Journal of Chromatography B 1162 (2021) 122466

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

Review

Chromatographic bioanalytical assays for targeted covalent kinase inhibitors and their metabolites

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ARTICLE INFO

Keywords:
Covalent targeted kinase inhibitors
Metabolites
Biomatrices
Chromatography
Mass spectrometry

ABSTRACT

Deriving from targeted kinase inhibitors (TKIs), targeted covalent kinase inhibitors (TCKIs) are a new class of TKIs that are covalently bound to their target residue of kinase receptors. Currently, there are many new TCKIs under clinical development besides afatinib, ibrutinib, osimertinib, neratinib, acalabrutinib, dacomitinib, and zanubrutinib that are already approved by the FDA. Subsequently, there is an increasing demand for bioanalytical methods to qualitatively and quantitively investigate those compounds, leading to a number of papers reporting the development, validation, and use of bioanalytical methods for TCKIs. Most publications describe the technological set up of analytical methods that allow quantification of TCKIs in various biomatrices such as plasma, cerebrospinal fluid, urine, tissue, and liver microsomes. In addition, the identification of metabolites and biotransformation pathways of new TCKIs has gained more interest in recent years. We provide an overview of bioanalytical methods of this new class of TCKIs. The included issues are sample pretreatment, chromatographic separation, detection, and method validation. In the scope of bioanalysis of TCKIs, protein precipitation is mostly applied to treat the biological matrices sample. Liquid chromatographic in reversed-phase mode (RPLC) and mass detection with triple quadrupole (QqQ) are the most often utilized separation and quantitative detection modes, respectively. There may be a possibility of increased use of the high-resolution mass spectrometry (HRMS) for qualitative investigation purposes in the future. We also found that US FDA and EMA guidelines are the most common guidelines employed as validation framework for the bioanalytical methods of TCKIs.

1. Introduction

A protein kinase is an enzyme that modifies other proteins via phosphorylation. In kinase phosphorylation, the terminal γ-phosphate group from adenosine triphosphate (ATP) is chemically added to serine, threonine, or tyrosine residues [1,2]. Most protein kinases promote cell proliferation, migration, and survival via the phosphorylation process [2]. This process is tightly regulated, and any disruption in this regulation may lead to disease states [1,3].

It has been well received that the dysregulation of kinases enhancing oncogenic potential [4,5]. Several mechanisms triggering kinase dysregulation are overexpression, relocation, and fusion, point mutations, or dysregulation of upstream signaling [6,7]. Following the fundamental finding on the protein kinase role in cancer, the development of small molecule targeting protein kinase as a cancer therapy is emerging and has been proven successful in clinical therapy [2]. The first targeted kinase inhibitor (TKI), imatinib, was approved by the US FDA in 2001 to treat chronic myeloid leukemia. To date, the US FDA has approved fifty-two (52) small molecule compound kinase inhibitors [8].

In general, TKIs are less toxic than conventional chemotherapy. They are also more potent in the right selected patient population. However, similar to conventional chemotherapy, their limitations are the possibility of resistance development and unwanted side effects [1]. TKIs can be covalently or noncovalently bound to their target protein subsequently differently affect their clinical outcome. Covalently bound TKIs tend to have an enhanced potency and an extended duration of action due to its irreversible bound and longer drug-target interaction compared to noncovalently bound (reversible) kinase inhibitors [9].

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https://doi.org/10.1016/j.jchromb.2020.122466
Received 24 September 2020; Received in revised form 17 November 2020; Accepted 19 November 2020
Available online 30 November 2020

This has initiated a new class of TKIs, targeted covalent kinase inhibitors (TCKIs).

TCKIs have been proven better reducing the risk of drug resistance than the reversible kinase inhibitors [10]. TCKIs, unlike the reversible kinase inhibitors, have a scaffold, usually an electrophile warhead, capable of accommodating a reaction moiety. This electrophile warhead plays a pivotal role in improving the selectivity and binding affinity of TCKIs by forming covalent interaction with a kinase residue [5]. However, it has been described that the covalent binding of TCKIs is different across the species [11] and therefore requires specific investigation.

TCKIs have rapidly emerged since afatinib is approved by US FDA in 2013 to treat non-small cell lung carcinoma (NSCLC) due to their higher selectivity and reduced risk of toxicity. To date, there are seven TCKIs that have been approved by US FDA (Table 1), with zanubrutinib being the latest drug approved in November 2019 to treat mantle cell leukemia [12], and many compounds are still under clinical investigation. With many attempts are made to investigate more TCKI candidates, bioanalysis is also becoming an indispensable tool for drug development of this new class of agents, specifically for the pharmacokinetic and pharmacodynamic investigation of those compounds [12-14]. According to our knowledge, approved TCKIs and those under development stage to date target only three families of protein kinase, i.e., Epidermal Growth Factor Receptor (EGFR) [15], Bruton's Tyrosine Kinase (BTK) receptor [16], and Fibroblast Growth Factor Receptor (FGFR) [17].

It is known that TKIs, in general, are associated with adverse effects that may be caused by the formation of reactive metabolites during TKIs metabolism [18]. Following this proposition, recent research on the biotransformation of TCKIs has been moving to the spotlight. Moreover, in recent times where triple quadrupole-mass spectrometry (QqQ-MS) becomes more accessible, more bioanalysis of TCKIs and their metabolites have been reported both quantitatively and qualitatively.

It is also well established that therapy with TKIs, including TCKIs, shows a high variability of the response in patients. It can be caused not only by the heterogeneity of the drug targets determining the sensitivity of the tumor but also by the pharmacogenetic background of the patients (e.g., polymorphisms of Cytochrome P450 (CYP)), patient characteristics (the adherence to treatment), and environmental factors [19]. For this specific reason, bioanalysis is often used for therapeutic drug monitoring (TDM) of TKIs and TCKIs for patient management, emphasizing the importance of TCKIs bioanalysis in a routine clinical setting [20]. Due to the rapid emergence and development of TCKIs, bioanalytical methods of this class of drugs are essential, and more may be required in the future. Therefore, this review reports the current bioanalytical assays of TCKIs and their metabolites, aiming to offer an insight into the recent bioanalytical trends in this oncologic field and to provide preliminary information maybe useful to develop bioanalytical method for upcoming promising compounds in TCKIs group

2. Methods

This review focuses only on TCKIs targeting EGFR [15], BTK receptor [16], and FGFR [17]. The website of EMA and US FDA, as well as ClinicalTrials.gov [21], were searched to confirm both registration status and clinical development of the eighteen TCKIs (Table 1). The molecular structure of those compounds, including several physical and chemical characteristics along with the electrophile warhead moiety, are depicted in Fig. 1. Pubmed database [22] was used to find and to collect bioanalytical papers utilizing chromatography techniques on listed TCKIs. Only original research articles published until June 2020 and describing the bioanalytical assays were included in this review. Labeled-compound analysis (mass-balanced study) and covalent binding analysis were not included in this review since different discussion and approach will be needed for that specific analysis. Papers describing both validated and unvalidated analytical assays were included due to the limitation of published papers, especially for TCKIs in the development stage.

From the initial search (["name of TCKI"] and ["chromatography"]), one hundred and twelve papers were included. Only those that mentioned sufficient details of the analytical method, i.e., sample pretreatment, separation conditions, and detection mode, were included for this review. After carefully assessing all of the articles, sixty articles in total for thirteen TCKIs remained, in which five of them contained more than one TCKI. From those research papers, information on the sample extraction, detection method, calibration range, and details of separation conditions and stability data were extracted (Table 2 & 3).

No bioanalytical assay article was found for tirabrutinib (BTK receptor inhibitor) to date. On the other hand, fisogatinib is the only FGFR inhibitor that has a published bioanalytical assay. From the sixty collected articles, two reported unvalidated methods, eight did not report whether the assay used is validated or not, and three papers indicated that the method used was validated by external partners without declaring which guideline was followed for the validation. The remaining forty-seven articles reported validated assays of the TCKIs both alone or together with other compounds, including their metabolites or other TKIs. Several guidelines are used for laboratory framework for the bioanalytical validation method by listed papers (Table 2) published by US FDA [23], EMA [24], ICH [25], SFSTP [26], Japan BMV [27], and China FDA/China Pharmacopeia [28] with US FDA and EMA

Table 1
List of Targeted Covalent Kinase Inhibitors (TCKIs).

Name / Research Code (Trade name)	Target	Indication	FDA Approval Year / CT Progress
Acalabrutinib / ACP-196 (Calquence)	BTK receptor	CLL, MCL	2017
Afatinib / BIBW2992 (Gilotrif)	EGFR (ErbB 1/2/4)	NSCLC	2013
Avitinib / AC0010	EGFR	NSCLC	Phase I & II CT
Dacomitinib / PF-00299804 (Vizimpro)	EGFR (Pan-HER)	NSCLC	2018
Fisogatinib / BLU-554	FGFR4	Hepatocellular carcinoma	Phase I & II CT
Futibatinib / TAS-120	Pan-FGFR	Advanced solid tumor	Phase I & II CT
H3B-6527	FGFR4	Hepatocellular carcinoma	Phase I CT
Ibrutinib / PCI-32765 (Imbruvica)	BTK receptor	CLL, MCL, WM	2013
Naquotinib / ASP8273	EGFR	NSCLC	Phase II CT
Neratinib / HKI-272 (Nerlynx)	EGFR (ErbB2/HER)	Breast cancer	2015
Olmutinib / HM61713	EGFR	NSCLC	Phase II CT
Osimertinib / AZD-92921 (Tagrisso)	EGFR	NSCLC	2015
Pelitinib / EKB-569	EGFR	NSCLC, CRC	Phase I CT
PRN1371	Pan-FGFR, CSF1R	Metastatic urothelial carcinoma	Phase I CT
Roblitinib / FGF401	FGFR4	Hepatocellular carcinoma	Phase I CT
Spebrutinib / AVL-292	BTK receptor	CLL	Phase I CT
Tirabrutinib / GS-4059	BTK receptor	WM	Phase II CT
Zanubrutinib / BGB-3111 (Burkinsa)	BTK receptor	CLL, MCL	2019 (MCL), Phase III CT (CLL)

BTK = Bruton's Tyrosine Kinase, EGFR = Epidermal Growth Factor Receptor, FGFR = Fibroblast Growth Factor Receptor, CLL = Chronic Lymphocytic Leukemia, MCL = Mantle cell lymphoma, NSCLC = Non-small cell lung cancer, WM = Waldenstorm macroglobulinemia, FDA = Food and Drug Administration; CT = Clinical trial.

Table 2
Information on TCKIs bioanalysis.

Drugs	Other drug/ metabolites	Matrix	Sample Preparation	Internal Standard	Separation Conditions				Detector	Validation Status	Dynamic Range (ng/ mL)	Ref
					Column	Mobile phase	Elution type	pH Cond.				
Acalabrutinib	No	Rat plasma	SPE; adsorbent: Orochem Celerity deluxe DVB-LP (RP); eluent: 80% ACN in water	Carbamezepine	Eclipse Plus C8	A: 5 mM NH_4COOH B: ACN	Gradient	N.R.	QqQ	US FDA	0.2 – 199	[88]
Afatinib	No	Human plasma	LLE with diethyl ether	Cyclobenzaprine	Luna PFP	ACN-water (40:60) containing 10 mM NH_4COOH	Isocratic	Acid (pH 4.5)	QqQ	US FDA & ICH	0.5 – 500	[68]
	No	Mouse plasma	SALLE with ACN and $MgCl_2$	$^{13}C_6$-afatinib	BEH C18	A: 0.1% NH_4OH in water B: ACN	Gradient	Basic	QqQ	US FDA & EMA	0.5 – 500	[94]
	No	Mouse plasma	PP with ACN	Carbutamide	Luna C18	A: 0.1% FA in water B: 0.1% FA in ACN	Gradient	Acid	QqQ	Not validated	103 – 51,400	[131]
	No	Rat plasma	PP with methanol	Gefitinib	Eclipse XDB-CN	water : methanol (15:85 v/v) containing 0.1% FA	Isocratic	Acid	QqQ	US FDA	0.5 – 200	[65]
	No	Human plasma	SPE; adsorbent: monomerically bounded DSC-18 (RP); eluent: methanol/ ACN/water	$^{13}C_6$-afatinib	C18	N.R.	Gradient	N.R.	QqQ	external parties	0.1-NR	[113]
	Yes	Human plasma	PP with ACN (containing 1% (v/v) FA)	$^{13}C_6$-afatinib	Accucore C18	A: 0.1% FA in water B: 0.1% FA in ACN	Gradient	Acid	QqQ	US FDA & EMA	5 – 250	[57]
	Yes	Human plasma	Dilution: 1/5 with solution of SDS and $NaH_2PO_4{\cdot}H_2O$ pH 7	N.R.	Kromasil C18	0.07 M SDS – 6% v/v 1-pentanol, buffered at pH 7 with 0.01 M phosphate salt	Isocratic	Neutral (pH 7.0)	UV-DAD	EMA	500,000 – 10,000,000	[82]
	Yes	Human plasma	PP with ACN	$^{13}C_6$-afatinib	BEH C18	A: 10 mM NH_4COOH B: 0.1% FA in ACN	Gradient	Acid (pH A 4.5)	QqQ	US FDA	1 – 100	[49]
	Yes	Human plasma	SPE; adsorbent: Oasis MCX (RP-IE); eluent: ACN/ methanol/ ammonia 25%	$^{13}C_6$-afatinib	Cortec C18 + UPLC	A: acetic acid buffer 0.01% in water B: ACN added with 10% A	Gradient	Acid	QqQ	US FDA & EMA	4 – 800	[121]
	Yes	Human plasma	PP with ACN	$^{13}C_6$-afatinib	Gemini C18	A: 10 mM $NH_4(HCO_3)$ in water B: 10 mM $NH_4(HCO_3)$ in methanol–water (1:9)	Gradient	N.R.	QqQ	US FDA & EMA	2 – 200	[132]
	Yes	Human Plasma	PP with ACN	$^{13}C_6$-afatinib	BEH C18	A: 0.1% NH_4OH & 0.01% FA in water B: ACN C: methanol	Gradient	Basic (pH A 9.8)	QqQ	US FDA & EMA	1 – 1000	[93]
	Yes	Human plasma	LLE with diethyl ether*	Cyclobenzaprine	Luna PFP	ACN : 0.01 M NH_4COOH in water (50:50 v/v)	Isocratic	Acid (pH B 4.1)	QqQ	US FDA & ICH	5 –100	[69]
	Yes	Human plasma	LLE with TBME*	Imatinib	Xbridge Shield RP18	10 mM NH_4OH in ACN : 1 mM NH_4OH in water (70:30 v/v)	Isocratic	Basic (pH aqueous 10.5)	QqQ	US FDA	0.1 – 206	[70]
	Yes	Human plasma	PP with ACN	N.R.	C18	Methanol : 0.1% FA in water (65:35 v/v)	Isocratic	Acid	DAD	N.R.	700 – 7000	[89]
	Yes	Human plasma	SPE; adsorbent: Oasis MCX (RP-IE); eluent: ACN/ methanol/ ammonia 25%	Diclofenac sodium	BEH C18	A: NH_4COOH buffer B: ACN	Gradient	Acid (pH A 3.2)	DAD	SFSTP	5 – 200	[80]
Avitinib (Abivertinib)	Yes (m)	Human CSF	PP with ACN*	IS-0010	BEH C18	A: water containing 10 mM $NH_4CH_3CO_2$ and 0.1% FA B: ACN:methanol (8:2 v/v) containing 0.1% FA	Gradient	Acid	QqQ	US FDA, EMA, China FDA	0.05 – 50	[34]
	Yes (m)	Rat liver microsomes	PP with ACN*	N.R.	Eclipse C18	A: 10 mM NH_4COOH in water B: ACN	Gradient	N.R.	QqQ	N.R.	N.R.	[45]
	Yes (only metabolites)	Human plasma	SPE; adsorbent: Oasis HLB (RP); eluent: methanol*	IS-0010	BEH C18	A: 10 mM $NH_4CH_3CO_2$ with 0.1% FA B: methanol-ACN (2:8 v/v) with 0.1% FA	Gradient	Acid	QqQ	US FDA, EMA, China FDA	0.5 – 500	[119]

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Table 2 (*continued*)

Drugs	Other drug/ metabolites	Matrix	Sample Preparation	Internal Standard	Separation Conditions Column	Mobile phase	Elution type	pH Cond.	Detector	Validation Status	Dynamic Range (ng/ mL)	Ref
Dacomitinib	No	Rat plasma	PP with ACN	Ibrutinib	BEH C18	A: ACN B: 0.1% FA in water	Gradient	Acid	QqQ	US FDA & EMA	1 – 150	[133]
	No	Rat liver microsomes	PP with ACN	Lapatinib	C18	10 mM NH_4COOH : ACN (30:70 v/v)	Isocratic	Acid (pH A 4.2)	QqQ	US FDA	2 – 500	[43]
Fisogatinib	Yes	Mouse plasma, homogenate mouse tissue***	PP with ACN	Erlotinib	BEH C18	A: 0.2% NH_4OH in water B: Methanol	Gradient	Basic	QqQ	US FDA & EMA	1 – 500	[41]
Ibrutinib	No	Human plasma	SPE; adsorbent: Oasis HLB cartridge (RP); eluent: methanol*	Nilotinib	Capcel Pack C18	ACN : 0.5% KH_2PO_4 in water (52:48% v/v)	Isocratic	Acid (pH 3.0)	UV	US FDA	10 – 500	[108]
	No	Rat plasma	LLE with ethyl acetate containing 1% triethylamine*	Evobrutinib	BEH C18	A: 0.1% FA in water B: ACN	Gradient	Acid	Q-Orbitrap	N.R.	1 – 1000	[73]
	No	Rat plasma	PP with ACN	Carbamazepine	BEH C18	A: 0.1% FA in water B: ACN	Gradient	Acid	QqQ	N.R.	1 – 1000	[134]
	No	Rat plasma	PP with ACN	Vilazodone	SB-C18	10 mM $NH_4CH_3CO_2$ + 0.1% FA in water : ACN (10:90 v/v)	Isocratic	Acid	QqQ	US FDA	1 – 2000	[135]
	Yes	Human plasma	LLE with a mixture of ethyl acetate and TBME*	Quizartinib	BEH C18	0.1% FA in ACN : 20 mM $NH_4CH_3CO_2$ (95:5 v/v)	Isocratic	Acid	QqQ	US FDA & EMA	5 – 1000	[71]
	Yes (m)	Human plasma	SLE; adsorbent: ISOLUTE SLE (Biotage); eluent: MTBE*	$^2H_5$-ibrutinib	L-column3 C18	10 mM NH_4COOH + 0.1% FA in water : 0.1% FA in ACN (55:45 v/v)	Isocratic	Acid	QqQ	US FDA & EMA	1 – 3000	[79]
	Yes	Human plasma	SALLE with ACN and zinc sulphate	$^2H_5$-ibrutinib	BEH C18	A: 10 mM NH_4COOH containing 0.1% FA B: ACN containing 0.1% FA	Gradient	Acid	QqQ	US FDA	1 – 500	[122]
	Yes	Human plasma	SPE; adsorbent: Oasis PRiME MCX (RP- IE); eluent: 5% NH_4OH in methanol and water	$^2H_5$-ibrutinib	Poroshell 120 ECC18	A: 0.1% FA in water B: 0.1% FA in ACN	Gradient	Acid (final pH 2)	QqQ	US FDA, EMA, ICH	0.3 – 400	[123]
	Yes	Human plasma	SPE; adsorbent: Oasis MCX (RP-IE); eluent: ACN/ methanol/ ammonia 25%	Diclofenac sodium	BEH C18	A: NH_4COOH buffer B: ACN	Gradient	Acid (pH A 3.2)	DAD	SFSTP	5 – 400	[80]
	Yes (m)	Rat plasma	LLE with ethyl acetate/ dichloromethane (90/10)*	Tolbutamide	YMC Pack ODS (C18) AM	A: ACN B: 0.1% FA buffer	Gradient	Acid	QqQ	US FDA	0.7 – 183	[72]
	Yes	Rat plasma	PP with methanol/water	Diazepam	X-Bridge C18	A: methanol B: water Both containing 5 mM $NH_4CH_3CO_2$ −0.2% FA	Gradient	Acid	QqQ	US FDA, EMA, Chinese FDA	1 – 500	[136]
	Yes	Mouse blood	PP with ACN	Labetalol, indomethacin, loperamide	Kinetex XB-C18	A: water with 0.1% FA B: ACN with 0.1% FA	Gradient	Acid	QqQ	Not validated	N.R.	[129]
	Yes (m)	Human plasma	PP with ACN*	$^2H_5$-ibrutinib	CSH C18	A: 0.1%FA in water B: Methanol	Gradient	Acid	QqQ	US FDA & EMA	1.1 – 1135	[87]
	Yes (m)	Human plasma	PP with ACN	$^2H_5$-ibrutinib	Method A Waters X-bridge C18 Method B Waters XSelect CSH Phenyl-Hexyl	Method A A: 0.01 M ammonium carbonate in water B: methanol:isopropanol (80:20 v/v) Method B A: 0.01 M ammonium carbonate in water B: ACN	Gradient	Acid	QqQ	US FDA & EMA	0.5 – 100	[50]
	Yes (m)	Human and mouse plasma	PP with ACN	$^2H_5$-ibrutinib	XBridge BEH300 C18	A: 0.1% FA in water B: Methanol	Gradient	Acid	QqQ	US FDA & EMA	5 – 5000	[47]

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Table 2 (*continued*)

Drugs	Other drug/ metabolites	Matrix	Sample Preparation	Internal Standard	Separation Conditions Column	Mobile phase	Elution type	pH Cond.	Detector	Validation Status	Dynamic Range (ng/ mL)	Ref
	Yes (m)	Human CSF & plasma	PP with ACN*	$^{2}H_5$-ibrutinib	BEH C18	A: 5 mM NH_4COOH buffer B: ACN containing 0.1% FA	Gradient	Acid (pH A 3.2)	QqQ	SFSTP	5 – 491**	[36]
	Yes (m)	Rat, dog, human hepatocytes	PP with ACN*	N.R.	BEH C18	A: 2 mM $NH_4CH_3CO_2$ B: ACN containing 0.1% FA	Gradient	Acid	DAD-Q-Orbitrap	N.R.	N.R.	[106]
	No	Huma plasma	LLE with ethyl acetate*	Lidocaine	C18 Hypersil Gold	ACN : Methanol : buffer solution of sodium acetate 10 mM & acetic acid 10 mM (40 : 20 : 40 v/ v/v)	Isocratic	Acid (pH buffer 4.7)	DAD	Validation guideline not reported	1 – 50	[111]
Naquotinib	No	Human liver microsomes	PP with ACN	Foretinib	Eclipse Plus C18	ACN : 10 mM NH_4COOH (45:55 v/v)	Isocratic	Acid (pH aqueous 4.2)	QqQ	US FDA	5 – 500	[137]
Neratinib	No	Human plasma	PP with ACN*	$^{2}H_6$-neratinib	BEH Shield RP18	A: methanol:water (90:10 v/v) with 10 mM $NH_4CH_3CO_2$ and 0.1% FA B: water with 10 mM $NH_4CH_3CO_2$ buffer and 0.1% FA	Gradient	Acid	QqQ	US FDA	2 – 1000	[138]
	Yes	Rat plasma	SPE; adsorbent: C18 cartridge (RP); eluent: methanol*	Domperidone	BEH C18	ACN containing 0.1% FA : water with 0.1% FA (70:30 v/v)	Isocratic	Acid	QqQ	US FDA	0.5 – 200	[139]
	Yes (m)	Rat hepatocytes, urine, bile	SPE; adsorbent: Oasis HLB (RP), eluent: methanol*	N.R.	HSS T3	A: 0.1% FA in water B: ACN	Gradient	Acid	DAD-Q-Orbitrap	N.R.	N.R.	[54]
Olmutinib	No	Rat plasma	PP with ACN	Dasatinib	Eclipse Plus RRHD C18	A: 0.1% FA in water B: ACN	Gradient	Acid	QqQ	US FDA & EMA	1 – 500	[85]
	No	Human plasma	PP with ACN	Ponatinib	Eclipse Plus C18	ACN and 0.1% FA in water (40:60 v/v)	Isocratic	Acid	QqQ	US FDA	5 – 500	[84]
	Yes (m)	Rat liver microsomes	PP with ACN*	N.R.	Eclipse Plus C18	A: 0.1% FA in water B: ACN	Gradient	Acid	QqQ	N.R.	N.R.	[46]
Osimertinib	No	Human plasma/ CSF	PP with ACN	Gefitinib D6	C18	10 mM $NH_4CH_3CO_2$ in water : ACN (50:50 v/v)	Isocratic	Acid	QqQ	Japan BMV	80 – 2000**	[35]
	No	Human plasma	PP with methanol	Pazopanib	C18	A: 2 mM $NH_4CH_3CO_2$ in water + 0.1% FA B: 2 mM $NH_4CH_3CO_2$ in methanol + 0.1% FA	Gradient	Acid	QqQ	EMA	25 – 500	[48]
	No	Human plasma	SALLE with ACN and magnesium sulphate	Pazopanib	BEH C18	A: 1.0% FA in water B: ACN	Gradient	Acid	QqQ	US FDA & EMA	1 – 1000	[77]
	No	Rat plasma	PP with ACN*	Sorafenib	Xterra MS C18	A: 0.1% ammonia water B: ACN	Gradient	Basic	Q-TOF	US FDA	1 – 500	[107]
	No	Rat plasma	PP with methanol/acetonitrile	Crizotinib	C18	A: 0.1% FA in water B: ACN	Gradient	Acid	QqQ	US FDA	1 – 1000	[66]
	Yes	Human plasma	PP with ACN with 1% (v/v) FA	^{13}C-$^{2}H_3$-osimertinib	Accucore C18	A: 0.1% FA in water B: 0.1% FA in ACN	Gradient	Acid	QqQ	US FDA & EMA	5 – 1000	[57]
	Yes	Human plasma	PP with ACN	^{13}C-$^{2}H_3$-osimertinib	Gemini C18	A: 10 mM $NH_4(HCO_3)$ in water B: 10 mM $NH_4(HCO_3)$ in methanol–water (1:9 v/v)	Gradient	Basic (pH A & B 10.5)	QqQ	US FDA & EMA	100 – 2000	[95]
	Yes	Human plasma	PP with ACN	$^{13}C_6$-afatinib, $^{2}H_6$-erlotinib, $^{2}H_5$-crizotinib	BEH C18	A: 10 mM NH_4COOH B: 0.1% FA in ACN	Gradient	Acid (pH A 4.5)	QqQ	US FDA	10 – 1000	[49]
	Yes	Human plasma	LLE with mixture of ethyl acetate and TBME*	Quizartinib	BEH C18	0.1% FA in ACN : 20 mM $NH_4CH_3CO_2$ (95:5 v/v)	Isocratic	Acid	QqQ	US FDA & EMA	5 – 1000	[71]
	Yes (m)	Human plasma	PP with ACN*	^{13}C-$^{2}H_3$-osimertinib	CSH-C18 column	A: 0.1% FA in water B: 0.1% FA in ACN	Gradient	Acid	QqQ	US FDA & EMA	4 – 4000	[114]
	Yes (m)	Human plasma/ urine/ CSF	PP with ACN*	^{13}C-$^{2}H_3$-osimertinib	Plasma: BEH C18	A: 10 mM NH_4COOH + 0.2% FA B: ACN	Gradient	Acid	QqQ	US FDA & EMA	0.025 – 25**	[38]
	Yes (m)	Human plasma	PP with ACN	IS-0741	BEH C18	A: FA – 10 mM $NH_4CH_3CO_2$ (1:1000 v/v) B: ACN	Gradient	Acid	QqQ	US FDA, EMA, China FDA	0.5 – 100	[115]
	Yes (m)	Human plasma	PP with ACN	Triazolam	Kinetex C18		Gradient	Acid	QqQ	N.R.	N.R.	[116]

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Table 2 (*continued*)

Drugs	Other drug/ metabolites	Matrix	Sample Preparation	Internal Standard	Separation Conditions Column	Mobile phase	Elution type	pH Cond.	Detector	Validation Status	Dynamic Range (ng/mL)	Ref
						A: 0.1% FA in water B: 0.1% FA in ACN						
	Yes (m)	Human plasma	PP with ACN*	^{13}C-$^{2}H_3$-osimertinib	BEH C18	A: 10 mM NH_4COOH containing 0.2% FA B: ACN	Gradient	Acid	QqQ	external parties	0.025 - NR	[140]
Pelitinib	No	Human plasma	PP with ACN	Erlotinib	BEH C18	A: 0.1% FA in water, B: ACN, C: water, D: methanol	Gradient	Acid	QqQ	US FDA & EMA	1 – 200	[141]
	Yes	Rat plasma	SPE; adsorbent: C18 cartridge (RP); eluent: methanol*	Domperidone	BEH C18	ACN containing 0.1% FA : water with 0.1% FA (70:30 v/v)	Isocratic	Acid	QqQ	US FDA	0.5 – 200	[139]
Spebrutinib	No	Human liver microsomes	PP with ACN	Naquotinib	Eclipse Plus C18	10 mM NH_4COOH in water : ACN (60:40 v/v)	Isocratic	Acid (pH aqueous 4.2)	QqQ	US FDA	5 – 500	[44]
Zanubrutinib	No	Human plasma	PP (solvent was not reported)	N.R.	Supelco Ascentis Express C18	A: 0.1% FA in water B: methanol:ACN (1:1 v/v)	Gradient	Acid	QqQ	external parties	1 – 1000	[128]

CSF = cerebrospinal fluid, PP = protein precipitation, LLE = liquid–liquid extraction, SLE = supported liquid–liquid extraction, SPE = solid-phase extraction, ACN = acetonitrile, TBME = *tert*-butyl-methyl-ether, RP = reversed-phase, RP-IE = reversed-phase ion exchange , SDS = sodium dodecyl sulphate, BEH = ethylene bridged hybrid, PFP = pentafluorophenyl, CSH = charged surface hybrid, HSS = high strength silica, NH_4COOH = ammonium formate, $NH_4CH_3CO_2$ = ammonium acetate, $NH_4(HCO_3)$ = ammonium bicarbonate , KH_2PO_4 = potassium dihydrogen phosphate , NH_4OH = ammonium hydroxide, mM = milimolar, M = molar, FA = Formic Acid, QqQ = triple quadrupole, Q = quadrupole, DAD = diode array detector, UV = Ultraviolet, US FDA = United States Food and Drug Agency, EMA = European Medicine Agency, SFSTP = The Société Française des Science et Technique Pharmaceutiques, Japan BMV = Japan Bioanalysis Method Validation, ICH = The International Council for Harmonization of Technical Requirement for Pharmaceuticals for Human Use, N.R. = Not reported (the intended information was not reported in the paper),

* sample preparation mentioned is followed by evaporation and reconstitution with suitable solvent.

** only the dynamic range in plasma is reported in this table for the readability.

*** homogenate mouse tissue tested are brain, kidney, liver, lung, small intestine, and spleen.

guidelines being the most commonly utilized.

3. Biological matrices

3.1. Species used

The listed assays were conducted in various biological matrices from several species including human (n = 44, 64.7%), rat (n = 18, 26.5%), mouse (n = 5, 7.4%), and dog (n = 1, 1.4%).

3.2. Plasma and serum

The knowledge of pharmacokinetics is essential in the development and characterization of new drugs and drug candidates, including TCKIs. Accurate and reproducible bioanalytical assays to measure the concentration of a drug, and their metabolites in biological matrices are required to establish their pharmacokinetic properties. This is frequently to be started from small rodents like mice and rats for preclinical studies and subsequently to human plasma for clinical applications. Most bioanalytical are performed in blood-derived samples (i.e., serum and plasma) because they provide basic data needed for research and monitoring patient compliance as well as compound effectiveness [29]. In the clinical setting, plasma is the most utilized biological specimen for drug quantification for TCKIs. It is shown in Table 2 that more than half of the listed assays use human plasma as the biological matrix.

To obtain plasma used for bioanalysis, anticoagulants are essential during the blood collection. Typical anticoagulants used in the bioanalytical field are EDTA, heparin, and citrate [30]. Specifically, in the bioanalysis of TCKIs, the type of anticoagulants used in blood collection may have a particular effect on TCKI stability. This will be discussed further in section 3.7 (Stability of TCKIs in biological matrices).

3.3. Cerebrospinal fluid

Brain metastasis is often found during the diagnosis of non-small cell lung cancer (NSCLC) in patients. It has been reported that brain metastasis is found in 40% of patients during their disease [31]. It has been confirmed that EGFR inhibitors, afatinib [32], and osimertinib [33] have a clinical effect for NSCLC-derived brain metastases in patients with EGFR mutation. Since the effect and duration of drug treatment depend on the concentration at sites of action, the investigation of drug levels in cerebrospinal fluid (CSF) is recommended. Because of this reason, Wang et al. [34] developed and validated a bioanalytical method for abivertinib (avitinib), a new EGFR inhibitor, in human cerebrospinal fluid (CSF).

Despite the importance of the TCKI concentrations in CSF, the number of published TCKI bioanalytical assays in CSF is far lower than plasma samples. To date, only three papers reported validated TCKIs bioanalytical methods in CSF [34-36]. The lower number of publications utilizing CSF may be caused by the difficulty of obtaining a sufficient quantity of CSF matrices for repeated measurement in small rodents and the cumbersome lumbar puncture procedures of taking CSF samples in patients compared to typical blood withdrawal proces to obtain plasma/serum matrices. Moreover, CSF has a lower protein level that can bind to a drug in comparison to plasma [35]. Therefore, it is expected that total drug concentrations in CSF are much lower than in plasma. Nevertheless, Irie et al. reported a method utilizing liquid chromatography tandem mass spectrometry capable of detecting down to 0.8 nM osimertinib in CSF as depicted by Fig. 2 [35].

Although the average protein concentration of CSF is about 1/200 of plasma, the salt level of CSF is almost at the same level as in plasma, imposing a particular risk of ion suppression in mass spectrometric detection [36,37]. Thus, an investigation of the matrix effect in MS detection should be carefully established in a bioanalytical assay involving both CSF and plasma.

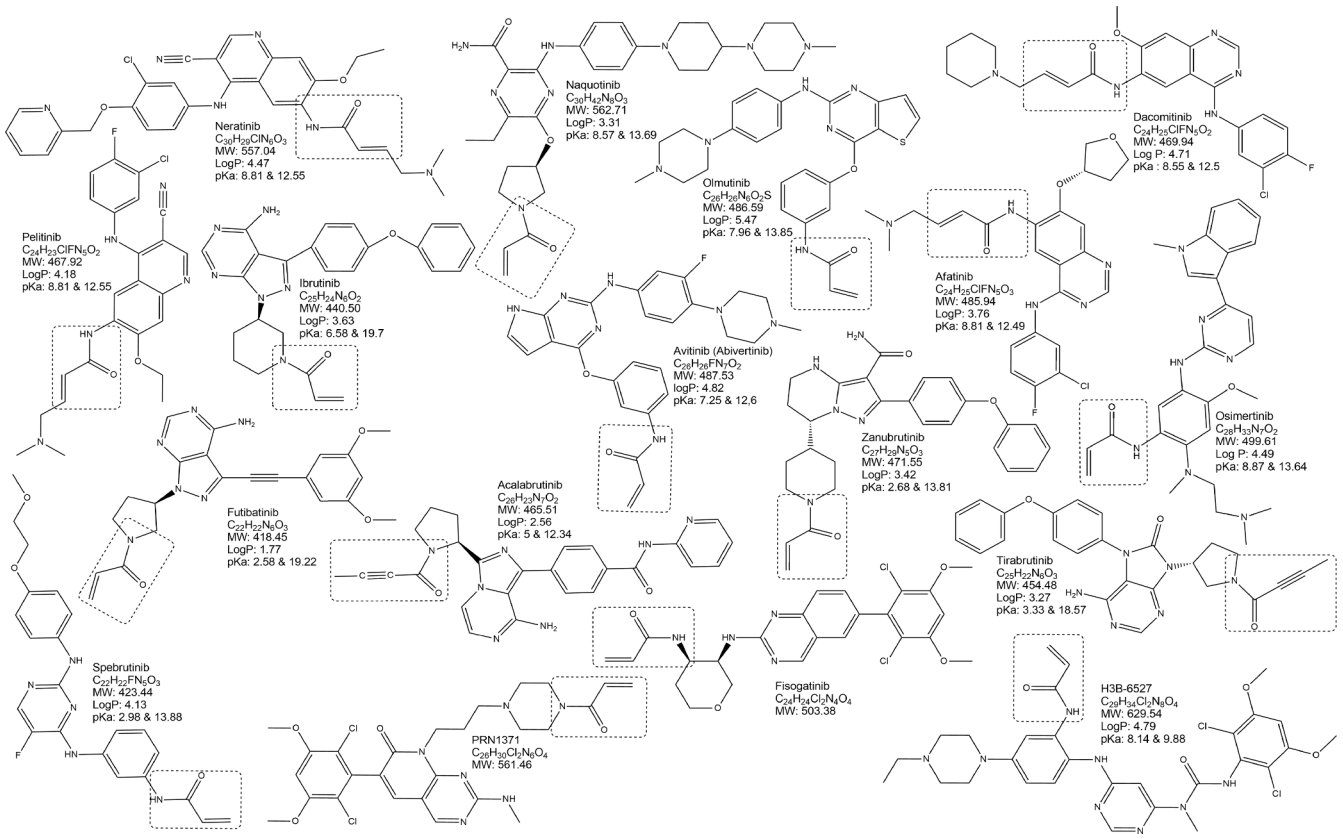

Fig. 1. Molecular structures of targeted covalent kinase inhibitors (TCKIs). The electrophile warhead is highlighted in a box. Log P and pK_a data are prediction data generated from Chemaxon® obtained from DrugBank (www.drugbank.ca [130], Canada) database.

3.4. Urine

The evaluation of drug and its metabolite quantities in urine play an integral role in understanding the excretory route of a drug. However, analyzing drug concentrations in urine bring its own challenge, which is called non-specific binding. Unlike plasma, normal urine contains no protein and fat that can bind the drug or a solubilized lipophilic compound. Thus, drug determination in urine may be underestimated due to the possibility of drug adsorption to the container wall. This problem may be counterpart with the employment of anti-adsorptive agents such as Tween 20, which was employed in the analysis of osimertinib and its metabolites [38].

Compared to plasma, TCKIs are usually present in higher concentration urine. Nevertheless, urine is characterized by high variability among humans depending on sex, age, hormonal status, diet, or physical activities [39]. Therefore, a standardized experimental protocol must be established beforehand. These reasons may cause the less popular application of bioanalysis of TCKIs in urine compared to plasma (Table 2).

3.5. Tissues

Bioanalysis of drugs in tissue is often conducted to elucidate the role of specific transporters or metabolism pathways in preclinical studies and to investigate the drug site target exposure in clinical applications. In a bioanalytical assay, tissue samples must be converted into a liquid form that is referred to as tissue homogenization. Compared to plasma, tissue homogenate contains complex cellular components leading to variable matrix effects [40]. Therefore, solid-phase extraction (SPE) and liquid–liquid extraction (LLE) often give better results than protein precipitation (PP) for these matrices. Tissue homogenates to be used in bioanalysis of TCKIs are only reported for fisogatinib so far: brain, kidney, liver, lung, small intestine, and spleen [41].

TCKI bioanalysis utilizing liver microsomes is also reported for new TCKIs, as shown in Table 2. Liver microsomes and hepatocytes contain various drug-metabolizing enzymes, especially the cytochrome P450 enzymes, making it an indispensable tool to support drug metabolism study, especially for orally administered drugs that undergo first-pass metabolism [42]. Unlike other TKIs that show rapid excretion, the quantitative metabolic stability determination of covalent EFGR inhibitor dacomitinib and covalent BTK inhibitor spebrutinib in liver microsomes showed that both compounds have a slower metabolic rate, possibly resulting in high bioaccumulation after multiple doses [43,44]. This is emphasizing the need of therapeutic drug monitoring for both compounds. On the other hand, Attwa et al. [45,46] qualitatively investigated the reactive metabolites and biotransformation pathways of other TCKI candidates olmutinib and avitinib utilizing rat liver microsomes. These findings underline the importance of the employment of liver microsomes in the bioanalysis of TCKI candidates to support metabolic investigations in the future.

3.6. Other biological matrices

Several known other biological matrices can also be used for bioanalytical assays of TCKIs; however, they are not fully utilized in the included articles, e.g., whole blood and dried blood spot (DBS) [47]. Whole blood within the scope of TCKIs bioanalysis was not used to validate the bioanalytical assays but to investigate the stability of osimertinib and ibrutinibin this matrix [38,48-50]. Concerning whole blood samples, one must consider the plasma-to-blood ratio since this may give different drug levels if compared to plasma concentrations. While DBS is gaining more recognition due to its simplicity in terms of sample collection, this matrix can give high analytical variation because of the interindividual variability of the hematocrit level [51].

Table 3
Stability data of TCKIs in biological matrices.

Compound	Matrix	Additives	Room temperature [h]	Refrigerator [h]	Freezer [day]	Ultra-low [month]	Sources
Acalabrutinib	**Rat Plasma**	K_2EDTA	≥6			≥0.5	[88]
Afatinib	**Human Plasma**	Na. citrate	≥24	≥48	≥60		[57]
		Li-hep	≥24	≥24		≥9	[49]
		Li-Hep	≥8		≥60		[93]
		K_2EDTA	≥48		≥30		[132]
		N.R.	≥8	≥24	≥30		[68]
		N.R.	≥8	≥24	≥30		[69]
		N.R.			≥30		[70]
	Rat Plasma	Heparin		≥96		≥1	[65]
	Mouse Plasma	Li-hep	≥24		≥60		[94]
Avitinib	**Human CSF**	N.R.	≥6			≥1	[34]
Dacomatinib	**Rat plasma**	N.R.	≥3		≥35		[133]
	Rat Liver Microsomes	Phosphate buffer	≥8		≥30		[43]
Fisogatinib	**Mouse plasma**	Li-hep	≥24		≥105		[41]
	Mouse brain	N.R.	≥6				
	Mouse kidney	N.R.	≥6				
	Mouse liver	N.R.	≥6				
	Mouse lung	N.R.	≥6				
	Mouse small intestine	N.R.	≥6				
	Mouse spleen	N.R.	≥6				
Ibrutinib	**Human plasma**	K_2EDTA	≥5		≥90		[79]
		Li-hep		≥2		≥2	[87]
		Li-hep	3 < 12		≥90		[47]
		Heparin	4 < 24	≥48			[122]
		Heparin	≥6	≥16	≥451	≥15	[50]
		N.R.	24 < 48	≥72		≥2	[123]
		N.R.	≥6			≥1	[71]
		N.R.	≥6		≥28		[108]
		K_2EDTA	N.R.	N.R.	≥90	N.R.	[111]
	Human blood	Heparin	≥2	≥2			[50]
	Rat plasma	N.R.	≥12			≥1	[73]
		N.R.	≥6		≥60		[135]
		N.R.	≥24			≥1	[136]
		N.R.	≥6			≥1	[72]
Neratinib	**Human plasma**	EDTA	≥4			≥12	[138]
	Rat plasma	N.R.	≥6		≥30		[139]
Osimertinib	**Human Plasma**	Na. Citrate	≥6	≥24	≥60		[57]
		Li-hep	1 < 2 (LQC) 5 < 24 (HQCl)	≥24		≥9	[49]
		Li-hep	≥4		≥75		[77]
		Heparin	2 < 4 (LQC) 0 < 2 (HQC)				[48]
		Na-EDTA		≥4	≥90		[114]
		K_2EDTA	≥6		≥37	≥5	[38]
		K_2EDTA	≥4	≥6.5	≥35		[95]
		EDTA	4 < 8 (LQC) 8 < 24 (HQC)				[48]
		N.R.	≥6			≥1	[71]
		N.R.				≥3	[35]
	Human CSF	BSA				≥3	[35]
		BSA	≥24		≥183	≥30	[38]
	Human Urine	Tween 20	≥24		≥417	≥13	[38]
	Human serum	Free of additives	< 24	<24		≥3	[48]
		Acidified	2 < 4 (LQC) ≥4 (HQC)				
		Alkalized	0 < 2 (LQC) 0 < 2 (HQC)				
	Rat plasma	N.R.	≥24			≥1	[107]
		N.R.	≥6		≥10		[66]
	Human Whole blood	N.R.		≥2			[38]
		N.R.	5 < 24	≥24			[49]
		N.R.	4 < 8 (LQC) ≥24 (HQC)				[48]
Olmutinib	**Human plasma**	N.R.	≥8		≥30		[84]
	Rat plasma	N.R.	≥4		≥15		[85]
Pelitinib	**Rat plasma**	N.R.	≥6		≥30		[139]
	Human plasma	Na_2EDTA	≥8		≥60		[141]
Spebrutinib	**Human liver microsomes**	N.R.	≥8		≥30		[44]

N.R. = Not reported, Room Temperature (20–25 °C), Refrigerator (0–8 °C), Freezer (-30 up to −20 °C), Ultra low (-80 up to −60 °C), LQC = Low QC, HQC = High QC.

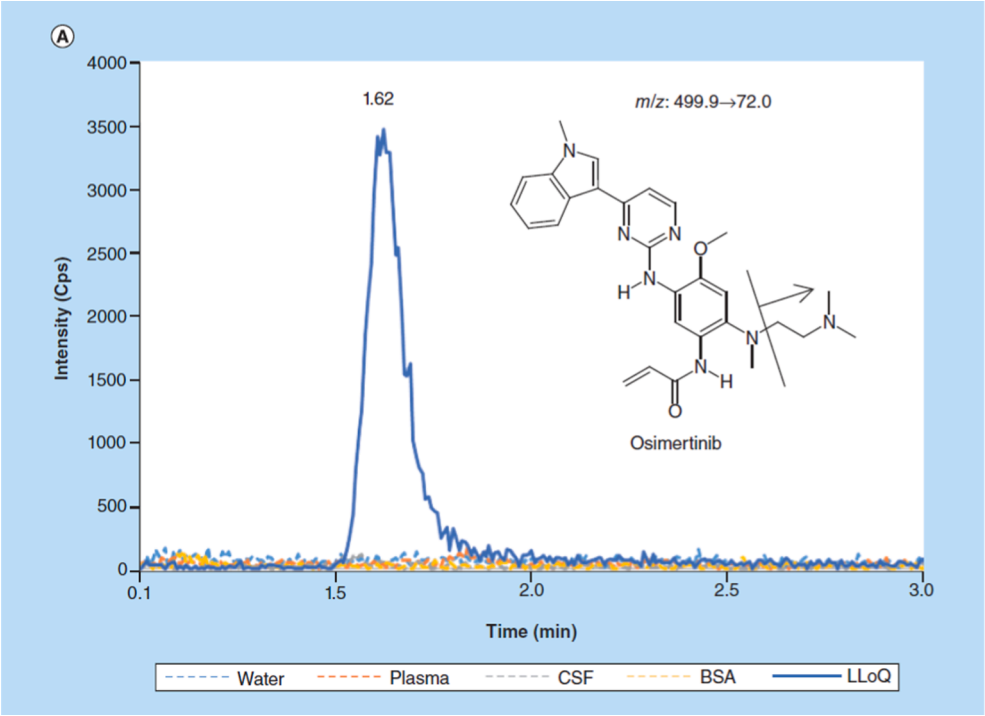

Fig. 2. Chromatogram of osimertinib in human cerebrospinal fluid and several blanks. The concentration of osimertinib was 0.8 nM (LLoQ). Republished with permission of Future Science Ltd. from [35], copyright (2020); permission conveyed through Copyright Clearance Center, Inc.

Other matrices such as feces, intestinal content, and bile are often used to investigate the toxicokinetics of a drug [52]. Feces samples generally are not homogeneous; thus, homogenization of the whole sample is required before analyzing a compound in this matrix. Bile tends to have a higher concentration of drugs and metabolites than other biofluids, which can be a good option when a lower response is observed in plasma samples [53]. Liu et al. [54] employed bile, hepatocytes, and urine of rats to investigate the metabolism of covalent EFGR inhibitor neratinib. To date, there is no paper reporting TCKI bioanalysis utilizing feces or intestinal content yet.

3.7. Stability of TCKIs in biological matrices

Drug and metabolite stability must be assessed during the validation process of quantitative bioanalytical procedures. Results for TCKIs in this review in the biological matrices studied are shown in Table 3, in which apart from osimertinib, all listed TCKIs were at least stable for 6 h at room temperature. Osimertinib, similar to other TCKIs, has an electrophilic warhead capable of reacting with nucleophilic residues in the targeted enzymes [55]. The acrylamide moiety (Fig. 1) of osimertinib is able to react and covalently bond with a cysteine residue in tyrosine kinase receptors leading to its enhanced potency and prolonged duration of action compared to reversible kinase inhibitors [56]. It is, however, hypothesized that the same electrophilic warhead also could react with other naturally present nucleophiles in plasma, such as thiol or amine functions, mainly via glutathione (GSH) conjugation pathway, contributed to its low stability [57,58]. In contradiction, afatinib has better stability despite having similar acrylamide moiety. This may be caused by sterical interference provided by the dimethylamino group on the double bond of the acrylamide moiety (Fig. 1). A mass balance study showed the acrylamide moiety of labeled-osimertinib also covalently bound to plasma proteins, mostly albumin, and resulted in a low plasma concentration of osimertinib and its two major metabolites [11]. These findings underline that the covalent binding of the electrophile warhead of TCKIs can also determine the pharmacokinetic profile of TCKIs in addition to its role in their stability.

Besides the acrylamide moiety that belongs to most of the listed TCKIs in this paper, 2-butynamide is another warhead moiety possessed by acalubrutinib and tirabrutinib only among this group of drugs (Fig. 1). It has been reported that employing 2-butynamide instead of acrylamide improved the selectivity of acalabrutinib and decreased its off-target site binding [59]. Still, similar to acrylamide, 2-butynamide can react with the natural-present nucleophiles in a biological system that may play a role in the stability of TCKIs. However, unlike acrylamide, the metabolic pathway of 2-butynamide has not been previously delineated [60].

In general, all listed TCKIs have good stability at lower temperatures, but stability can highly vary at room temperature (Table 3). It is believed that TCKI stability at room temperature can be influenced by the biological matrices and the additives used. Several papers investigated the stability of osimertinib and ibrutinib -most reported TCKIs showing instability at room temperature- in a variety of human biological matrices such as plasma, serum, whole blood, CSF, and urine [38,48-50]. Van Veelen et al. [48] extensively investigated the stability of osimertinib in several matrices with different additives. He reported that osimertinib has short-term stability at room temperature. Still, the degradation rate of osimertinib is lower in EDTA-plasma and in an acidic environment compared to a neutral one. As for ibrutinib, it is reported that heparinized plasma gives better stability at room temperature than heparinized blood [50]. Following this finding, exposure of ibrutinib and osimertinib to room temperature should be minimized. Thus, special measures during sample collection, transportation, and pretreatment should be established when dealing with these compounds. Since all TCKIs share a similar structure of electrophile warhead, imposing them to the risk of reactivity and instability, a careful investigation on their stability should be done during the validation step of the bioanalysis method.

4. Sample pretreatment

The most often used sample preparation techniques in bioanalysis are protein precipitation (PP), liquid–liquid extraction (LLE), and solid-

phase extraction (SPE). Sample pretreatment for UV or Diode array detection (DAD) has to eliminate any spectrometrically interfering substances. While for a more selective detector such as a mass spectrometer (MS), these interferences may play a smaller role. However, potential isobaric interferences still can occur in MS detection-based analysis, as observed in the ibrutinib bioanalysis [50], which will be discussed further on in Section 9 (Future Perspective). Still, in MS detection, sample preparation plays a crucial role since the most frequently used ionization mode is electrospray ionization (ESI), which is prone to ion suppression. Thus, sample preparation in MS detection aims to remove any contaminants that have the potency to affect the ionization efficiency. Often occurring ion suppressants are endogenous compounds typically found in biological matrices such as proteins, (phospho)lipids, and salts.

4.1. Protein precipitation

Protein precipitation (PP) can be included in a dilution step to alleviate matrix effects from protein content, adsorption to the analytical device, and carry-over [61]. Typical PP is performed by diluting a sample with a highly acidic solution or an organic solvent [62,63]. PP with organic solvents is based on the replacement of water by organic solvents on the surface of the protein, which reduces the hydration layer of protein [64]. This results in protein aggregation by attractive electrostatic and dipole forces, subsequently decreasing the solubility of the protein. The use of PP with acetonitrile, the most efficient organic solvent for PP [74], is reported in more than half of the bioanalytical methods for TCKIs (Table 2). Some papers described the use of methanol or a combination of methanol and acetonitrile as the organic solvent [48,65,66].

PP is considered as a crude technique and still leaves many contaminants in the sample. Thus, this technique is not frequently suitable for less selective detection methods such as UV–Vis. However, PP usually has a higher recovery compared to LLE and SPE. PP is also the most often technique used in bioanalytical assays of TCKIs due it is simplicity and its compatibility with MS detection (Table 2). When PP is performed in bioanalytical assays, especially in a method where ESI is used together with MS as the detection method as frequently employed in TCKIs bioanalysis, the matrix effect should be carefully considered during the validation; moreover, the low level of sample cleanliness in PP could also give a raise in analytical drift with multiple injections of the sample [61], which is also needed to be addressed during the validation process.

4.2. Liquid-liquid extraction

Liquid-liquid extraction (LLE) is a separation technique employing two immiscible phases (solvents), typically aqueous and organic. The extraction of the analytes occurs due to distribution differences between both phases [67]. This method can produce a cleaner extract compared to PP, and this method is relatively simple compared to SPE. However, developing the LLE method can be quite challenging for simultaneous investigation of multiple TCKIs, as these compounds commonly have different pK_a values and do not share similar "non-ionized" moieties. Moreover, most solvents used for LLE are non-polar and raise the need for solvent removal for the compatibility with the most commonly used reversed-phase liquid chromatography (RPLC). Evaporating the solvent and subsequently reconstituting the residue in a more compatible aqueous solvent mixture can tackle this downside. Typical solvents used for reconstitution in RPLC system are acetonitrile, methanol, water, and their combinations or the mobile phase as reported for the bioanalysis of afatinib [68-70], osimertinib [71], and ibrutinib [71-73].

Salting–out assisted liquid–liquid extraction (SALLE) is a special form of LLE in which salt is added besides the original organic solvent. Salt is added in a high concentration as the salting-out agent, which helps to precipitate proteins or other present macromolecules and creating a phase separation between the normally water-miscible organic solvent and the aqueous phase. This method results in better recoveries compared to LLE for relatively polar compounds [74,75]. SALLE has the same merits as LLE but simultaneously eliminates the troublesome step of evaporation and reconstitution in an RPLC-compatible solvent [76]. The use of salts such as sodium chloride and magnesium sulfate are not "MS-friendly" and can interfere with the ionization or even damage the ion optics. Magnesium sulfate has been reported to provide a clearer phase separation in osimertinib extraction, which is practically helpful during the sample extraction step [77]. Although carrying a risk for the MS detector, only the low salt containing organic phase is injected, and the use of a divert valve can prevent salt from entering the MS.

Solid-supported liquid–liquid extraction or supported liquid extraction (SLE), another form of LLE, utilizes the same aqueous phase as LLE, which is coated onto inert diatomaceous earth support. This system has a better and efficient extraction, uses less organic solvent without emulsion formation compared to LLE [78]. One paper reported the use of SLE for ibrunitib quantification, which yielded around 90% recovery and fewer steps than those performed in solid-phase extraction, confirming its extraction efficiency [79].

4.3. Solid-phase extraction

Solid-phase extraction (SPE) separates the analytes based on their distribution between the solid phase and the sample solvent. This versatile extraction method is similar to liquid chromatography but uses larger solid-phase particles. SPE overcomes the matrix effect issues in PP and poor recovery due to incomplete phase separations in LLE. It is reported that the use of SPE in a simultaneous quantification of afatinib and ibrutinib with a diode array detector was capable to quantitatively detect both compounds down to 2 ng/ml [80].

However, SPE employs many steps of repeated addition and elution of fluid that takes a longer time for sample preparation compared to LLE [81]. In addition, these many steps can contribute to its low reproducibility of the response of analyte(s) compared to PP [63]. This can be circumvented by adding an internal standard (IS), preferably a stable isotope-labeled (SIL) IS, which is discussed further in Section 5 (Internal Standard). Typical SPE often used in LC-MS bioanalysis can be classified into reversed-phase (RP) SPE, ion-exchange (IE) SPE, and mixed-mode SPE (combination between RP and IE) [63]. Only RP SPE and mixed-mode SPE have been used for bioanalysis TCKIs to date (Table 2).

4.4. Dilution and filtration

Dilution and filtration are the simplest sample pretreatment methods. These techniques, however, are rarely performed for TCKI bioanalysis without combination with a more selective pretreatment technique. A simultaneous quantification method of afatinib, axitinib, and lapatinib was reported to be treated with a simple 1/5 dilution with the mobile phase by Albiol-Chiva et al. [82], being the only article reported the use of this simple technique in TCKI bioanalysis. Due to the capability of the micellar mobile phase to solubilize macromolecules in plasma, the sample could directly be injected into the chromatographic column after simple dilution. This method also has been reported to have a low impact on personal health and the environment. However, this method cannot be coupled with MS detection due to its high risk of matrix effects. Moreover, the reported dilution method by Albiol-Chiva et al. has relatively low sensitivity with 0.5 mg/L as the lower limit of quantification (LLoQ).

5. Internal standard

The employment of internal standards in chromatographic bioanalysis, especially in liquid chromatography-tandem mass spectrometry (LC-MS/MS), is a common practice. The addition of IS in bioanalysis is intended to compensate for the analytical variation in each step of

bioanalysis procedures, i.e., sample preparation, separation, and detection. Moreover, a good IS will also provide qualitative information such as the confirmation of a shift in the retention time of analyte(s) or declension of peak shape [83].

There are two types of IS used in bioanalysis: an analog compound or stable isotope-labeled (SIL) version of the analyte(s). An analog IS is often used for a new compound where the SIL version is not yet commercially available or for a non-mass spectrometric detection method. A compound can be used as an analog IS if it has a similar size and logD value as the analytes and shares the key functional group that is likely playing a role during the steps of extraction, chromatographic separation, and ionization [83]. Therefore, it is a common practice to use commercially available TKI(s) as the analog IS to quantify newly developed TCKIs. This is observed in several papers included in this review such as the employment of gefitinib and imatinib to quantify afatinib [65,70]; the utilization of pazopanib to determine osimertinib concentration in human plasma [48,77]; the use of lapatinib in dacomitinib quantification [43]; and dasatinib and ponatinib to quantify olmutinib [84,85].

To date, SIL is considered as the best option for IS in LC-MS/MS methods because SIL ISs are capable of compensating more analytical variabilities of the analytes from sample extraction until the detection step including matrix factor due to its higher similarity to the analytes [83]. Despite their excellency compared to analog IS, only several approved TCKIs, namely afatinib, osimertinib, neratinib, and ibrutinib used SIL, as illustrated by Table 2, probably caused by the limited availability of their SIL.

6. Separation

6.1. Liquid chromatography

The most common separation technique used for the bioanalytical assay, including TCKIs, is liquid chromatography, notably RPLC. Unlike normal-phase liquid chromatography (NPLC), RPLC employs a more polar mobile phase than the stationary phase [86].

Typical stationary phases for RPLC methods are C8 (octyl) and C18 (octadecyl) silica-based columns. In RPLC bioanalysis of TCKIs, C18 is more popular than C8, as shown in Table 2. C18 has a higher hydrophobicity property in comparison to C8 due to its longer alkyl chain backbone, making it more favorable for hydrophobic and organic compound retention such as TCKIs. The addition of certain groups such as fluorophenyl or phenyl in the silica surface of C18 can offer improved column selectivity as observed in the analysis of afatinib and ibrutinib, which utilize PFP (pentafluoro phenyl) C18 [68,69] and CSH (charged surface hybrid) C18 [87] respectively. Although less popular, the C8 column was used for the determination of the new BTK inhibitor acalabrutinib in rat plasma [88]. Another commercially available stationary phase for RPLC is the C6 (hexyl) column. De Vries et al. [50] showed that the determination of ibrutinib and its dihydrodiol metabolite employing both C18 and C6 (CSH-phenyl-hexyl) columns give similar results.

In addition to the stationary phase, the mobile phase also plays an essential role in the chromatographic analysis [89]. In the RP mode, combinations of water and organic modifier are used. pH modifier is often used in one or both solvents used as the mobile phase. Typical pH modifiers used in bioanalysis are acetic acid, formic acid, and ammonium acetate with formic acid being the most popular pH modifier [90]. The frequent used of formic acid as the pH modifier is also observed in TCKIs bioanalysis (Table 2). As for the organic modifier, acetonitrile is the most often employed in RPLC bioanalysis of TCKIs, as shown in Table 2. Acetonitrile has a stronger elution strength than methanol in RPLC. Therefore, it is expected that in the same proportion of water, acetonitrile will give a shorter retention time than methanol. In addition, acetonitrile has a lower viscosity than methanol offering a lower pressure in RPLC system when it is used as a mobile phase. This maybe be the reason for the frequent employment of acetonitrile as organic modifier in bioanalysis of TCKIs, especially for those that utilizing a column with particle size $\leq$ 2.0 μm.

6.2. Gradient vs. isocratic elution

The elution type, namely gradient and isocratic, is also to be decided after choosing a suitable column and mobile phase. While isocratic elution is simpler, gradient elution is more capable of removing strong retaining contaminants and facilitating the separation of wide range analytes with different retention factors such as multi-compound analysis. Thus, gradient elution is more often used in multianalytes of TCKIs bioanalysis, including the analysis of their metabolites (Table 2). Nevertheless, around 1/3 of the reported papers utilized isocratic elution for TCKIs bioanalysis, with a similar proportion of single and multianalyte analysis probably due to its simplicity.

6.3. Acidic vs. basic eluent

The low pH (acidic) mobile phase is predominantly used in RPLC bioanalysis, including TCKIs bioanalysis, as illustrated in Table 2. It shows that more than 90% of the listed TCKIs bioanalysis is conducted in an acidic environment. This may have many reasons. Most drug substances, including TCKIs, are basic compounds. Specifically, most of the listed TCKIs under this review have two values of pK_a ranging from 2 – 20, underlining their weak acid and basic properties (Fig. 1). Shifting the eluent into an acidic environment with an acid modifier such as formic acid, as utilized by the majority of the bioanalytical assays for TCKIs in this review (Table 2), is widely believed may facilitate the ionization process of TCKIs. This is subsequently linked to the improvement of detection sensitivity [91,92]. Another reason is the volatility properties of formic acid that makes it compatible with MS detection [91].

Despite the major use of the acidic mobile phase, several papers reported using a basic mobile phase with a C18 column to determine concentrations of afatinib and osimertinib [70,93-95]. Employing high-pH, even though less popular in bioanalysis, has been reported to provide a better chromatographic peak shape and higher signal-to-noise ratio for basic compounds [92]. These merits, contradictory with general knowledge of the use of acidic mobile phase, is capable of increasing the detection sensitivity. Thus, an investigation to choose either acidic or basic eluent should be carefully performed for each TCKIs.

6.4. Micellar chromatography

Albiol-Chiva et al. [82] reported the use of micellar chromatography with a UV-diode array detector to simultaneously quantify axitinib, afatinib, and lapatinib in human plasma. This system uses sodium dodecyl sulfate (SDS) as a surfactant and a hybrid micellar (SDS/1-pentanol) mobile phase. The SDS-monomers and SDS-micelles bind to protein, promoting their denaturation and solubilization while small hydrophobic molecules are also solubilized in this system. Therefore, proteins are eluted near the dead time instead of precipitating inside the column and do not interact with the analyte. Thus, after a simple dilution, the plasma sample can be directly injected into the column, which saving the analysis time. Besides eliminating the need for the sample cleanup and purification steps, which are tedious and often have an incomplete recovery, micellar chromatography also does not require the use of IS. However, this method reported the dynamic range 0.5 – 10 mg/L, which is much higher than the ng/mL range that is obtained by a typical LC-tandem mass spectrometric method.

7. Detection

7.1. LC-MS detection

A mass spectrometer (MS) measures the abundance of a molecule that has been converted to an ion and or subsequently formed fragments.

In an MS spectrum, a *m/z* (mass to charge ratio) is used as the dimensionless independent variable. Mass spectrometry, specifically tandem mass spectrometry (MS/MS), is extensively used in TCKI bioanalytical assays (Table 2). MS has some benefits compared to other techniques, such as its capability to be coupled to both gas-phase (GC–MS) and liquid-phase (LC-MS) separation and its high sensitivity and specificity. Moreover, current MS can provide not only quantitative targeted analysis but also qualitative, simultaneously quantitative/qualitative and untargeted (omics) analysis, which are gaining more interest recently [96].

MS/MS describes mass spectrometry experiments where mass-selected ions are subjected to a second mass spectrometric analysis. Thus, MS/MS requires at least two stages of *m/z* analysis. There are many types of MS/MS commercially available. However, only triple quadrupole (QqQ), quadrupole-orbitrap (Q-Orbitrap), and quadrupole-TOF (Q-TOF) will be discussed in this review since only these MS/MS instruments are used in TCKIs bioanalysis to date.

7.1.1. Ionization method

The ionization process is a crucial part of MS. There are several ionization methods employed for MS, such as electron impact (EI), chemical ionization (CI), atmospheric pressure ionization (APCI), and electrospray ionization (ESI) [97]. To date, APCI and ESI are the most common ionization modes for coupling LC-MS/MS [98].

It was reported that both APCI and ESI are the most frequently used in the bioanalysis of TKI [99]. Hence, only ESI in positive ionization is employed as the ionization source for the bioanalysis of TCKIs. The predominant use of positive ionization in TCKIs bioanalysis is caused by the molecular structures of TCKIs that have aromatic and nitrogen atoms, as depicted by Fig. 1. It has been proven that protonation plays a paramount role in the ion formation of aromatic, nitrogen-containing compounds [100]. Furthermore, ESI provides higher sensitivity compared to APCI, which is critical for drug quantification [101].

The use of ESI as the ionization source in a mass spectrometry-based bioanalytical method comes with a downside since the ESI method is prone to ion suppression from nonvolatile or less volatile compounds such as salts, lipids, and proteins that are normally present in the biomatrices. This risk may be alleviated by switching a divert valve to waste until the elution of the “dead-volume” components to ensure that accumulated non-volatile contaminants such as salts residue will not reach the ionization interface. Switching the divert valve can also be used to avoid late elution of interferences such as lipids that may occur during the flushing period.

7.1.2. Triple quadrupole

Triple quadrupole mass spectrometry (QqQ) is tandem mass spectrometry utilizing three quadrupoles in which two quadrupoles act as mass analyzer (Q) and is separated by another quadrupole acting as collision cell (q)[97]. QqQ is purely driven by radio frequency (RF) and AC-DC components to adjust the *m/z* range, which enables this instrument to change operational parameters at very high rates. However, QqQ generally operates at a resolution unit which is not adequate for accurate mass measurement [102].

QqQ in selected reaction monitoring (SRM) mode is the method of choice for small molecules quantification in biological matrices due to its excellence in selectivity, sensitivity, and broad dynamic range [103]. QqQ is also used in most bioanalytical methods for TCKIs (Table 2). The downside of SRM in QqQ is that this approach needs some optimizations for each compound to be measured. Thus, it requires extensive time to develop bioanalytical assays for multiple analytes, especially those that have similar molecular structures as a metabolite. Moreover, during the optimization process, possible crosstalk, adduct formation, and specific ion transitions should be carefully considered [103].

7.1.3. Q-orbitrap-mass spectrometer

The Q-orbitrap-MS is a tandem mass spectrometry combining a quadrupole and an orbitrap mass analyzer. Orbitrap mass analyzer has a spindle-like central electrode and a barrel-like outer electrode. The *m/z* and abundance of the ions in orbitrap are calculated based on the ionic motions and oscillation of the ion inside the mass analyzer [102].

Contradictory with QqQ, Q-Orbitrap’s main feature is the capability to obtain a high mass accuracy (1–2 ppm) [104]. Therefore Q-Orbitrap is classified as a high-resolution mass spectrometer (HRMS). This technique is generally associated with qualitative research despite its capability to perform both quantitative and qualitative analysis. While orbitrap’s capability to perform a qualitative analysis is well recognized, its ability in quantitative analysis is still debatable. Some papers reported that the quantitative analysis performance of Q-orbitrap and QqQ is comparable for several compounds [103,105]. To date, three papers reported the use of a Q-orbitrap-MS detector in TCKI bioanalysis. One article employed the Q-orbitrap-MS for quantitative analysis of ibrutinib when administered with naringenin [73]. The other papers utilize the Q-orbitrap-MS in combination with DAD detection to investigate the metabolite profiles of ibrutinib [106] and neratinib [54].

7.1.4. Quadrupole-time-of-flight-mass spectrometer

Quadrupole-Time-of-Flight (Q-TOF)-MS is a tandem mass spectrometer combining quadrupole technology and a time-of-flight mass analyzer. Inside the TOF, ions with equal kinetic energy but different masses are moving at different velocities. When those ions are traveling down a field-free drift path of a given length, their times-of-flight depends on their masses. Thus, measuring the times-of-flight of those ions allowing calculation of their masses [102].

Q-TOF is also classified as HRMS. Although it generally has a lower mass accuracy ($\leq$10 ppm) than Q-orbitrap, Q-TOF offers a theoretically unlimited *m/z* range and a higher acquisition rate of the mass spectra [102,104]. Therefore, Q-TOF is also often employed to perform full-scans of product ions of metabolites (qualitative analysis) [104]. Hitherto, only one paper reported the use of Q-TOF-MS to quantify a single analyte, osimertinib, among all articles on bioanalysis of TCKIs [107].

7.2. LC-UV/DAD

Despite the advantages of MS detection, which enables to obtain relatively low limits of detection (LOD), it requires the availability of an IS and optimization of parameters for all analytes such as cone voltage, collision energy, and ion suppression [89]. Moreover, bioanalytical assays utilizing MS detectors require a relatively expensive detector compared to the light spectroscopic detection methods, and such equipment is not always available in every clinical laboratory or hospital. Thus, in such a situation, another detector such as UV–Vis/diode array detector (DAD) may be preferable to MS/MS [80,89,108].

It is known that the sensitivity of DAD is lower than low-resolution MS by at least one or two orders of magnitude [109,110]. To overcome this problem, higher sample volumes (*ca.* 100–500 μL) to have higher analyte amounts can be an option [99]. Croitoru et al. [111] reported that sensitivity down to 1 ng/ml can be achieved by DAD in ibrutinib quantitation with a minimum of 1 ml human plasma sample treated by LLE. Unlike humans, small rodents, i.e., mice and rats, generally only have up to 1 ml and 15 ml of total blood volume, respectively [112]. Thus, the requirement of 1 ml of plasma sample will be a hurdle in preclinical studies using small rodents. This is probably the reason behind the fact that only human plasma is used for TCKIs bioanalysis employing a UV-DAD detector in this review (Table 2). Another way to improve the quantification limit of TCKIs measured by UV–Vis/DAD is to use SPE as sample pretreatment instead of rather crude PP, as observed for afatinib [80,89].

8. Metabolite analysis

The increased biotransformation of a parent compound is associated with lower plasma levels of that compound and subsequently can lead to

a subtherapeutic effect. Since TCKIs naturally poses an electrophile as a warhead moiety that is reactive to neutrophils, TCKIs can be susceptible to rapid biodegradation, as observed in the instability of osimertinib at room temperature (Section 3.6.). Therefore, investigation on metabolites or metabolic pathways of prospective TCKIs is gaining interest, as shown in Table 2.

Several authors have included one or more metabolites in their bioanalytical assays (Table 2). Afatinib, the first registered TCKI, is known to have negligible metabolism. Stopfer et al. [113] reported in a mass balance study that the parent compound of afatinib was found for more than 90% in human plasma and was found around 89% in human urine and feces. Among registered TCKIs, osimertinib and ibrutinib are the most frequent TCKIs investigated for their metabolites. N-desmethyl-osimertinib (AZ5104 and AZ7550) are major metabolites of osimertinib frequently quantitatively analyzed [38,114-116]. Those compounds are major pharmacologically active circulating metabolites in humans and are formed mainly by CYP3A4 via N-demethylation at two different positions of the molecule [11,38]. All bioanalysis methods within this review employ PP as sample pretreatment and a QqQ detector to quantify osimertinib along with its well-characterized metabolites.

Besides N-desmethyl-osimertinib, dihydrodiol-ibrutinib (PCI 45227, DHI) is a metabolite often quantitively measured in ibrutinib bioanalysis [36,47,50,87]. DHI is also a pharmacologically active metabolite produced by CYP3A4. Hence, it reversibly binds to the BTK receptor with a lower potency up to 15 times compared to ibrutinib [117]. Contradictory with its lower potency compared to ibrutinib, DHI is reported to have a higher plasma level than ibrutinib. Thus, DHI monitoring for clinical purposes becomes more prominent [50,117]. Both ibrutinib and osimertinib utilize a combination of PP as sample pretreatment and QqQ as a detection method to quantify their well-characterized metabolites. The excellency of QqQ in quantitative measurement and the compatibility of PP for ESI-MS detection mode probably cause their frequent utilization to fit this purpose.

On the other hand, Liu et al. [54] qualitatively reported twelve metabolites of neratinib, another approved TCKI, in which the primary metabolic site was the α-β-unsaturated ketone of neratinib via GSH conjugation as the major metabolic pathway. In this investigation, Liu et al. utilized DAD-Q-Orbitrap detection [54] since Q-Orbitrap offers better mass accuracy, which may provide better results in the qualitative exploration of the metabolism pathway. Shibata et al. [58] also reported that neratinib underwent extensive glutathione conjugation, highlighting the potential contribution of non-oxidative metabolism to the total body clearance and subsequently to the drug exposure in humans, especially in cases where oxidative metabolism is impaired as in the case of drug-drug interaction [118]. Besides neratinib, glutathione conjugation of ibrutinib and osimertinib have been investigated [11,58]. Quantitative assays for glutathione conjugates of both osimertinib and ibrutinib have been reported by Rood et al. [87,114]. According to our current knowledge, there is no analytical paper investigating the metabolites of the latest approved covalent targeted BTK inhibitor zanubrutinib available yet.

As stated before, metabolite studies of new TCKIs (both qualitative and quantitative) are gaining more interest. This has been observed in new candidates of covalent targeted EGFR inhibitors abivertinib and olmutinib. Currently, seven reactive metabolites of olmutinib have been identified, including one aldehyde, three iminoquinones, and three immino ions with a hydroxylation as the primary phase I metabolic pathway [46]. Whereas, both qualitative and quantitative metabolite investigations have been conducted for abivertinib [34,45,119]. Attwa et al. [45] reported ten reactive metabolites of abivertinib, including four iminoquinones, three aldehydes, and three iminium ions. On the other hand, Zheng et al. [119] quantitatively reported two primary metabolites of abivertinib, namely M7 (double bond reduction) and MII-6 (N-dealkylation followed by acetylation) [120], both had plasma concentrations around 10% of the parent drug. Several metabolites of new TCKIs have been identified and reported. Hence, the extent of their contribution to the efficacy and toxicity of the drugs has not been established yet. Since the presence of the reactive metabolites and pharmacologically active metabolites of TCKIs capable of shifting the balance of efficacy-toxicity of TCKIs and subsequently affect their clinical outcome, more investigations in this direction will be required in the near future. This may most likely lead to the further employment of HRMS in the bioanalysis of TCKIs.

9. Future perspectives

Generally, bioanalysis preferably involves simple and high throughput assays with a minimum number of manual steps.

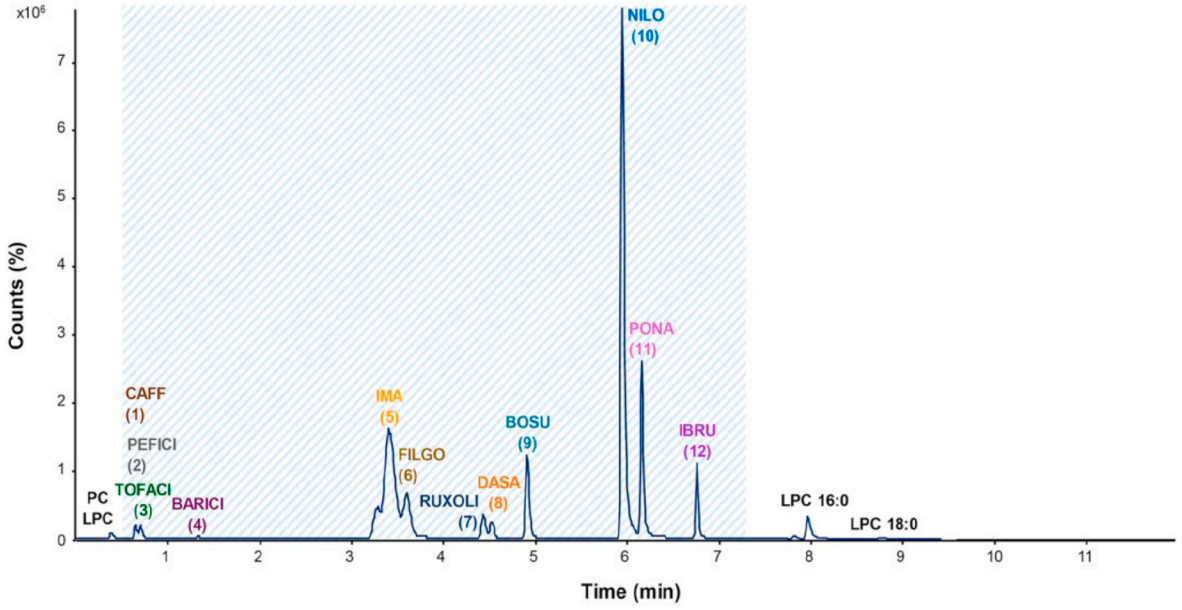

Fig. 3. Chromatogram of eleven TKIs in human plasma. PEFICI : peficitinib; TOFACI : tofacitinib; BARICI : baricitinib; IMA : imatinib; FLIGO : filgotinib; RUXOLI : ruxolitinib; DASA : dasatinib; BOSU : bosutinib; NILO : nilotinib; PONA : ponatinib; IBRU : ibrutinib; CAFF : caffeine; LPC16:10 : lysophosphatidylcholine 16:10; LPC18:10 : lysophosphatidylcholine 18:10. Reprinted from [123], copyright (2020), with permission from Elsevier.

Chromatographic separation still plays a significant role in this field with a distinguishable shortening of analysis time over decades. A recent paper showed a reliable analysis of multiple TKIs with up to 17 compounds in less than four minutes of run time could be achieved with LC-MS/MS [121]. Bioanalysis of multianalytes in oncology is often observed due to the clinical relevance as many cancer patients receive many medications. In such cases, with more than ten analytes, gradient elution is commonly used to obtain a reliable separation in the shortest run time possible [121-123]. An example of such a separation is showed by Fig. 3 where eleven TKIs can be separated within eight minutes [123]. Gradient elution systems provide various solvent strengths depending on the percentage of organic solvent during the analytical run. This facilitates the chromatographic separation of multiple analytes that often have different retention factors [124]. Gradient elution may also offer a better separation for the simultaneous analysis of (unmapped) metabolites for new TCKIs, underlining the opportunity of the increased use of gradient elution in TCKIs bioanalysis in the near future.

We envision that MS detection will remain the preferred detection system in bioanalysis, especially for quantitative analysis. MS detectors are readily available in more research and academic institutes nowadays since the price is more affordable than in previous years. Some researchers fancy the utilization of high-resolution mass spectrometry, e.g., Q-TOF or Q-Orbitrap systems [103,125]. HRMS has ideal properties for quantitative bioanalysis, e.g., high dynamic range and mass accuracy. Besides the equal quantitative performance of HRMS and triple quadrupole MS, HRMS has superior capability in the presence of potential isobaric interferences compared to triple quadrupole MS [125]. It has been reported that isobaric interferences are observed in the bioanalysis of ibrutinib and its metabolites in severe hepatically impaired patients. Taurocholic acid is detected in the same SRM channel as the internal standard of the ibrutinib active metabolite (d_5-dihydrodiol ibrutinib) with an m/z 480 → 309 in tandem mass spectrometry. This interference can be easily alleviated with HRMS since taurocholic acid will give an m/z 480.2778 → 309.2557, while d_5-dihydriol ibrutinib shows an m/z 480.2402 → 309.1507 [50]. HRMS also offers flexibility when an unknown compound is encountered as these instruments have better analytical capabilities for qualitative and simultaneously qualitative/quantitative analysis [96]. Thus, Q-TOF and Q-Orbitrap are more favorable to use in a qualitative study on the unknown metabolite(s) and biotransformation pathways of the new TCKIs, which also have gained more attention lately. Still, further investigation on the comparison of HRMS to golden standard QqQ should be established prior to conducting a quantitative analysis of TCKIs and their metabolites by HRMS.

Bioanalysis of TCKIs is essential in drug and cancer research. The assays that were developed over the last periods provide a better insight into PK/PD profile [126,127], drug metabolism [11,54,58], drug-drug interactions [128,129], and drug-food interaction [73]. Together with the investigation on the therapeutic target level and clinical exposure-effect relationship, those assays can improve clinical efficacy and reduce the toxicity of TCKIs via therapeutic drug monitoring (TDM).

10. Conclusions

Bioanalysis of TCKIs is an essential tool to provide a better insight into overall balance efficacy – toxicity of TCKIs, and subsequently can be used to improve the clinical outcomes of TCKIs. In respect of bioanalytical assays of TCKIs, chromatographic separation still is the foremost choice of separation mode with gradient elution used more frequently due to the trend of multianalytes and metabolite analysis. As for the detection mode, triple quadrupole mass spectrometry is still being the most used one for the quantification of TCKIs in biomatrices. Following the growing interest in the clinical implication of the metabolite(s) of TCKIs, HRMS such as Q-orbitrap and Q-TOF may become more popular than current state due to its superior capability in qualitative and untargeted analysis and its reported comparable quantitative performance to the gold standard triple quadrupole mass spectrometry for some compounds. However, further use of HRMS in quantitative analysis in TCKIs should preferably be preceded by an investigation on its comparison to QqQ as the gold standard.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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