Journal of Pharmaceutical and Biomedical Analysis 233 (2023) 115452

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.journals.elsevier.com/journal-of-pharmaceutical-and-biomedical-analysis

Quantitation of total antibody (tAb) from antibody drug conjugate (ADC) PYX-201 in rat and monkey plasma using an enzyme-linked immunosorbent assay (ELISA) and its application in preclinical studies

Feng Yin [a], Chris DeCiantis [b], Jan Pinkas [a,*], Biplab Das [a], Frank Wang [a], Nancy Zheng [c], David Hahn [c], Aniruddha Amrite [d], Jianwen Feng [a], Diana Adhikari [a], Cheikh Kane [b], Jack Sikora [e], Justin Pittman [b], Rebecca Wates [b], Elizabeth Shaheen [e], Shawn Harriman [a,*]

[a] Department of Nonclinical Research, Pyxis Oncology, Inc., 321 Harrison Avenue, Suite 1, Boston, MA 02118, USA
[b] Department of Biopharma Services, KCAS Bioanalytical and Biomarker Services, 10830 South Clay Blair Boulevard, Olathe, KS 66061, USA
[c] Department of Clinical Pharmacology, Amador Bioscience, Inc., 4695 Chabot Drive, Pleasanton, CA 94588, USA
[d] Department of Clinical Pharmacology, Pyxis Oncology, Inc., 321 Harrison Avenue, Suite 1, Boston, MA 02118, USA
[e] Department of Project Management, Pyxis Oncology, Inc., 321 Harrison Avenue, Suite 1, Boston, MA 02118, USA

ARTICLE INFO

Keywords:
PYX-201
ADC
Total antibody analysis
Preclinical
ELISA
Validation

ABSTRACT

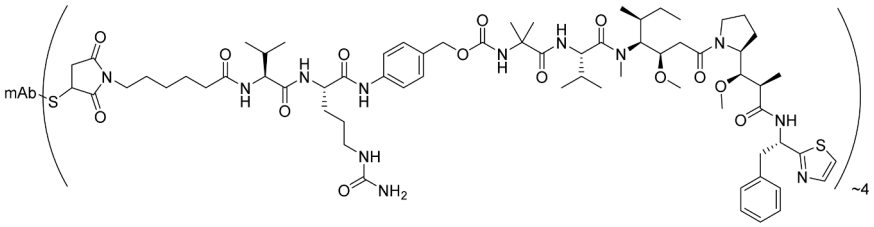
PYX-201 is an investigative ADC oncology drug composed of a monoclonal human immunoglobulin G (IgG) antibody targeting the extra domain B splice variant of fibronectin (EDB + FN) conjugated to an auristatin payload through a cleavable linker. Effective measurement of PYX-201 tAb is the key to ADC drug PYX-201 preclinical pharmacokinetics (PK) assessment. PYX-201 monoclonal antibody (mAb) was used as the reference standard, goat anti-human IgG polyclonal antibody (pAb) or rabbit anti-human Kappa light chain mAb was employed as the capture antibody, and mouse mAb or goat pAb anti-human IgG the crystallizable fragment (Fc) (horseradish peroxidase (HRP)) was utilized as the detection antibody in this ELISA. This assay was validated with a dynamic range 250 – 10,000 ng/mL and 250 – 6000 ng/mL in rat and monkey K_2EDTA plasma, respectively. PYX-201 tAb bioanalytical ELISA assay was reported for the first time in any biological matrix. This is the first time for a bioanalytical method to be validated for a tAb from an ADC drug targeting EDB + FN in any biological matrix.

1. Introduction

Antibody drug conjugate (ADC) drugs are a multicomponent drug product comprised of an mAb linked to a cytotoxic drug (payload or warhead) via a chemical linker [1]. Unlike chemotherapy, ADCs target and kill specific tumor cells while reducing the off-target effects in healthy cells due to their capability of directly conveying the cytotoxic drug into a targeted tumor space [2–4]. ADC drugs bind to the tumor cell surface antigen targeted by the antibody, then are internalized by the tumor cells. The linker is cleaved by the endo-lysosomal system inside the tumor cells and the toxic payload is released into the cytoplasm to induce tumor cell apoptosis [2,3]. Since the first U.S. Food and Drug Administration (FDA) approved ADC drug Mylotarg™ for the treatment of CD33-positive acute myeloid leukemia (AML) in 2000 [5,6], 13 ADC products have been approved by U.S. FDA [7–9]. To date, all ADC drugs are approved in the oncology space with 6 drugs in solid tumors and the rest for hematological malignancies [7–9]. The antibody-payload conjugation is balanced by the unique linker modification so that the overall efficacy in the intended indication could be optimized [3, 10–12]. PYX-201 is an investigational anti-extra domain B splice variant of fibronectin (anti-EDB + FN) ADC with a fully human immunoglobulin G1 (IgG1) antibody engineered with 4 cytotoxic Auristatin 0101 (Aur0101) payloads via a cleavable linker mcValCitPABC (Fig. 1). PYX-201 is currently being investigated in a phase I clinical trial in patients with advanced solid tumors (phase I study: NCT05720117, www.clinicaltrials.gov, EudraCT Number: 2022-002284-30). The mAb portion of PYX-201 targets the EDB + FN that is abundant in almost all human solid tumor microenvironment including breast cancer, non-small cell lung cancer (NSCLC), and pancreatic ductal adenocarcinoma (PDAC), etc. while expressed significantly lower in most healthy

* Corresponding authors.
E-mail addresses: jpinkas@pyxisoncology.com (J. Pinkas), sharriman@pyxisoncology.com (S. Harriman).

https://doi.org/10.1016/j.jpba.2023.115452
Received 30 March 2023; Received in revised form 2 May 2023; Accepted 7 May 2023
Available online 8 May 2023



PYX-201

Fig. 1. PYX-201 drug substance structure. mAb = monoclonal antibody.

adult tissues [13–16]. The PYX-201 payload, Aur0101, is an auristatin derivative (Fig. 1) and can be cleaved from the ADC by proteolytic enzymes, resulting in cell cycle arrest and cell toxicity [17,18].

Oncology drugs, as well as their metabolites, catabolites, and biomarkers have been extensively researched in small molecules, monoclonal antibodies (mAbs), and ADCs recently. Quite a few oncology drugs related bioanalytical methods have been developed and published using different analytical techniques, including LC-MS/MS and ELISA, etc. [19–29]. Recently, we published a bioanalytical assay development and validation for an ADC drug PYX-201 in rat and monkey K_2EDTA plasma [30]. In this manuscript, we report a bioanalytical assay validated under regulatory guidance [31] to quantitate PYX-201 total antibody (tAb) in rat and monkey K_2EDTA plasma. This is the first time for a bioanalytical method to be validated for a tAb from an ADC drug targeting EDB + FN in any biological matrix. This fully validated assay was also successfully applied to preclinical rat and monkey plasma sample analysis to support investigational new drug (IND) filing (IND 161622). Primary toxicokinetic (TK) parameters were provided and mean plasma concentration-time profiles of PYX-201 tAb by dose group were also plotted based on the bioanalytical data analyzed with this assay.

2. Materials and methods

2.1. Chemicals & reagents

PYX-201 mAb at 31.0 mg/mL was produced by WuXi Biologics (Shanghai, China). Goat anti-human IgG pAb and goat polyclonal anti-human IgG Fc (HRP) were ordered from Southern Biotech (Birmingham, AL, USA). Rabbit anti-human Kappa light chain mAb and mouse monoclonal anti-human IgG Fc (HRP) were acquired from Abcam (Cambridge, UK). Rat and monkey K_2EDTA plasma was obtained from BioIVT (Westbury, NY, USA). Acetic acid, 1 x phosphate buffered saline (PBS), 20 x PBS with Tween 20, and blocker Casein in PBS were purchased from ThermoFisher Scientific (Waltham, MA, USA). 3,3′,5,5′-tetramethylbenzidine (TMB) microwell peroxidase substrate and TMB stop solution were received from SeraCare (Milford, MA, USA). Tween 20, PBS tablets, and Trizma base were got from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid was provided by Capitol Scientific (Austin, TX, USA).

2.2. Software for data acquisition and processing

Data acquired from SpectraMax™ i3 or i3x microplate readers (Molecular Devices, San Jose, CA, USA) were processed and analyzed with SoftMax Pro 6.5.1 GxP (Molecular Devices, San Jose, CA, USA), Microsoft Office Excel 365 (Microsoft, Redmond, WA, USA), and Watson LIMS (Version 7.5, Thermo Scientific, Waltham, MA, USA). The standard calibration was regressed using a weighted ($1/y^2$) 5-parameter logistic (Marquardt) curve for rat K_2EDTA plasma method or a weighted ($1/y^2$) 4-parameter logistic (Marquardt) curve for monkey K_2EDTA plasma method. Excel and Watson LIMS were used to calculate the statistical data (Mean, S.D., %RE, %CV, etc.).

2.3. Calibration standards and quality control (QC) samples preparation

31.0 mg/mL of PYX-201 mAb stock solution was received in 20 mM L-glutamic acid and sodium hydroxide buffer with 6 % (w/v) sucrose at pH 5.0 and were kept at −80 °C when not in use. Calibration standards and QC samples were prepared by spiking PYX-201 mAb stock solution into rat and monkey K_2EDTA plasma, respectively. Calibration ranges of 250–10,000 ng/mL in rat K_2EDTA plasma and 250–6000 ng/mL in monkey K_2EDTA plasma were validated in this assay for PYX-201 tAb. 100-μL of the sample was required in this assay after the minimum required dilution (MRD) 1:20 of rat K_2EDTA plasma samples in assay dilution buffer-blocker Casein in PBS or after the MRD 1:100 of monkey K_2EDTA plasma samples with 1:10 dilution in 100 mM acetic acid then 1:10 dilution in the neutralization buffer 150 mM Tris in block Casein in PBS at pH 9. Rat K_2EDTA plasma calibration standards were prepared at 250, 400, 600, 800, 1000, 2500, 4000, 6000, 8000, and 10,000 ng/mL with two anchor points at 100 and 20,000ng/mL. Monkey K_2EDTA plasma calibration standards were prepared at 250, 400, 800, 1000, 2000, 3500, 5000, and 6000ng/mL with two anchor points at 100 and 8000 ng/mL. In addition, a matrix blank was included in all assay runs but is not used in any computation and does not have any acceptance criteria nor subtracted or included in the standard curve calculation when processing data in regression software. Calibration standards were evaluated fresh in at least one accepted run, then frozen for the subsequent use in the validation.

QC samples were prepared by spiking PYX-201 mAb stock solution into rat or monkey K_2EDTA plasma and QC samples were analyzed throughout the validation runs with standards. Five QC levels in rat K_2EDTA plasma were arranged: 250 (LLOQ), 750 (LQC), 2000 (MQC), 7500 (HQC), and 10,000 ng/mL (ULOQ) and five QC levels in monkey K_2EDTA plasma were applied: 250 (LLOQ), 750 (LQC), 1500 (MQC), 4500 (HQC), and 6000 ng/mL (ULOQ). Assay accuracy and precision were evaluated by three QC replicates, at all five levels, in at least six batches. Assay stability was tested by LQC and HQC samples kept at different temperatures (e.g. room temperature, refrigeration temperature, or −80 °C).

2.4. Assay procedure

100 μL coating solution goat anti-human IgG pAb in blocker Casein in PBS at 1.0 μg/mL for rat K_2EDTA plasma method or rabbit anti-human Kappa light chain mAb in PBS at 1.0 μg/mL for monkey K_2EDTA plasma method was added in a 96-well Nunc Maxisorp ELISA plate and incubated overnight at 2–8 °C for rat K_2EDTA plasma method or 70 min at 37 °C for monkey K_2EDTA plasma method. The ELISA plate was washed 3 times with each time 300 μL (3 × 300 μL) of wash buffer 10 mM PBS with 0.05 % Tween 20, blocked with 250 μL of blocker Casein in PBS, then washed again with 3 × 300 μL of wash buffer. 100 μL of study samples, QC samples, or standards was added to the coated ELISA plate after the MRD of 1:20 dilution of rat K_2EDTA plasma samples in blocker Casein in PBS or 1:10 dilution of monkey K_2EDTA plasma samples in 100 mM acetic acid then 1:10 dilution in the neutralization buffer 150 mM Tris in block Casein in PBS at pH 9. The

ELISA plate was incubated for approximately 60 min at room temperature on a plate shaker with shaking and washed again with 3 × 300 μL of wash buffer. 100 L of detection antibody mouse monoclonal anti-human IgG Fc (HRP) diluted 1:5000 in assay dilution buffer (ADB) blocker Casein in PBS in rat K_2EDTA plasma method or goat polyclonal anti-human IgG Fc (HRP) diluted 1:2000 in ADB blocker Casein in PBS in monkey K_2EDTA plasma method was added, then the plate was sealed and incubated at room temperature for about 60 min with shaking. 100 μL of TMB was added into each well after the plate was washed with 3 × 300 μL of wash buffer. The rat K_2EDTA plasma assay plate was incubated at room temperature with shaking until standard 1 reached an optical density (O.D.) of 1.0–1.2 at 650 nm and the monkey K_2EDTA plasma assay plate was incubated at room temperature for approximately 11 min. 100 μL of TMB stop solution was added in each sample, and the plate was read at 450 nm with a reference wavelength of 650 nm on SpectraMax™ i3 or i3x.

3. Results and discussion

3.1. Assay selectivity

Ten individual lots of rat K_2EDTA plasma and ten individual lots of monkey K_2EDTA plasma were evaluated at unspiked, LLOQ, and HQC levels to assess the ability of the assay to quantify PYX-201 tAb in the presence of other constituents in the sample matrix. According to the bioanalytical regulatory guidance [31], the mean response observed in at least 80 % of the sources of unspiked matrix must be BQL, the mean % RE of spiked samples was required to be within ± 25.0 % at LLOQ, and the mean %RE of spiked samples was required to be within ± 20.0 % at HQC. Selectivity of PYX-201 tAb was demonstrated since the data met the acceptance criteria as presented in Supplementary Table S1 and Supplementary Table S2 for rat and monkey K_2EDTA plasma method, respectively.

3.2. Linearity and analytical range

Rat K_2EDTA plasma calibration curve contained ten non-zero standard concentrations from 250 to 10,000 ng/mL with 2 anchor points at 100 and 20,000 ng/mL while monkey K_2EDTA plasma calibration curve contained eight non-zero standard concentrations from 250 to 6000 ng/mL with 2 anchor points at 100 and 8000 ng/mL. Each calibration standard was prepared and processed in duplicate. The standard point would be excluded from the calculation if the %CV between duplicates is > 25.0 % at LLOQ and ULOQ or the %CV between duplicates is > 20.0 % at the other standards. All LLOQ and ULOQ samples would be deactivated if the %RE is > 25.0 % or < −25.0 % of the nominal concentrations. All the other standards would be deactivated if the %RE is > 20.0 % or < −20.0 % of the nominal concentrations. Calibration curves were evaluated using 5-parameter logistic with $1/y^2$ weighting in rat K_2EDTA plasma or using 4-parameter logistic with $1/y^2$ weighting in monkey K_2EDTA plasma. For the calibration curve to be accepted, at least 75 % (minimum 8 points in rat K_2EDTA plasma method or minimum 6 points in monkey K_2EDTA plasma method) of the initial number of standards, including LLOQ and ULOQ, in the quantifiable range were required to remain in the regression. The standard curve data are depicted in Supplementary Table S3 for rat K_2EDTA plasma method and in Supplementary Table S4 for monkey K_2EDTA plasma method.

3.3. Accuracy and precision (A&P)

Accuracy and precision are the two most important indicators to evaluate a bioanalytical assay with accuracy of an assay measuring the closeness of a determined value to its true or nominal value and precision of an assay measuring the closeness of replicate analyses. Usually, accuracy is reported as %RE or %bias and precision is reported as %CV. Three replicates of quality control (QC) samples at 5 levels (LLOQ, LQC,

Table 1
Accuracy and precision for PYX-201 tAb in rat K_2EDTA plasma.

Run Number	LLOQ QC 250 ng/mL	LQC 750 ng/mL	MQC 2000 ng/mL	HQC 7500 ng/mL	ULOQ 10,000 ng/mL
3	238	688	1990	6430	##12800
	233	689	1980	8180	10400
	232	679	1970	7490	10800
Intra-run Mean	234	685	1980	7370	11300
Intra-run S.D.	3.21	5.51	10.0	881	1290
Intra-run %CV	1.4	0.8	0.5	12.0	11.4
Intra-run %RE	-6.4	-8.7	-1.0	-1.7	13.0
7	232	695	2050	7360	8580
	219	702	2020	6820	8610
	226	680	1960	7740	8240
Intra-run Mean	226	692	2010	7310	8480
Intra-run S.D.	6.51	11.2	45.8	462	206
Intra-run %CV	2.9	1.6	2.3	6.3	2.4
Intra-run %RE	-9.6	-7.7	0.5	-2.5	-15.2
8	262	795	2210	7750	10400
	258	775	2170	7440	12100
	253	755	2160	8450	##13500
Intra-run Mean	258	775	2180	7880	12000
Intra-run S.D.	4.51	20.0	26.5	517	1550
Intra-run %CV	1.7	2.6	1.2	6.6	12.9
Intra-run %RE	3.2	3.3	9.0	5.1	20.0
9	262	785	2160	7500	10900
	263	780	2140	7440	9300
	254	780	2190	#9950	9350
Intra-run Mean	260	782	2160	8300	9850
Intra-run S.D.	4.93	2.89	25.2	1430	910
Intra-run %CV	1.9	0.4	1.2	17.2	9.2
Intra-run %RE	4.0	4.3	8.0	10.7	-1.5
Run Number	LLOQ QC 250 ng/mL	LQC 750 ng/mL	MQC 2000 ng/mL	HQC 7500 ng/mL	ULOQ 10000 ng/mL
11	251	*	2070	6810	11500
	247	733	2060	6850	10000
	233	716	2050	#9190	9900
Intra-run Mean	244	725	2060	7620	10500
Intra-run S.D.	9.45	12.0	10.0	1360	896
Intra-run %CV	3.9	1.7	0.5	17.8	8.5
Intra-run %RE	-2.4	-3.3	3.0	1.6	5.0
12	265	835	#2430	8110	8770
	273	828	2220	7930	9210
	257	782	2290	8210	9860
Intra-run Mean	265	815	2310	8080	9280
Intra-run S.D.	8.00	28.8	107	142	548
Intra-run %CV	3.0	3.5	4.6	1.8	5.9
Intra-run %RE	6.0	8.7	15.5	7.7	-7.2
13	283	816	#2410	7690	8580
	270	801	2160	6240	7610
	251	862	2240	6680	8130

(continued on next page)

Table 1 (*continued*)

Run Number	LLOQ QC 250 ng/mL	LQC 750 ng/mL	MQC 2000 ng/mL	HQC 7500 ng/mL	ULOQ 10,000 ng/mL
Intra-run Mean	268	826	2270	6870	8110
Intra-run S.D.	16.1	31.8	128	743	485
Intra-run %CV	6.0	3.8	5.6	10.8	6.0
Intra-run %RE	7.2	10.1	13.5	-8.4	-18.9
Inter-Run Mean	251	759	2140	7630	9930
Inter-Run S.D.	17.0	57.2	134	891	1580
Inter-Run %CV	6.8	7.5	6.3	11.7	15.9
Inter-Run %RE	0.4	1.2	7.0	1.7	-0.7
Inter-Run %Total Error	7.2	8.7	13.3	13.4	16.6
n	21	20	21	21	21

*No value due to %CV of replicates >20 % # >20.0 %RE ## >25.0 %RE.

MQC, HQC, and ULOQ) in 7 accepted batches were utilized to assess the intra-run and inter-run A&P. Based on FDA requirements [31] for intra-run and inter-runs, the %CV of LQC, MQC, and HQC was required to be ≤ 20.0 %, the %CV of LLOQ and ULOQ was required to be ≤ 25.0 %, the %RE of LQC, MQC, and HQC was required to be within ± 20.0 % from the nominal concentrations, and the %RE of LLOQ and ULOQ was required to be within ± 25.0 % from the nominal concentrations. In the inter-run, %total error (|%RE| + %CV) of LQC, MQC, and HQC must be ≤ 30.0 %, and total error of LLOQ and ULOQ must be ≤ 40.0 %. The intra-run and inter-run A&P data and total error data for this assay are depicted in Table 1 for PYX-201 tAb in rat K_2EDTA plasma and Table 2 for PYX-201 tAb in monkey K_2EDTA plasma. In the PYX-201 tAb bioanalytical method in rat K_2EDTA plasma, the intra-run %RE was from −18.9 to 20.0 % with %CV between 0.4 % and 17.8 % and the inter-run %RE ranged from −0.7 to 7.0 % with %CV between 6.3 % and 15.9 % for all QC levels. The inter-run %total error has values between 7.2 % and 16.6 %. In the PYX-201 tAb bioanalytical method in monkey K_2EDTA plasma, the intra-run %RE was from −16.0 to 8.7 % with %CV between 0.8 % and 25.3 % and the inter-run %RE ranged from −9.6 % to −3.1 % with %CV between 8.2 % and 12.0 % for all QC levels. The inter-run % total error has values between 11.3 % and 21.6 %. It was noticed that a little more scattered values were observed in run 1 at the LLOQ level in monkey K_2EDTA plasma method with a %CV value at 25.3 % due to the complexity of the assay. This slightly high %CV value in only one run was deemed as no impact to the integrity of the assay.

3.4. Dilution linearity and prozone

Dilution linearity was proven to enable this assay to analyze pre-clinical rat or monkey K_2EDTA plasma samples at concentrations higher than the ULOQ. An ultra-high QC (UHQC) sample with PYX-201 mAb in rat or monkey K_2EDTA samples at 1,000,000 ng/mL was serially diluted in the same matrix to have 2 samples with dilution factors 20 and 50 above the range of quantitation to evaluate the possible prozone or hook effect and 3 samples with dilution factors 200, 500, and 2000 within the range of quantitation to investigate the dilution linearity. The UHQC sample was frozen at −80 ℃ overnight, diluted in rat or monkey K_2EDTA plasma on the day of assay, then processed according to the assay procedure. Five replicates for each dilution factor were tested in the experiment. To be deemed acceptable, the mean absolute %RE and %CV, for at least 2/3 of samples at each dilution factor of the QC expected within the range of quantitation, were required to be within

Table 2
Accuracy and precision for PYX-201 tAb in monkey K_2EDTA plasma.

Run Number	LLOQ QC 250 ng/mL	LQC 750 ng/mL	MQC 1500 ng/mL	HQC 4500 ng/mL	ULOQ 6000 ng/mL
1	##178	649	1420	3910	5010
	189	732	*	5180	6540
	277	805	1660	4820	5360
Intra-run Mean	215	729	1540	4640	5640
Intra-run S.D.	54.3	78.1	170	655	802
Intra-run %CV	25.3	10.7	11.0	14.1	14.2
Intra-run %RE	-14.0	-2.8	2.7	3.1	-6.0
4	240	679	1440	4550	6300
	260	738	1450	4750	6660
	231	717	1390	4460	6600
Intra-run Mean	244	711	1430	4590	6520
Intra-run S.D.	14.8	29.9	32.1	148	193
Intra-run %CV	6.1	4.2	2.2	3.2	3.0
Intra-run %RE	-2.4	-5.2	-4.7	2.0	8.7
5	214	658	1360	4060	5700
	206	662	1390	4400	5780
	239	738	1450	4460	5680
Intra-run Mean	220	686	1400	4310	5720
Intra-run S.D.	17.2	45.1	45.8	216	52.9
Intra-run %CV	7.8	6.6	3.3	5.0	0.9
Intra-run %RE	-12.0	-8.5	-6.7	-4.2	-4.7
11	198	635	1300	3890	5260
	208	636	1260	4100	5330
	223	644	1250	3890	4810
Intra-run Mean	210	638	1270	3960	5130
Intra-run S.D.	12.6	4.93	26.5	121	282
Intra-run %CV	6.0	0.8	2.1	3.1	5.5
Intra-run %RE	-16.0	-14.9	-15.3	-12.0	-14.5
Run Number	LLOQ QC 250 ng/mL	LQC 750 ng/mL	MQC 1500 ng/mL	HQC 4500 ng/mL	ULOQ 6000 ng/mL
13	204	653	1320	4390	5930
	215	749	1380	4630	5800
	212	652	1240	4220	5520
Intra-run Mean	210	685	1310	4410	5750
Intra-run S.D.	5.69	55.7	70.2	206	210
Intra-run %CV	2.7	8.1	5.4	4.7	3.7
Intra-run %RE	-16.0	-8.7	-12.7	-2.0	-4.2
14	222	660	1330	4420	5630
	267	721	1570	4650	6420
	208	#593	1220	3800	5440
Intra-run Mean	232	658	1370	4290	5830
Intra-run S.D.	30.8	64.0	179	440	520
Intra-run %CV	13.3	9.7	13.1	10.3	8.9
Intra-run %RE	-7.2	-12.3	-8.7	-4.7	-2.8
15	233	*	1280	4060	5380
	271	795	1510	4640	5740
	243	709	1350	4200	5450

(*continued on next page*)

Table 2 (*continued*)

Run Number	LLOQ QC 250 ng/mL	LQC 750 ng/mL	MQC 1500 ng/mL	HQC 4500 ng/mL	ULOQ 6000 ng/mL
Intra-run Mean	249	752	1380	4300	5520
Intra-run S.D.	19.7	60.8	118	303	191
Intra-run %CV	7.9	8.1	8.6	7.0	3.5
Intra-run %RE	-0.4	0.3	-8.0	-4.4	-8.0
Inter-Run Mean	226	691	1380	4360	5730
Inter-Run S.D.	27.1	56.6	114	359	517
Inter-Run %CV	12.0	8.2	8.3	8.2	9.0
Inter-Run %RE	-9.6	-7.9	-8.0	-3.1	-4.5
Inter-Run %Total Error	21.6	16.1	16.3	11.3	13.5
n	21	20	20	21	21

*No value due to %CV of replicates> 20 % # > 20.0 %RE ## > 25.0 %RE.

± 20.0 % and ≤ 20.0 %, respectively. The highest acceptable dilution was defined to be the greatest workable dilution. Dilution linearity data were given in Table S5 and Table S6 for rat and monkey K_2EDTA plasma method, respectively. Dilution linearity, using dilution factors up to 1:2000 was proven to be valid in both rat and monkey K_2EDTA plasma methods. This assay doesn't have prozone effect based on the observation of above the quantitation limit (AQL) values in samples diluted above the ULOQ and interpolated concentrations in samples diluted below the ULOQ.

3.5. Stability assessment

Analyte stability was required [31] in the method validation to demonstrate PYX-201 tAb was intact during the sample collection, storage, and processing. Accordingly, freeze/thaw stability, bench-top stability, refrigeration stability, and long-term stability were assessed in this assay.

To evaluate the stability of PYX-201 tAb during sequential freeze/thaw cycles (frozen separately at −80 °C and thawed unassisted at ambient temperature), low and high concentration QC pools were frozen for at least 24 h before the first thawing. They were re-frozen more times for at least 12 h each time and were thawed prior to analysis along with a set of QCs and calibration standards. To be deemed stable, the mean % RE at each level (low and high QCs with n ≥ 2 reportable results) were to be within ± 20.0 % of nominal values with %CV ≤ 20.0 % between reportable results. PYX-201 tAb was found stable for 3 cycles of freeze (−80 °C) and thaw (room temperature) in rat K_2EDTA plasma and stable over 5 cycles of freeze (−80 °C) and thaw (room temperature) in monkey K_2EDTA plasma.

To evaluate the stability of PYX-201 tAb in rat and monkey K_2EDTA plasma during sample processing at ambient temperature, aliquots of low and high concentration QC samples were prepared and frozen at −80 °C for at least 24 h. Aliquots were then allowed to thaw under ambient or refrigerated conditions for some duration prior to analysis along with a set of QCs and calibration standards. To be deemed stable, the mean %RE at each level (low and high QCs with n ≥ 2 reportable results) were to be within ± 20.0 % of nominal values with %CV ≤ 20.0 % between reportable results. PYX-201 tAb was proven stable at room temperature for at least 7 h in rat K_2EDTA plasma. PYX-201 tAb refrigeration stability was not evaluated in rat K_2EDTA plasma as it was not needed in the sample analysis. PYX-201 tAb was proven stable at room temperature for at least 5.25 h and stable under refrigerated conditions for at least 17 h in monkey K_2EDTA plasma.

To evaluate the stability of PYX-201 tAb in rat and monkey K_2EDTA plasma during sample storage, aliquots of low and high concentration QC pools were prepared and frozen in polypropylene tubes at −80 °C for a period of time longer than the expected duration of preclinical rat and monkey K_2EDTA plasma samples stored in −80 °C freezers and analyzed with a set of freshly prepared QCs and calibration standards. To be deemed stable, the mean %RE at each level (low and high QCs with n ≥ 2 reportable results) were to be within ± 20.0 % of nominal values with %CV ≤ 20.0 % between reportable results. PYX-201 tAb was demonstrated stable at −80 °C for at least 104 days in rat K_2EDTA plasma and stable at −80 °C for at least 73 days in monkey K_2EDTA plasma.

3.6. Assay robustness and ruggedness

Assay robustness and ruggedness is to test the ability of the assay to endure random or deliberate changes, and this was verified by the evaluation of results from a second analyst and/or from a second instrument using the same assay in this bioanalytical assay validation study.

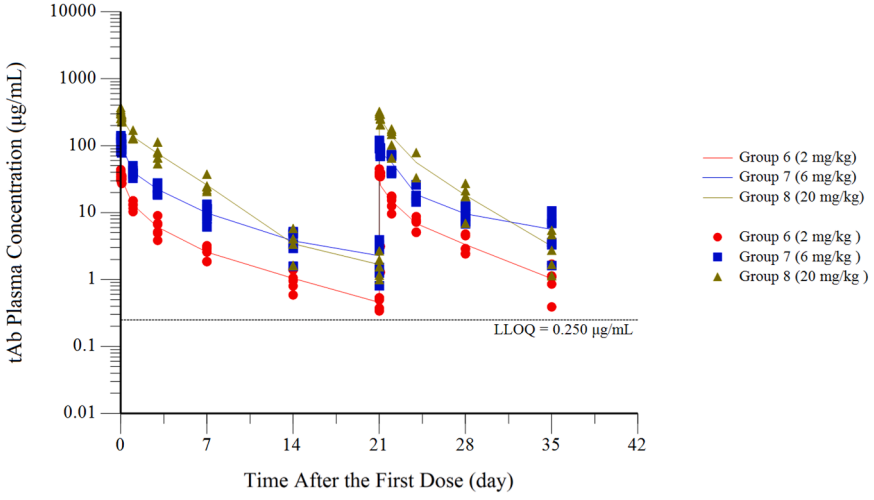


Fig. 2. Mean and individual concentration-time profiles of PYX-201 tAb by dose group following bolus intravenous administration of PYX-201 in rats. tAb = total antibody; LLOQ = lower limit of quantification. Solid lines represent mean values and symbols represent individual values.

Table 3
Summary of primary TK parameters for PYX-201 tAb by dose group in rat plasma.

Group (Dose)	Dose Day	T_{max} (h)	C_{max} (μg/mL)	AUC_{tau} (h*μg/mL)	AUC_{inf} (h*μg/mL)	CL[a] (mL/h/kg)	$t_{1/2}$ (h)
Group 6 (2 mg/kg)	1	0.0830	36.4	1760	1840	1.08	135
	22	0.0830	38.8	1890	NA	1.06	96.9
Group 7 (6 mg/kg)	1	0.0830	113	6120	6550	0.916	133
	22	0.0830	103	6480	NA	0.925	158
Group 8 (20 mg/kg)	1	0.0830	318	16500	16700	1.20	72.8
	22	0.0830	302	13900	NA	1.44	63.9

AUCinf = the area under the concentration versus time curve (AUC) from time 0 extrapolated to infinity; AUCtau = AUC from time 0 to dosing interval tau; CL = total body clearance; Cmax = the maximum observed concentration measured after dosing; NA = not applicable; t1/2 = terminal half-life; Tmax = the time of maximum observed concentration after dosing.
All TK parameters are rounded to 3 significant figures.
[a]CL_{ss} is presented for Day 22.

3.7. Assay application

Two preclinical studies 20360771 (A 4-Week Study of PYX-201 by Intravenous Injection in Sprague Dawley Rats with a 6-Week Recovery Period) and 20360770 (A 4 Week Toxicology Study of PYX-201 by Intravenous Infusion in Cynomolgus Monkeys with a 6 Week Recovery) were successfully supported by this assay. PYX-201 tAb concentrations in rat and monkey K_2EDTA plasma samples were analyzed using this fully validated bioanalytical assay. Fig. 2 illustrated the mean and individual concentration-time profiles of PYX-201 tAb by dose group, and Table 3 summarized primary TK parameters for PYX-201 tAb in rat K_2EDTA plasma by dose group. Fig. 3 illustrated the mean plasma concentration-time profile of PYX-201 tAb after day 1 and day 22 dosing by dose group and sex, and Table 4 summarized statistics of primary TK parameters for PYX-201 tAb in monkey K_2EDTA plasma by dose group and sex.

4. Conclusion

A robust and fast ELISA bioanalytical assay was developed and validated in rat and monkey K_2EDTA plasma for the quantitation of tAb from an investigational ADC drug PYX-201 under 2018 U.S. FDA guidance [31]. Calibration range was validated over 250–10,000 ng/mL in rat K_2EDTA plasma and 250–6000 ng/mL in monkey K_2EDTA plasma. The inter-day %RE was from −0.7% to 7.0 % with %CV ≤ 15.9 % and % total error ≤ 16.6 % for all QC samples in rat K_2EDTA plasma. The inter-day %RE was from −9.6 % to −3.1 % with %CV ≤ 12.0 % and % total error ≤ 21.6 % for all QC samples in monkey K_2EDTA plasma. Dilution linearity for dilution factors up to 1:2000 was verified in this assay. PYX-201 tAb is stable after 3 freeze (−80 °C)/thaw (room temperature) cycles, being stored at room temperature for at least 7 h, and being stored at −80 °C for a minimum of 104 days in rat K_2EDTA plasma. PYX-201 tAb is stable after 5 freeze (−80 °C)/thaw (room temperature) cycles, being stored at room temperature for at least 5.25 h or refrigerated temperature for at least 17 h, and being stored at −80 °C for a minimum of 73 days in monkey K_2EDTA plasma.

This validated assay has been successfully applied in preclinical sample analysis to support the PYX-201 U.S. FDA IND submission and approval (IND 161622). We are currently working on the method development and validation for PYX-201 tAb in human plasma to support the ongoing PYX-201 first-in-human clinical trial.

Funding

This research was funded by Pyxis Oncology, Inc., USA.

CRediT authorship contribution statement

Feng Yin: Methodology, Investigation, Validation, Sample analysis, Writing – original draft. **Chris DeCiantis:** Methodology, Investigation, Validation, Sample analysis, Writing – review & editing. **Jan Pinkas:** Resources, Methodology, Investigation, Validation, Sample analysis, Writing – review & editing. **Biplab Das:** Methodology, Investigation, Sample analysis, Writing – review & editing. **Frank Wang:** Investigation, Sample analysis, Writing – review & editing. **Nancy Zheng:**

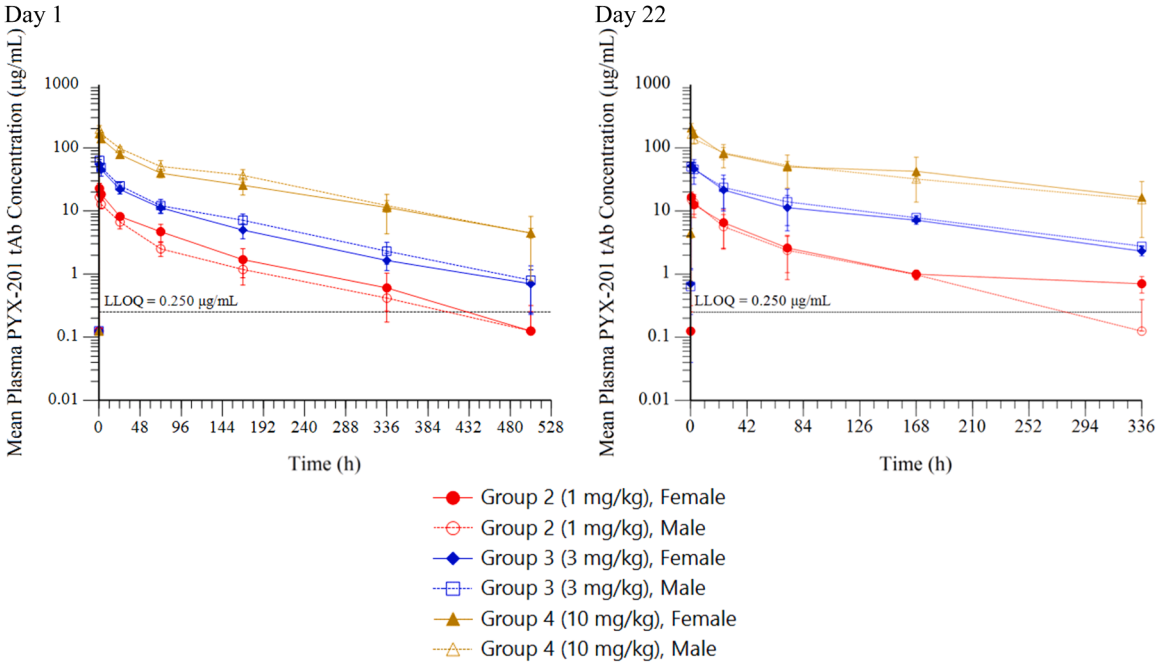


Fig. 3. Mean plasma concentration-time profiles of PYX-201 tAb after day 1 and day 22 dosing by dose group and sex in monkeys. tAb = total antibody; LLOQ = lower limit of quantification. Values below the LLOQ (0.250 μg/mL, as shown by dashed line) are plotted at half of LLOQ for illustrative purposes only.

Table 4
Summary statistics of primary TK parameters for PYX-201 tAb by dose group and sex in monkey plasma.

Group (Dose)	Sex	Dose Day	T_{max} (h)	C_{max} (μg/mL)	AUC_{tau} (h*μg/mL)	AUC_{inf} (h*μg/mL)	CL[a] (mL/h/kg)	$t_{1/2}$ (h)
Group 2	F	1	0.500	22.7	1140	1170	0.913	95.8
(1 mg/kg)	M	1	0.500	16.6	772	796	1.31	101
	F	22	0.900	17.0	838	NA	1.24	63.5
	M	22	1.30	15.6	682	NA	1.48	62.1
Group 3	F	1	0.500	54.6	3020	3150	0.989	118
(3 mg/kg)	M	1	1.00	63.8	3620	3760	0.820	111
	F	22	0.900	51.9	2740	NA	3.33	70.3
	M	22	0.900	52.6	4160	NA	0.725	101
Group 4	F	1	0.500	167	12600	13600	0.781	130
(10 mg/kg)	M	1	0.500	197	15700	16500	0.609	111
	F	22	0.500	206	12100	NA	1.42	81.2
	M	22	0.900	166	14000	NA	0.635	129

AUCinf = AUC from time 0 extrapolated to infinity; AUCtau = AUC from time 0 to dosing interval tau; CL = total body clearance; Cmax = the maximum observed concentration measured after dosing; F = female; M = male; NA = not applicable; t1/2 = terminal half-life; Tmax = the time of maximum observed concentration after dosing.
All TK parameters are presented with mean values and rounded to 3 significant figures.
[a]CL_{ss} is presented for Day 22.

Methodology, Investigation, Sample analysis, Writing – review & editing. **David Hahn:** Methodology, Investigation, Sample analysis, Writing – review & editing. **Aniruddha Amrite:** Sample analysis, Writing – review & editing. **Jianwen Feng:** Validation, Writing – review & editing. **Diana Adhikari:** Validation, Sample analysis, Writing – review & editing. **Cheikh Kane:** Resources, Investigation, Validation, Sample analysis, Writing – review & editing. **Jack Sikora:** Resources, Investigation, Writing – review & editing. **Justin Pittman:** Resources, Investigation, Writing – review & editing. **Rebecca Wates:** Resources, Investigation, Writing – review & editing. **Elizabeth Shaheen:** Resources, Writing – review & editing. **Shawn Harriman:** Resources, Investigation, Validation, Sample analysis, Writing – review & editing.

Declaration of Competing Interest

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

Acknowledgements

The authors greatly thank Francis Kajumo, Alvin Evans, and John Pirro from KCAS Bioanalytical and Biomarker Services in Olathe, KS for their contributions to the assay development as well as Dr. R. Criste from Amador Bioscience for his contribution to the TK analysis in support of this manuscript.

Author statement

Authors state that all data and results in this manuscript are original and real, and have never been published anywhere else.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2023.115452.

References

[1] A. Beck, L. Goetsch, C. Dumontet, N. Corvaia, Strategies and challenges for the next generation of antibody-drug conjugates, Nat. Rev. Drug Discov. 16 (2017) 315–337.
[2] A.H. Staudacher, M.P. Brown, Antibody drug conjugates and bystander killing: is antigen-dependent internalization required? Br. J. Cancer 117 (2017) 1736–1742.
[3] C. Criscitiello, S. Morganti, G. Curigliano, Antibody-drug conjugates in solid tumors: a look into novel targets, J. Hematol. Oncol. 14 (2021) 20.
[4] C.H. Chau, P.S. Steeg, W.D. Figg, Antibody-drug conjugates for cancer, Lancet 394 (2019) 793–804.
[5] N. Joubert, A. Beck, C. Dumontet, C. Denevault-Sabourin, Antibody-drug conjugates: the last decade, Pharmaceuticals 13 (9) (2020) 245.
[6] S. Coats, M. William, B. Kebble, R. Dixit, L. Tseng, N. Yao, D.A. Tice, J. Soria, Antibody-drug conjugates: future directions in clinical and translational strategies to improve the therapeutic index, Clin. Cancer Res. 25 (18) (2019) 5441–5448.
[7] C. Pazo, K. Nawaz, R.M. Webster, The oncology market for antibody-drug conjugates, Nat. Rev. Drug Discov. 20 (8) (2021) 583–584.
[8] A. Pandit. Antibody-drug conjugates in oncology: key considerations and future trends, Premier-research.com/blog-antibody-drug-conjugates, 2022.
[9] Axispharm, Antibody-drug conjugates (ADCs) list approved by FDA (2000-2023), Axispharm.com/antibody-drug-conjugatesadcs-list-approved-by-fda2000-2022 (2023).
[10] R.V. Chari, Targeted cancer therapy: conferring specificity to cytotoxic drugs, Acc. Chem. Res. 41 (1) (2008) 98–107.
[11] E.L. Sievers, P.D. Senter, Antibody-drug conjugates in cancer therapy, Annu. Rev. Med. 64 (2013) 15–29.
[12] S. Panowski, S. Bhakta, H. Raab, P. Polakis, J.R. Junutula, Site-specific antibody-drug conjugates for cancer therapy, mAbs 6 (1) (2014) 34–45.
[13] E. Matsumoto, T. Yoshida, Y. Kawarada, T. Sakakura, Expression of fibronectin isoforms in human breast tissue: production of extra domain A+/extra domain B+ by cancer cells and extra domain A+ by stromal cells, Jpn. J. Cancer Res. 90 (3) (1999) 320–325.
[14] Z.A. Khan, J. Cartero, Y.P. Barbi, B.M. Chan, S. Uniyal, S. Chakrabarti, ED-B fibronectin in non-small cell lung carcinoma, Exp. Lung Res. 31 (7) (2005) 701–711.
[15] S. Sauer, P.A. Erba, M. Petrini, A. Menrad, L. Giovannoni, C. Grana, B. Hirsch, L. Zardi, G. Paganelli, G. Mariani, D. Neri, H. Durkop, H.D. Menssen, Expression of the oncofetal ED-B-containing fibronectin isoform in hematologic tumors enables ED-B-targeted 131I-L19SIP radioimmunotherapy in Hodgkin lymphoma patients, Blood 113 (10) (2009) 2265–2274.
[16] P.L. Qiao, M. Gargesha, Y. Liu, V.E.A. Laney, R.C. Hall, A.M. Vaidya, H. Gilmore, K. Gawelek, B.B. Scott, D. Roy, D.L. Wilson, Z. Lu, Magnetic resonance molecular imaging of extradomain B fibronectin enables detection of pancreatic ductal adenocarcinoma metastasis, Magn. Reason. Imaging 86 (2022) 37–45.
[17] M.T. Birchler, D. Milisavlijevic, M. Pfaltz, D. Neri, B. Odermatt, S. Schmid, S. J. Stoeckli, Expression of the extra domain B of fibronectin, a marker of angiogenesis, in head and neck tumors, Laryngoscope 113 (7) (2003) 1231–1237.
[18] E.I. Graziani, M. Sung, D. Ma, B. Narayanan, K. Marquette, S. Puthenveetil, L. N. Tumey, J. Bikker, J. Casavant, E.M. Bennett, M.B. Charati, J. Golas, C. Hosselet, C.M. Rohde, G. Hu, M. Guffroy, H. Falahatpisheh, M. Finkelstein, T. Clark, F. Barletta, L. Tchistiakova, J. Lucas, E. Rosfjord, F. Loganzo, C.J. O'Donnell, H. Gerber, P. Sapra, PF-06804103, a site-specific anti-HER2 antibody-drug conjugate for the treatment of HER2-expressing breast, gastric, and lung cancers, Mol. Cancer Ther. 19 (10) (2020) 2068–2078.
[19] B. Gorovits, S.C. Alley, S. Bilic, B. Booth, S. Kaur, P. Oldfield, S. Purushothama, C. Rao, S. Shord, P. Siguenza, Bioanalysis of antibody-drug conjugates: American association of pharmaceutical scientists antibody-drug conjugate working group position paper, Bioanalysis 5 (9) (2013) 997–1006.
[20] P.L. Salomon, R. Singh, Sensitive ELISA method for the measurement of catabolites of antibody-drug conjugates (ADCs) in target cancer cells, Mol. Pharm. 12 (6) (2015) 1752–1761.
[21] X. Li, X. Chen, D. Zhong, Bioanalysis in the development of antibody-drug conjugates, Yao Xue Xue Bao 51 (4) (2016) 517–528.
[22] J. Wang, H. Gu, A. Liu, A. Kozhich, V. Rangan, H. Myler, L. Luo, R. Wong, H. Sun, B. Wang, H.E. Vezina, S. Deshpande, Y. Zhang, Z. Yang, T.V. Olah, A. Aubry, M. E. Arnold, R. Pillutla, B. DeSilva, Antibody–drug conjugate bioanalysis using LB-

LC–MS/MS hybrid assays: strategies, methodology and correlation to ligand-binding assays, Bioanalysis 8 (13) (2016) 1383–1401.
[23] H. Sugimoto, D. Wei, L. Dong, D. Ghosh, S. Chen, M.G. Qian, Perspectives on potentiating immunocapture-LC–MS for the bioanalysis of biotherapeutics and biomarkers, Bioanalysis 10 (20) (2018) 1679–1690.
[24] H. Neubert, T. Olah, A. Lee, S. Fraser, R. Dodge, O. Laterza, M. Szapacs, S.C. Alley, O.M. Saad, S. Amur, L. Chen, E. Cherry, S.J. Cho, I. Cludts, L.D. Donato, A. Edmison, L. Ferrari, F. Garofolo, S. Haidar, S. Hopper, S. Hottenstein, A. Ishii-Watabe, S. Kassim, P. Kurki, G.M.L. Santos, G. Miscoria, J. Palandra, J. Pedras-Vasconcelos, S. Peccoli, S. Rogstad, Y. Saito, N. Savoie, T. Sikorski, S. Spitz, L. Staelens, D. Verthelyi, S. Vinter, M. Wadhwa, Y. Wang, J. Welink, N. Weng, E. Whale, E. Woolf, J. Wu, H. Yan, H. Yu, S. Zhou, 2018 White paper on recent issues in bioanalysis: focus on immunogenicity assays by hybrid LBA/LCMS and regulatory feedback (Part 2 – PK, PD & ADA assays by hybrid LBA/LCMS & regulatory agencies' inputs on bioanalysis, biomarkers and immunogenicity), Bioanalysis 10 (23) (2018) 1897–1917.
[25] M. Faria, M. Peay, B. Lam, E. Ma, M. Yuan, M. Waldron, W.R. Mylott Jr, M. Liang, A.I. Rosenbaum, Multiplex LC-MS/MS assays for clinical bioanalysis of MEDI4276, an antibody-drug conjugate of Tubulysin analogue attached via cleavable linker to a biparatopic humanized antibody against HER-2, Antibodies 8 (1) (2019) 11.
[26] F. Yin, Y. Ling, J. Keller, D. Kraus, R. Narayanaswamy, H. Mangus, F. Li, H. Yang, G. Liu, Quantitation of 2-hydroxyglutarate in human plasma via LC-MS/MS using a surrogate analyte approach, Bioanalysis 12 (16) (2020) 1149–1159.
[27] F. Yin, J. Keller, D. Kraus, H. Mangus, F. Li, G. Liu, A double surrogate approach for the quantitation of 2-Hydroxyglutarate – an oncometabolite in human brain tumors via LC-MS/MS, J. Pharm. Biomed. Anal. 179 (2020), 112916.
[28] F. Yin, Y. Ling, J. Martin, R. Narayanaswamy, L. McIntosh, F. Li, G. Liu, Quantitation of uridine and L-dihydroorotic acid in human plasma by LC-MS/MS using a surrogate matrix approach, J. Pharm. Biomed. Anal. 192 (2021), 113669.
[29] F. Yin, S. Yu, R. Narayanaswamy, H. Mangus, E. McCourt, G. Liu, Quantitation of ivosidenib in human plasma via LC-MS/MS and its application in clinical trials, Bioanalysis 13 (11) (2021) 875–889.
[30] F. Yin, C. DeCiantis, J. Pinkas, B. Das, F. Wang, N. Zheng, D. Hahn, A. Amrite, D. Adhikari, C. Kane, J. Sikora, J. Pittman, R. Wates, E. Shaheen, S. Harriman, Quantitation of an ADC drug PYX-201 in rat and monkey plasma via ELISA and its application in preclinical studies, Bioanalysis 15 (1) (2023) 43–52.
[31] US FDA, Guidance for industry, bioanalytical method validation, May 2018. ⟨(https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf)⟩.