solvent evaporation and reconstitution. The most often used format in high throughput bioanalysis is the 96-well format microtiter plate. Most sample preparative methods can be employed on the 96-well format, and the aid of liquid handling robots makes processing the plates even faster, whilst requiring less human operation [91]. Of the reviewed TKI assays, some make use of the format for PP [31,72], SPE [21,92,94] and SLE [91]. The 96-well format provides a universal format for analytical sample preparation, plates can be handled through devices like vacuum or pressurizing systems aiding in the flow through SPE or SLE columns or filters.

4.7. Internal standards

Internal standards are deemed indispensable in chromatographic bioanalysis, especially when using MS detection [106]. Each step along the way in sample processing, separation and detection

adds variation to the analysis. In bioanalysis, stable isotopically labeled (SIL) internal standards are regarded as the best choice. However, these are not always available, sometimes expensive and unsuitable for non-MS detection techniques, as they show very similar chemical and physicochemical properties compared to the analyte. Requirements for a non-SIL internal standard can be similar logP and logD, solubility in mobile phase, polarity of ionization in MS, and stability in experimental conditions [26]. For instance, the use of sorafenib as an internal standard for gefitinib and erlotinib was based on the structural similarities between the drugs [107]. Additionally, when a drug is chosen as the internal standard it should not be used concomitantly with the analyte(s), which is the case in the example. Some authors justify the use of non-SIL internal standards on a cost-basis [26,108], while this statement might hold when these standards are not available commercially, the cost-per-sample is not that high when you consider the amounts used. Most assays use the SIL internal standards at the ng/ml range. For most MS methods, 100 μ l of 250 ng/ml would suffice. If 1 mg would cost 1000 euros, this would come to 2.5 cents per sample.

5. Chromatographic separation

5.1. Separation principles

The most widespread separation technique in biomedical analysis is reversed-phase chromatography on C18 columns. All but a few of the recent assays use some form of reversed phase C18 column, most notably the bridged ethylene hybrid cross-linked columns of Waters (Acquity BEH or Xbridge columns). Some assays use C8 reversed phase chromatography, one group stated that the use of a C8 column instead of a C18 column (along with decreasing the pH) significantly improved the peak shape and resolution of gefitinib in order to separate an interfering peak originating from human plasma in a LC-UV assay [107]. In TKI bioanalysis, only one paper recently described the use of hydrophilic interaction liquid chromatography (HILIC), which in other fields becomes increasingly popular, mainly because it provides good retention for polar analytes [91]. An advantage of HILIC over reversed-phase is direct injection of organic extracts into the LC-MS/MS system, so that the drying-down and reconstitution or dilution steps can be eliminated without the compromise of chromatographic peak shape [91]. This can in theory provide much benefit when using SALLE pre-treatment, since the salting out gives an almost complete organic (mostly acetonitrile) extract [71]. Although HILIC is not equipped routinely in the recent years for TKIs, a method from 2009 employed the technique successfully with 1.0 min run-times for dasatinib, nilotinib and imatinib [109]. Uses of other column-chemistries, such as phenyl-columns [52,95,100], strong cation exchange [7], or PLRP macroporous spherical polystyrene/divinylbenzene particles [24] have been employed in TKI bioanalysis, although these reports do not state any justification for their choice.

5.2. HPLC vs UHPLC

UHPLC is being used for approximately 10–12 years now [110]. Although it is gradually becoming the standard in chromatography, the initial TKI assays were developed on regular HPLC. With UHPLC not only the analysis time can be reduced [94,111–113], also resolution and sensitivity can be increased by enhancing efficiency of the chromatographic separation. Narrow-bore (between 1 and 3.5 mm inner diameter) or micro-bore (sub 1-mm inner diameter columns) are increasingly used [32]. These columns increase sensitivity of concentration dependent detectors such as an electrospray-MS, FLD, or DAD/UV-VIS detectors due to reduced chromatographic dilution. The downside of these columns and the corresponding

narrow diameter tubing is the increased internal system pressure, frictional heating of the column, and an increased effect of system dead-volumes and gradient delays [110].

5.3. Hyphenated chromatography

Most methods focus on getting accurate quantitative results, whilst requiring minimal sample size and analytical run time, maximizing throughput, and thus reducing cost-per-sample. Because off-line sample extraction techniques such as LLE, SLE or SPE can be time consuming and labor intensive or even impractical with water-soluble analytes, two research papers described a semi-on-line methodology [114,115]. These methods are based on two-dimensional liquid chromatography, where the first dimension is a high-turbulence [114] or perfusion [115] LC column, which through a valve-switching arrangement transfers retained analytes to a traditional C18 HPLC column (second dimension). By using multiple first-dimension-HPLC channels, they further increased the capacity for high-throughput analysis [114]. Although the researchers claim a full on-line sample preparation, the method still uses a protein precipitation step, with adding stable isotope labeled internal standards.

5.4. Whole plasma chromatography

One paper described the direct injection of diluted plasma into the chromatographic system, quantifying five TKIs (erlotinib, imatinib, sunitinib, sorafenib, and lapatinib). Micellar liquid chromatography (MLC), using sodium dodecyl sulfate (SDS) as the surfactant and a C18 column, where the SDS solubilizes proteins and other macromolecules that are non-soluble in the mobile phase. SDS reduces retention of these molecules, so less modifier is needed. Four percent 1-butanol was added to improve resolution. Since the use of surfactants is not compatible with MS, UV-detection was used. The detection limit was sufficient, at 50 ng/ml for all compounds [105].

6. Detection

6.1. Mass spectrometry

MS based detection provides some distinct advantage over other techniques, such as its easy coupling to both gas-phase and liquid-phase separation, high sensitivity and specificity, and relatively low sample consumption (see Table 2). Moreover, the MS detector is capable of gaining spectral data for structural elucidation and identification of unknown metabolites [116].

6.1.1. Ionization methods

The ionization method that is most often used in LC–MS(/MS) bioanalysis still is electrospray ionization (ESI), followed by atmospheric pressure chemical ionization (APCI) [7,114]. As it is known to be less susceptible for ion saturation or suppression in the source, APCI is often employed to overcome these effects [61,75,114,117]. Less common techniques like atmospheric pressure photoionization have not been used in TKI bioanalysis. All MS methods used positive ionization for TKIs, with one method using positive/negative ionization switching for the simultaneous analysis of celecoxib [118].

6.1.2. Tandem mass spectrometry (Triple quadrupole)

Triple Quadrupole (QqQ) mass spectrometry remains the method of choice for quantitative analysis. These instruments have a high linear range, due to which they can cover a wide dynamic range. The multiple reaction monitoring (MRM) methods are highly specific and sensitive for the selected compounds.

The first quadrupole filters out most background, while the second quadrupole filters out unwanted fragments generated by the intermediate (quadrupole) collision cell by collision induced dissociation (CID). As said, the MRM-mode of QqQ-MS is highly selective and sensitive, the downside of this selectivity is that QqQ can only 'see' what is selected [119].

6.1.3. Time-Of-Flight

Time-of-Flight mass spectrometers differ from (triple-) quadrupole mass spectrometers on the basis that TOF instruments do not possess a mass filter, but separate m/z 's over time and thus can record whole spectra more sensitively. This can be used to find unexpected masses. The disadvantages are that TOF instruments generally are more suited for pulsed sources (e.g. Matrix Assisted Laser Desorption (MALDI)), and that the detector-response is less linear than is the case with QqQ. Hyphenation with other mass spectrometers like quadrupoles is often done to improve the quantitative performance, as well as introducing the ability to perform CID. Recently only one paper used TOF for quantitative analysis of TKIs by MALDI-MS-imaging [62]. In quantitative analysis in the more traditional way, TOF is not used at all recently.

6.1.4. Orbitrap

Most methods in quantitative analysis use MRM-mode triple quadrupole MS, as they excel in selectivity and sensitivity when compared to other types of MS's. However, the optimization of MRM is rather time consuming and requires a skilled technician, as cross-talk, adducts and non-specific CID-transitions have to be accounted for [119]. Co-eluting matrix-components with a very close m/z value to that of the analyzed parent drug can also be problematic in SRM acquisition [72,120,121]. Due to its selectivity, with SRM acquisition of additional information like degradation products or adducts of the analytes of interest is lost. Recently, Orbitrap was compared to a QqQ mass spectrometer. Both mass analyzers performed comparably, although the researchers claim a significant ease in method development and the QqQ's limitation to analyze a large panel of xenobiotics. The limitation of the analysis of large panels would be even exacerbated with UHPLC due to the rapid separation and overall short run-times [119]. This statement is not a strong argument, as QqQ's are capable of performing large pesticide panels [122], and quantitative assays for multiple drugs almost never analyze more than 10–15 components (not including toxicological screening, which are often semi-quantitative).

6.1.5. Mass interference

One concern in the bioanalysis of drugs is the interference caused by endogenous compounds or concomitant drug administration. While endogenous compounds are screened for by analyzing single and double blanks, concomitant administration of drugs is often overlooked. With MS and especially MS/MS detection, the potential for interference by other drugs is possibly neglected due to the acclaimed high specificity. With single quadrupole MS, at a unit mass resolution the signal for some TKIs could overlap, like the BCR-ABL targeting TKIs nilotinib ($^{12}\text{C}_{28}^{1}\text{H}_{22}^{19}\text{F}_3^{14}\text{N}_7^{16}\text{O}$) and bosutinib ($^{12}\text{C}_{26}^{1}\text{H}_{29}^{35}\text{Cl}_2^{14}\text{N}_5^{16}\text{O}_3$). For these two TKIs the mass difference is 0.0191, which is not distinguishable at that resolution. For tandem instruments, the fragments produced are different (288.9 for nilotinib, and 141.2 for bosutinib), and high resolution instruments can distinguish the masses [123,124]. And while the molecular weight of dasatinib and vemurafenib differs by 1.9168 mass units, the intensity of ^{37}Cl -dasatinib ($^{12}\text{C}_{22}^{1}\text{H}_{26}^{37}\text{Cl}^{14}\text{N}_7^{16}\text{O}_2^{32}\text{S}$) is 40% of the ^{35}Cl -vemurafenib, and differs 0.0810 mass units from ^{35}Cl -vemurafenib ($^{12}\text{C}_{23}^{1}\text{H}_{18}^{35}\text{Cl}^{19}\text{F}_2^{14}\text{N}_3^{16}\text{O}_3^{32}\text{S}$). Here again the fragments provide the assurance that tandem instruments can distinguish both com-

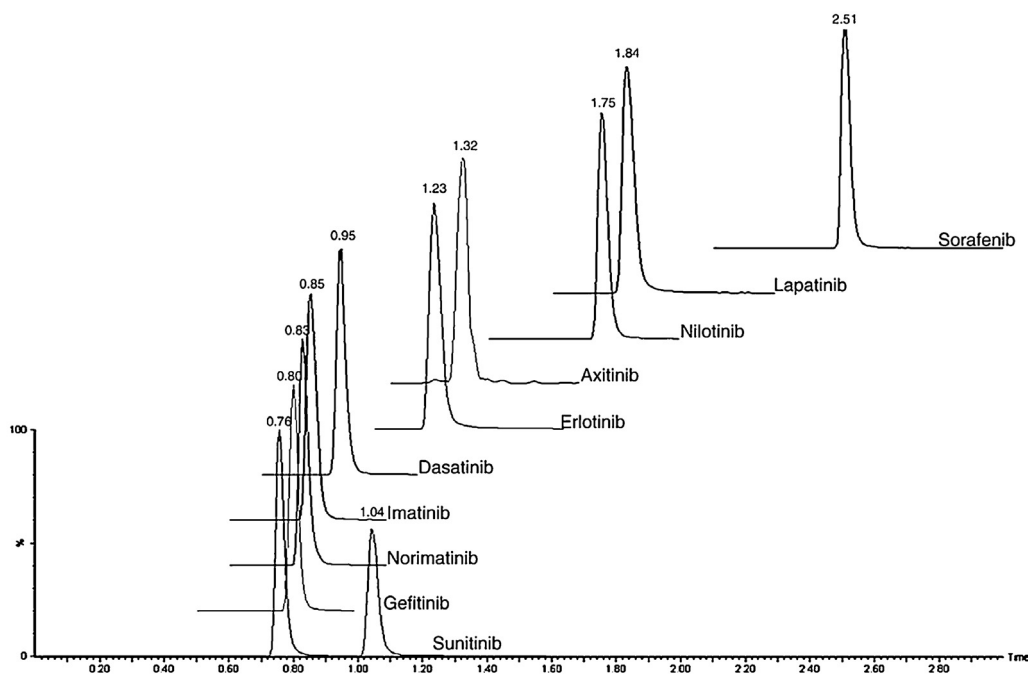


Fig. 3. Chromatogram of a plasma sample spiked with 10 compounds, 100 ng/mL imatinib, *N*-desmethyl-imatinib (norimatinib), nilotinib, lapatinib, erlotinib and sorafenib and 4 ng/mL for dasatinib, axitinib, gefitinib and sunitinib. Reprinted with permission from [15].

pounds (231.95 and 400.97 for dasatinib [7], and 255.0 and 383.1 for vemurafenib [66]), as well as being used for different targets, making concomitant use very unlikely and chromatographic separation. Isobaric metabolites and in-source metabolite conversion have been described in literature, and are discussed in more detail in Section 8.

6.2. Light spectroscopy

Whilst MS-detection excels in selectivity and sensitivity, and frequently produces faster analytical performance than light spectroscopic detection methods, it requires relatively expensive detectors, ranging from approximately 100,000 to 1,000,000 euros, and highly skilled technical expertise. These may not be readily available and/or affordable for most laboratories, particularly in resource-limited settings. In such settings, other detectors might be preferable to MS/MS [83,125,126].

6.2.1. UV-VIS/Diode array detector

When using UV-vis or diode array detection, the ranges usually increase to roughly 10-fold of the quantifying range of MS/MS detection. To lower these limits, usually a relatively large amount of sample is needed, around 100–500 μ l. For human or large animal samples this is feasible, but for small rodents these amounts require lethal exsanguination [77]. A major advantage of MS detection over photometric detection is the ability to drastically reduce analytical time per sample due to higher selectivity, especially when multiple analytes per run are quantified. The main downside of light spectroscopy is the lower quantification limit, which in some cases is not sufficient to measure the in TDM commonly used trough-levels. This is true for most TKIs for which a relatively low trough level is required, such as afatinib [98], dasatinib [98,99,127], lapatinib, and sunitinib [105]. For ponatinib, and trametinib, the required trough levels are also expected to range below the detection limit for LV-UV (see Table 2). Multiple-analyte quantitation is routinely performed by multiple laboratories using UV or DAD detectors for up to four TKIs [98,100,101,107,127,128], or the simultaneous quantitation of metabolites [96,108], compared to up to ten com-

ponents in TKI bioanalysis with MS-detection [15]. Recently, with increasing computer power, the selectivity of diode array detectors is increased by using principal component analysis methods such as alternating trilinear decomposition algorithms. This algorithm can be used to decompose mixed or overlapping signals from DADs to pure spectra [83].

6.2.2. Fluorescence detection (FLD)

In the absence of mass spectrometric detection, fluorescence detection (FLD) can provide a more selective detection method if the compound possesses fluorescent properties. Although FLD has some advantages over UV detection, FLD detectors are much less common. Recently FLD was only used for the quantitation of dasatinib, with a relatively low detection limit (50 ng/ml) with a 200 μ l sample- and 50 μ l injection-volume [126].

7. Simultaneous analysis of kinase inhibitors

A current trend in kinase inhibitor bioanalysis is quantification of various analytes in one assay. This is mostly done in clinical practice, where multi-target analysis can prove practical in daily routine [23,83,113,124,129,130]. For the TKIs targeting BCR-ABL (imatinib, dasatinib, nilotinib, bosutinib, ponatinib), a number of assays have been published, most notably for imatinib, dasatinib, and nilotinib, using either HPLC-MS or -UV [47,99,131]. There are no published assays yet for simultaneous analysis of ponatinib. Assays analyzing multiple TKIs are most employed in clinical setting for TDM, providing a platform for analysis of multiple analytes in one go. These assays are not limited to one subset of TKI's (unlike the assays described above). The difficulty in developing such assays lies in the extraction-efficiency, chromatographic separation, and selectivity and specificity of the detector [15,23,124,125,129,130]. With UHPLC-MS/MS (QqQ) up to 10 components (9 TKIs and one metabolite) could be quantified within a 4 min chromatographic run (see Fig. 3) [15]. The main issue with these short run-times in combination with MS-detection lies in the potential suppression of ionization. This can be overcome by the appropriate sample pretreatment, like SPE [15], or using a less

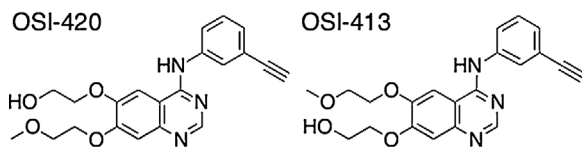


Fig. 4. Structures of isomeric O-desmethyl-erlotinib metabolites OSI420 (left) and OSI-413 (right) [84,135].

suppression-sensitive ionization technique like APCI [114]. With the less selective UV-detection, better separation is often required. All eluting components should be base-line separated, requiring 20–35 min run-times [100,105,127] for up to 9 different drugs [127].

8. Metabolite analysis

For a number of the registered TKIs one or more metabolites have been included in the bioanalytical assay. In Table 4 the Phase-I metabolites in these assays are noted. In recent years, the quantitative analysis of kinase inhibitor metabolites is playing a more prominent role in bioanalysis of TKIs. For some kinase inhibitors one or more active metabolites are known. These metabolites are in some cases equally potent when compared to the parent compound, as is the case for desmethyl-imatinib or N-desethyl-sunitinib [5]. For a number of known active metabolites, a quantitative assay has been reported, except for O-debenzyl-lapatinib, N-desmethyl-osimertinib, hydroxy- and N-desmethyl-pazopanib, N-deacetyl-trametinib, and hydroxy-ruxolitinib metabolite M5 (see Table 4). One of the main reasons for the absence of metabolite quantification is the lack of commercial standards at the time of development of the assay [84]. Alternatively, papers from Vikingsson et al. (hydroxy-vemurafenib (M1-3)) [132], Svedberg et al. (multiple erlotinib metabolites) [84], and Sparidans et al. (multiple crizotinib metabolites) [133] reported quantification of the metabolites relative to their parent compound, or a related metabolite of which an analytical standard is readily available [84,132,133]. For sunitinib, it has been demonstrated that the active metabolite is equally potent, but less dermatotoxic. Whilst concomitant administration of an CYP3A4 inhibitor would put patients at a higher risk of development of these toxicities, the use of an inducer might lower sunitinib toxicity without diminishing potency [9]. For sunitinib, multiple assays that focus on the active metabolite as well as the parent compound have been published [24,57,65,104,114,130,134]. The active O-desmethyl-metabolite of erlotinib, OSI420, is in most literature noted as the most abundant metabolite, while in fact the peak described as OSI420 actually were the isomeric OSI420 and OSI413 (Fig. 4) combined [84,135]. The development of high resolution chromatographic columns has improved the separation of these metabolites [84,135]. In these recent papers OSI413 was identified as the most dominant isomer. The OSI413 metabolite is not new, as it was already mentioned in the public registration documents, where it is stated that these metabolites were not separable by the chromatographic method. According to this, the two metabolites should be mentioned as the combined OSI413/420 [136]. Articles after that time all only mentioned the OSI420 metabolite, except for one article, which showed OSI413 in feces, but not in plasma or urine [137], and the two recent papers [84,135]. Although Phase-II conjugate drug metabolism does occur for TKIs [96,115,132,133,136], no recent analytical papers describing quantitative assays for these metabolites were published. Vikingsson et al. described that a glucuronide and a glucosylation metabolite of vemurafenib were excluded from their method due to low concentrations and poor stability [132]. Glucuronide and N-oxide metabolites can be an important source of interference, as they

can be converted back to the parent drug due to degradation in the ion source, thereby leading to cross-analyte interference in the transition window of the parent drug when these analytes are not chromatographically separated [138].

9. Advances in chromatography-less tyrosine kinase inhibitor bioanalysis

Recent advances in bioanalysis of kinase inhibitors focus on non-chromatographic quantitation of these drugs, of which three techniques have been introduced in the past five years. Two of which use laser assisted ionization.

9.1. Direct injection MS

Since the beginning of mass-spectrometry it has been hypothesized that LC-separation might be unnecessary due to the high selectivity of mass-detectors. Due to multiple effects this is fairly impractical, although some researchers are focusing on direct injection MS, or flow-injection analysis. In this technique there is great need for a stable isotope labeled internal standard, since all matrix-components that were not removed by the sample components will enter the ionization source at the same time as the analytes, causing ionization-suppression [121,139]. Since most analyses are done on triple-quadrupole mass analyzers, components with the same unit-resolution M/Z value can cause interference in the MRM channel of the component. One paper describes the use of the MRM³ (which is not actually MRM to the third power, but in fact a second fragmentation with a third mass-selection) technique in a Sciex QTRAP, in which the third separating quadrupole can function as a linear ion-trap. With this technique, the selectivity of the instrument can be augmented. Whilst the ionization-conditions are far from ideal with LC-less analysis, still a high sensitivity can be achieved with a lower limit of quantitation around 1 ng/ml for dasatinib to 5 ng/ml for imatinib, nilotinib and lapatinib in a single injection [121].

9.2. Direct DBS MS–MS analysis

A recent development is paper spray ionization, a direct, fast and low-cost combined sampling and ionization method. The method can be used for both qualitative and quantitative MS analysis of DBS. Ions are generated by applying a high voltage and a small volume of around ten microliters of spray solvent onto a porous substrate. The sample can be preloaded onto the paper or mixed into the spray solution. A disposable sample cartridge can be made for paper spray MS, aiding outpatient TDM analysis and point-of-care diagnostics [35,36]. The paper spray MS technique has been used for sunitinib [35] and imatinib [36] in a sensitive manner, with silica-coated paper a lower quantitation limit of 10 ng/ml was achieved for sunitinib. The substrate coating has a large impact on sensitivity, as the lower limit with uncoated chromatography paper is 500 ng/ml [35]. Also the shape and angle of the paper-tip, combined with the spray-voltage has a great influence on the sensitivity, as was described by Yang et al. [36].

9.3. Quantitative MS imaging

MALDI has been used to quantify dasatinib in mouse kidney medulla. Schultz et al. demonstrated that with normalization against a deuterated dasatinib internal standard, it is possible to quantify the drug relatively. The quantitative data correlated well with liquid chromatography–tandem mass spectrometry based drug quantification of dasatinib in mouse kidney medulla [62].

Table 4
table of relevant tyrosine kinase inhibitor Phase-I metabolites.

Drug	Metabolites (research code)	Source
afatinib	Negligible ¹ *	[145,146]
axitinib	-sulfoxide*	[191]
bosutinib	oxydechloro-*, <i>N</i> -desmethyl-*, - <i>N</i> -oxide*	[192]
cabozantinib	- <i>N</i> -oxide*	[193]
ceritinib	Negligible ¹ *	[180]
crizotinib	-lactam, - <i>N</i> -oxide, Hydroxy-	[133]
dabrafenib	Hydroxy-, Desmethyl-, Carboxy-	[194]
dasatinib	<i>N</i> -desalkyl, - <i>N</i> -oxide, -acid, Hydroxy-	[95,114]
erlotinib	O -desmethyl- (OSI413 and OSI420), <i>O,O</i> -Didesmethyl-	[61,84,114,118,135,150]
gefitinib	O -desmethyl-	[114,152]
ibrutinib	Dihydrodiol - (PCI45227)	[72,76,113,145]
imatinib	<i>N</i> -desmethyl- (CGP74588)	[10,15,100,108,119,130]
lapatinib	O -debenzyl-*	[114]
lenvatinib	Decyclopropyl-, Dimethyl-, - <i>N</i> -oxide <i>O</i> -dearyl-	[138]
nilotinib	- <i>N</i> -Oxide*, Hydroxy-*	[195,196]
nintedanib	<i>O</i> -demethyl-	[159]
osimertinib	<i>N</i> -desmethyl-*	[197]
pazopanib	Hydroxy *, <i>N</i> -demethyl-*	[198]
ponatinib	<i>N</i> -desmethyl-*	[199]
regorafenib	<i>N</i> -oxide- (BAY757495), <i>N</i> -oxide- <i>O</i> -desmethyl- (BAY818752)	[163]
ruxolitinib	Hydroxy *,	[200]
sorafenib	- <i>N</i> -oxide	[2,114]
sunitinib	<i>N</i> -desethyl- <i>N,N</i> -didesethyl-	[24,57,65,104,114,130,134]
trametinib	<i>N</i> -deacetyl- (M5)*	[201]
vandetanib	<i>N</i> -desmethyl-*, <i>O</i> -desmethyl-*	[202]
vemurafenib	Hydroxy- (M1-3)	[203]

Bold: confirmed active major metabolite, *curstive*: confirmed inactive metabolite, *:no bioanalytical paper available for metabolite (since 2010), ¹: (>90% of AUC^{0–24h} of total drug related material in patients is parent).

9.4. Ultra-high throughput bioanalysis

Recently, a non-chromatographic method has been published using laser diode thermal desorption (LDTD). One example of its practical use is the ultrafast analysis of ceritinib in human plasma by LDTD–APCI–MS/MS [117]. LDTD uses a laser diode to thermally desorb a dried sample into gas phase, somewhat similar to MALDI. The main difference to MALDI is that LDTD produces neutral species, which are transferred through to an APCI source using compressed air. This direct method achieves a very fast and efficient sample introduction into a mass spectrometer in a short period of time (usually less than 10 s), but still required sample preparation. The method performs well, with a validated range from 5 to 1000 ng/ml with good linearity and good accuracies and precisions of the QC samples. The method was compared to a validated LC–ESI–MS/MS method, and showed a high level of agreement with low bias [117].

9.5. Micellar enhanced spectrofluorimetry

An assay for ponatinib using only fluorimetric detection human plasma and urine has been published. The fluorescent properties of ponatinib in a micellar system of Cremophor RH 40 were significantly enhanced. The enhanced fluorescent properties give a linear relationship between the fluorescence intensity of PTB and its concentration over the range 5–120 ng/ml, with a limit of quantification of 2.7 ng/ml [140]. Unfortunately, this method will not be able to distinguish the parent compound from the active *N*-desmethyl metabolite and other metabolites. Although the use of surfactants to enhance the sensitivity of spectrofluorimetry is not new [141], until recently it was never used in TKI bioanalysis [140].

9.6. Immunoassay

Most non-chromatographic methods addressed in this review focus on MS-based detection. In routine lab settings, simple automated methods are preferred. Most methods in this review require skilled laboratory-technicians to prepare samples and handle

equipment. Immuno-assay based analyzers are routinely used for any number of assays in directly in biological fluids, not only drugs. These machines mostly work as ‘ready-to-use black-boxes’ with standardized assays, and require little skill to operate. A method for the analysis of imatinib on such an analyzer is recently described [27]. With a lower limit quantification of 296 ng/ml, it is no match for most LC assays, which have limits up to 8.4 ng/ml for MS [102] or 50 ng/ml for UV [105]. And although these methods are easy to operate, the required reagent-kits often make the cost per sample relatively high. One other concern in the development and use of these assays is the cross-reactivity towards metabolites of the drug. The authors claim that their method is adequately selective [27].

10. Future

The field of bioanalysis desires simple, high throughput assays that require a minimum number of (manual) steps. Chromatography still plays an important role in this, though chromatographic run times are considerably shortened. Where the field of chromatography used to focus on the most efficient separation of all (residual) components in a sample when using spectroscopic detection, with MS-detection, the high selectivity of the detector usually is able to accurately distinguish between components and filter out any co-eluting (non-isobaric) components. The main objective is providing enough separation, creating an elution window in which the ionization is least harmed, for instance ballistic gradient separation of 1 minute LC–MS [109], or high throughput SPE–MS devices used in drug discovery like the Agilent RapidFire system [142]. Recently, ultrafast chromatography-less analysis with minimal sample preparation was achieved for multiple TKIs using flow-injection [139], paper-spray [35,36], or LDTD–APCI [117]. Techniques like these remove the relatively slow chromatography, increasing throughput of the equipment while techniques like APCI inherently suffer less from ionization suppressive effects [114,117]. For clinical settings, automated (immuno-)assays allow easy method-implementation [27]. The other techniques require skilled personnel, and cumbersome method development and val-

idation. We envisage that MS detection will become the standard detection system, as MS detectors are becoming readily available to more-and-more institutes, as the prices become lower and more vendors enter the market. Some researchers predict the use of high mass-resolution instruments, such as hybrid-TOF or –Orbitrap systems [119,120]. The Orbitrap mass analyzer was introduced commercially in 2005 and possesses ideal properties for bioanalysis, like a high dynamic range and mass accuracy [116]. The problem with taurocholic acid described earlier could have been resolved using a high resolution mass-analyzer, as the taurocholic acid with the in-source loss of $2\text{H}_2\text{O}$ giving a m/z of 480.2778 ($\text{C}_{26}\text{H}_{42}\text{NO}_5\text{S}^+$), and the m/z of the internal standard of the metabolite at 480.2402 ($\text{C}_{25}\text{H}_{42}\text{D}_5\text{N}_6\text{O}_4^+$) [72]. The required resolution to distinguish between the two is around 12,500, which is well below the limits of either TOF or Orbitrap. These instruments also offer flexibility when an unknown component is encountered, as the high-resolution limits the search-window.

Another separation technique, that is not noted earlier in this review, is the ion-mobility interface which could aid in enhancing chromatography-less bioanalysis, also being able to possibly separate isobaric compounds, or even chiral compounds [143].

Bioanalysis of TKIs is an indispensable tool in drug and cancer-research. The assays that were developed over the last few years helped gaining insight in not only PK/PD modeling, but also into drug-drug and drug-food interactions, drug-metabolism, and drug-induced toxicity. With the elucidation of clinical exposure-effect relationships, and therapeutic target levels, these assays can be used for the improvement of individual dosing, reducing side effects and improving clinical efficacy through TDM. Even for TKIs like erlotinib, the assays still have an added value, creating new insights in metabolism [84,135] and even making local headlines by research about oral absorption enhancement by something simple as a soda [144]. It is envisioned that research using these bioanalytical tools can improve the effectiveness and safety of TKIs.

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