Preliminary Communication

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Bioanalysis

Quantification of antibody–drug conjugate PYX-201 in rat and monkey plasma via ELISA and its application in preclinical studies

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Aim: PYX-201 is a novel antibody–drug conjugate targeting the extra domain B splice variant of fibronectin in the tumor microenvironment. Accurate quantification of PYX-201 is critical for PYX-201 pharmacokinetics profiling in preclinical studies. **Materials & methods:** ELISA was performed using reference standard PYX-201, mouse monoclonal anti-monomethyl auristatin E antibody, mouse IgG1, mouse monoclonal anti-human IgG horseradish peroxidase and donkey anti-human IgG horseradish peroxidase. **Results:** This assay was validated at 50.0–10,000 ng/ml in rat dipotassium EDTA plasma and 250–10,000 ng/ml in monkey dipotassium EDTA plasma. **Conclusion:** This is the first time for a PYX-201 bioanalytical assay in any matrix to be reported.

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The extra domain B splice variant of fibronectin (EDB + FN) is an extracellular matrix protein. EDB + FN is a complete type III repeat of 91 amino acids and is abundant in most human solid tumor tissues [1]. EDB + FN is a promising tumor target since it is low in normal adult vasculature, whereas it selectively accumulates in stroma around new blood vessels in tumors [2]. EDB + FN is highly expressed in various human cancer types, such as non-small-cell lung cancer, head and neck cancer, breast cancer and pancreatic ductal adenocarcinoma [3–8]. PYX-201 (Figure 1) is a novel investigational antibody–drug conjugate (ADC) targeting EDB + FN in the tumor microenvironment for the treatment of advanced solid tumors. PYX-201 is composed of an EDB targeting mAb L19-kK183C-K94R-K290C-hG1, a fully human IgG1 antibody engineered with reactive cysteines to enable drug-to-antibody ratio 4 site-specific conjugation of a cleavable linker payload mcValCitPABC_Aur0101 (pelidotin). PYX-201 binds specifically to EDB + FN protein and delivers the potent cytotoxic payload Aur0101 precisely to the tumor. Internal *in vitro* cytotoxicity assay revealed potent cell killing induced by PYX-201 in the EDB + FN endogenously expressing human cell line WI-38 VA-13 subline 2RA. Internal *in vivo* pharmacology studies of PYX-201 showed robust antitumor activity against non-small-cell lung cancer, pancreatic cancer, and breast cancer. Further, PYX-201 enhanced expression of PD-L1 in a syngeneic mouse breast cancer model with sustained tumor regression [9]. Combination therapies of PYX-201 and PD-L1 checkpoint inhibitor antibodies induced further tumor regression compared with a single agent *in vivo*.

A few bioanalytical LC-MS/MS and ELISA methods have been validated for ADCs and their catabolites as well as oncology drugs and their biomarkers in animal or human plasma and serum [10–20]. To date, no bioanalytical method has been established in any matrix for PYX-201. As of the time of this writing, there are no approved treatments for targeting EDB + FN on tumor cells. Pyxis Oncology and KCAS recently developed and validated a robust ELISA assay to quantify PYX-201 in rat and monkey plasma to support preclinical studies. This assay was validated under regulatory guidance [21,22] and was smoothly applied in rat and monkey plasma sample analysis.

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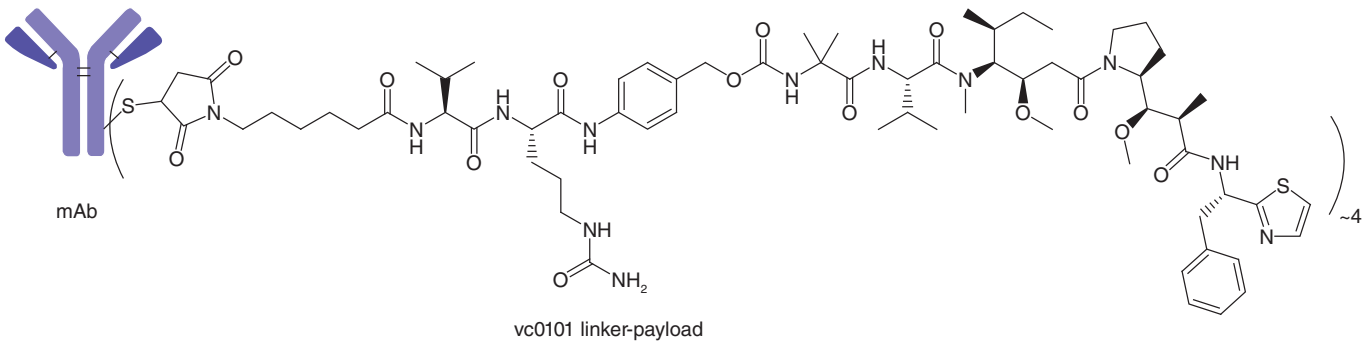

Figure 1. PYX-201 drug substance structure.
vc0101 linker-payload: Valine–citrulline linker to Aur0101 payload.

Here the authors share the first published bioanalytical analysis of PYX-201, a novel investigational ADC targeting EDB + FN to deliver an antimitotic payload to tumor cells.

Materials & methods

Chemicals & reagents

The authors purchased 1× phosphate-buffered saline (PBS), 20× PBS with Tween 20, Pierce protein-free PBS blocking buffer and Blocker Casein in PBS blocking buffer from Thermo Fisher Scientific (MA, USA). The authors obtained 3,3′,5,5′-tetramethylbenzidine (TMB) microwell peroxidase substrate and TMB stop solution from KPL International Limited (New Delhi, India). The authors acquired mouse anti-monomethyl auristatin E antibody from ACROBiosystems (DE, USA). The authors ordered mouse monoclonal anti-human IgG horseradish peroxidase (HRP) and donkey anti-human IgG HRP from Abcam (Cambridge, UK). The authors purchased dipotassium EDTA (K_2EDTA) rat and monkey plasma from BioIVT (NY, USA). PYX-201 ADC was produced by WuXi Biologics (Shanghai, China).

ELISA system

SpectraMax™ i3 and i3× microplate readers (Molecular Devices, CA, USA) were utilized to measure absorbance of the drug–antibody complex. SoftMax® Pro 6.5.1 GxP software (Molecular Devices) and Watson LIMS 7.5 (Thermo Fisher Scientific) were used for data acquisition and processing.

Calibration standards & quality control sample preparation

PYX-201 stock solution was provided at 14.8 mg/ml in 20 mM histidine with 6% (w/v) sucrose and 0.02% (w/v) PS80 at pH 5.5 and stored at -80°C when not in use. PYX-201 stock solution was directly spiked into rat or monkey K_2EDTA plasma to produce standard curves and quality control (QC) samples. PYX-201 was quantified within a nominal range of 50.0–10,000 ng/ml in rat K_2EDTA plasma and 250–10,000 ng/ml in monkey K_2EDTA plasma. This assay required 100 µl of sample after rat or monkey K_2EDTA plasma samples were diluted 1:50 as the minimum required dilution in assay dilution buffer (protein-free PBS in rat K_2EDTA plasma method and Blocker Casein in PBS in monkey K_2EDTA plasma method). Calibration standards were prepared in rat K_2EDTA plasma at 50.0, 100, 250, 500, 1000, 2500, 4000, 6000, 8000 and 10,000 ng/ml with two anchor points at 25.0 and 20,000 ng/ml. Calibration standards were also prepared in monkey K_2EDTA plasma at 250, 500, 750, 1000, 2000, 4000, 5000, 6500, 8000 and 10,000 ng/ml with two anchor points at 100 and 12,500 ng/ml. All calibration standard were prepared fresh on a daily basis.

PYX-201 was fortified in blank pools of matrix to make QC samples for analysis in each run. QCs were prepared in rat K_2EDTA plasma at five concentrations: 50.0 (lower limit of quantification [LLOQ]), 150 (low QC), 1500 (mid QC), 7500 (high QC [HQC]) and 10,000 (upper limit of quantification [ULOQ]) ng/ml. QCs were also prepared in monkey K_2EDTA plasma at five concentrations: 250 (LLOQ), 750 (low QC), 2500 (mid QC), 7500 (HQC) and 10,000 (ULOQ) ng/ml. Accuracy and precision were evaluated for the aforementioned five QCs in rat or monkey K_2EDTA plasma in three replicates. QC samples were prepared fresh or stored at desired temperatures (e.g., -80°C) for the purpose of stability testing.

Assay procedure

A 96-well Nunc Medisorp plate (Thermo Fisher Scientific) was incubated overnight at 2–8°C with 100 µl coating solution (mouse monoclonal anti-monomethyl auristatin E antibody in PBS at 2.0 µg/ml), washed with wash buffer (0.01 M PBS with 0.05% Tween 20), blocked with blocking buffer (Pierce protein-free PBS blocking buffer for rat K_2EDTA plasma assay and Blocker Casein in PBS for monkey K_2EDTA plasma assay) and washed again with wash buffer. Next, 100 µl of standards, QC samples or study samples was thawed, mixed upside down at room temperature, aliquoted, added to the prepared 96-well Nunc Medisorp plate and incubated for 1 h at room temperature on a plate shaker. After the plate was washed with wash buffer, 100 µl of detection antibody (mouse monoclonal anti-human IgG HRP 1:25,000 for rat K_2EDTA plasma assay and donkey anti-human IgG HRP 1:25,000 for monkey K_2EDTA plasma assay) was added and the samples were incubated for 1 h at room temperature on a plate shaker. Samples were washed with wash buffer, and 100 µl of TMB was then added followed by 100 µl of TMB stop solution. The plate was read using a SpectraMax™ i3 or i3× at 450 nm with a reference wavelength of 650 nm.

Software for data acquisition & processing

SoftMax® Pro 6.5.1 GxP and Watson LIMS 7.5 were used to acquire and process data from the SpectraMax™ i3 or i3× plate reader. The calibration curves were evaluated using five-parameter logistic (Marquardt) regression with $1/y^2$ weighting. Statistical data, including mean, standard deviation, percent relative error (%RE) and percent coefficient of variation (%CV), were calculated by Watson LIMS. Toxicokinetic noncompartmental analysis was performed on the concentration–time data from PYX-201-treated rats or monkeys using Phoenix WinNonlin 8.3.4 (Certara, NJ, USA).

Results & discussion

Assay selectivity

Selectivity is the ability of a method to quantify PYX-201 in the presence of other constituents in the sample matrix. Ten different sources of rat K_2EDTA plasma were evaluated both unspiked and spiked with PYX-201 at the LLOQ, and ten different sources of rat K_2EDTA plasma were evaluated at HQC levels. Eleven different sources of monkey K_2EDTA plasma were evaluated both unspiked and spiked with PYX-201 at the LLOQ and HQC levels. To be deemed acceptable, the mean response observed in at least 80% of samples of unspiked matrix was required to be below the limit of quantification, and %RE of the spiked sample was required to be ±25.0% at LLOQ and ±20.0% at HQC. Assay selectivity met the acceptance criteria in both rat K_2EDTA plasma and monkey K_2EDTA plasma (Supplementary Tables 1 & 2).

Linearity & analytical range

Ten different non-zero standard concentrations with an analytical range of 50.0–10,000 ng/ml and two anchor points at 25.0 and 20,000 ng/ml in rat K_2EDTA plasma as well as ten different non-zero standard concentrations with an analytical range of 250–10,000 ng/ml and two anchor points at 100 and 12,500 ng/ml in monkey K_2EDTA plasma were used to construct the calibration curves. Calibration standards were processed in duplicate. The allowable %RE from nominal for each standard was within ±20.0%, with the exception of LLOQ and ULOQ, where within ±25.0% was accepted. The allowable %CV of signal between replicates for each standard was ≤20.0%, with the exception of LLOQ and ULOQ, where ≤25.0% was accepted. Any unaccepted standard was required to be excluded from the regression model. Data for the respective standard curves in rat K_2EDTA plasma and monkey K_2EDTA plasma for accepted runs are presented in Supplementary Tables 3 & 4.

Accuracy & precision

Inter- and intra-assay accuracy and precision were assessed in six accepted batches by analyzing three replicates of each of the validated samples spiked at five separate concentrations (LLOQ, low QC, mid QC, HQC and ULOQ). Accuracy of an assay is to measure how close a set of determined values are to their true values and is usually reported as %RE or %bias. Precision of an assay is to assess how close the analyses are to each other and is usually presented as %CV. For intra- and inter-assay, the mean %RE at each validated sample level was required to be within ±20.0% of the nominal concentration, with the exception of LLOQ and ULOQ, where ±25.0% was acceptable. The %CV at each validated sample level was required to be ≤20.0%, with the exception of LLOQ and ULOQ, where ≤25.0% was acceptable. The total error (|%RE| + %CV) of the validated samples was required to be ≤30.0% at low QC,

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mid QC, HQC and ≤40.0% at LLOQ and ULOQ. The intra- and inter-assay accuracy and precision data for this assay met the required criteria. Data for PYX-201 in rat K_2EDTA plasma are presented in Table 1. Data for PYX-201 in monkey K_2EDTA plasma are presented in Table 2. Intra-day %RE ranged from -21.9% to 16.7% with %CV between 1.0% and 21.4% and inter-day %RE ranged from -5.9% to 5.0% with %CV between 6.4% and 15.3% for all QC levels in rat K_2EDTA plasma assay. Inter-day percent total error (%TE) ranged from 9.6% to 21.3% in rat K_2EDTA plasma assay. Intra-day %RE ranged from -26.1% to 23.6% with %CV between 0.5% and 24.5% and inter-day %RE ranged from -5.8% to 10.2% with %CV between 3.8% and 15.4% for every QC level in monkey K_2EDTA plasma assay. Inter-day %TE ranged from 7.9% to 19.0% in monkey K_2EDTA plasma assay. Because of the complexity of the assay, slightly higher %CV and %RE values were observed in three runs at the high end of the assay, with a %CV of 21.4% for HQC in run five of the rat K_2EDTA plasma method, %RE of -26.1% for ULOQ in run four of the monkey K_2EDTA plasma method and %CV of 24.5% for HQC in run six of the monkey K_2EDTA plasma method; however, the integrity of the assay was not impacted.

Dilution linearity & prozone

Dilution linearity was determined using an ultra-high QC (UHQC) sample spiked with PYX-201 at 1,000,000 ng/ml. The UHQC was serially diluted to have at least two samples expected above the range of quantification and three within the range of quantification. The use of samples above the range of quantification is employed to evaluate the possibility of a hook effect. The UHQC sample was prepared in rat or monkey K_2EDTA plasma and frozen at -80°C overnight. The UHQC was serially diluted in matrix, and then each dilution was further diluted to the minimum required dilution in assay dilution buffer and run in the assay. A total of five replicates per dilution factor in rat K_2EDTA plasma and ten replicates per dilution factor in monkey K_2EDTA plasma were evaluated. The greatest acceptable dilution for the method was defined as the highest dilution tested that was within the range of quantification and met the target acceptance criteria. Lack of prozone was demonstrated by the observation that samples prepared above the ULOQ of the method resulted in an above quality limit, whereas samples below the ULOQ resulted in an interpolated concentration. Dilution linearity using dilution factors between 1:125 and 1:10,000 in rat K_2EDTA plasma and 1:125 and 1:2500 in monkey K_2EDTA plasma was proven to be valid. Data for the rat K_2EDTA plasma method are presented in Supplementary Table 5 and data for the monkey K_2EDTA plasma method are presented in Supplementary Table 6.

Stability assessment

As required by regulatory guidance [21,22], freeze–thaw, bench-top, refrigeration and long-term stability was evaluated in this assay validation. Aliquots of low- and high-concentration QC samples were frozen for at least 24 h before the initial thawing and then refrozen for at least 12 h and thawed again. QC samples went through more freeze–thaw cycles and were analyzed with a set of QCs and calibration standards. PYX-201 in rat K_2EDTA plasma was proven stable over five cycles of freeze (-80°C) and thaw (room temperature). PYX-201 in monkey K_2EDTA plasma was proven stable over four cycles of freeze (-80°C) and thaw (room temperature).

Low- and high-concentration QC samples were frozen at -80°C for a minimum of 24 h and then thawed at ambient conditions or refrigerated temperatures prior to analysis with a set of QCs and calibration standards. PYX-201 in rat K_2EDTA plasma was proven stable at room temperature for at least 22.5 h and was also stable at refrigerated temperatures for at least 120.5 h. PYX-201 in monkey K_2EDTA plasma was proven stable at room temperature for at least 20 h and was also stable at refrigerated temperatures for at least 20 h.

To prove nonclinical plasma samples were still intact after being stored in the toxicology lab and the analytical lab before being analyzed, long-term stability was tested. QC samples were prepared, aliquoted and frozen in polypropylene tubes at -80°C and evaluated over time at various intervals along with a freshly prepared set of QCs and calibration standards. PYX-201 was stable in rat K_2EDTA plasma at -80°C for at least 99 days and was stable in monkey K_2EDTA plasma at -80°C for at least 86 days.

Assay robustness & ruggedness

Assay robustness is to test the ability of a method to withstand random changes. Assay robustness was approved by the evaluation of results from a second analyst using the same assay as well as the successful incurred sample reanalysis in preclinical sample analysis studies. Assay ruggedness is to test the ability of a method to withstand deliberate changes. Assay ruggedness was approved by the evaluation of different incubation time and equipment in the method validation studies.

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

Run number	LLOQ QC, 50.0 ng/ml	LQC, 150 ng/ml	MQC, 1500 ng/ml	HQC, 7500 ng/ml	ULOQ, 10,000 ng/ml
1	44.4	163	1380	7000	7700
	43.9	151	1330	5930†	8160
	42.7	148	1330	6420	8230
Intra-run, mean	43.7	154	1350	6450	8030
Intra-run, SD	0.874	7.94	28.9	536	288
Intra-run, %CV	2.0	5.2	2.1	8.3	3.6
Intra-run, %RE	-12.6	2.7	-10.0	-14.0	-19.7
2	55.2	163	1550	8400	9840
	52.7	165	1510	8530	9750
	52.7	169	1580	8810	9420
Intra-run, mean	53.5	166	1550	8580	9670
Intra-run, SD	1.44	3.06	35.1	210	221
Intra-run, %CV	2.7	1.8	2.3	2.4	2.3
Intra-run, %RE	7.0	10.7	3.3	14.4	-3.3
3	52.8	162	1520	7170	10,900
	53.9	155	1470	7500	11,200
	58.9	154	1490	8290	12,800‡
Intra-run, mean	55.2	157	1490	7650	11,600
Intra-run, SD	3.25	4.36	25.2	576	1020
Intra-run, %CV	5.9	2.8	1.7	7.5	8.8
Intra-run, %RE	10.4	4.7	-0.7	2.0	16.0
	LLOQ QC, 50.0 ng/ml	LQC, 150 ng/ml	MQC, 1500 ng/ml	HQC, 7500 ng/ml	ULOQ, 10,000 ng/ml
4	53.1	163	1430	8580	9720
	62.3	158	1630	9050†	9470
	59.6	159	1480	8620	9130
Intra-run, mean	58.3	160	1510	8750	9440
Intra-run, SD	4.73	2.65	104	261	296
Intra-run, %CV	8.1	1.7	6.9	3.0	3.1
Intra-run, %RE	16.6	6.7	0.7	16.7	-5.6
5	46.2	157	1450	8050	10,900
	44.9	138	1300	6300	9310
	42.4	133	1260	5290†	9350
Intra-run, mean	44.5	143	1340	6550	9850
Intra-run, SD	1.93	12.7	100	1400	907
Intra-run, %CV	4.3	8.9	7.5	21.4	9.2
Intra-run, %RE	-11.0	-4.7	-10.7	-12.7	-1.5
6	57.4	173	1630	7120	6650‡
	49.8	160	1610	8790	8200
	51.6	163	1600	7400	8570
Intra-run, mean	52.9	165	1610	7770	7810
Intra-run, SD	3.97	6.81	15.3	894	1020
Intra-run, %CV	7.5	4.1	1.0	11.5	13.1
Intra-run, %RE	5.8	10.0	7.3	3.6	-21.9
Inter-run, mean	51.4	157	1480	7630	9410
Inter-run, SD	6.14	10.0	117	1120	1440
Inter-run, %CV	12.0	6.4	7.9	14.6	15.3
Inter-run, %RE	2.7	5.0	-1.7	1.7	-5.9
Inter-run, %TE	14.7	11.3	9.6	16.3	21.3
n	18	18	18	18	18

†>20.0 %RE.
‡>25.0 %RE.
HQC: High-quality control; LLOQ: Lower limit of quantification; LQC: Low-quality control; MQC: Mid-quality control; %CV: Percent coefficient of variation; %RE: Percent relative error; %TE: Percent total error; QC: Quality control; SD: Standard deviation; ULOQ: Upper limit of quantification.

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

Run number	LLOQ QC, 250 ng/ml	LQC, 750 ng/ml	MQC, 2500 ng/ml	HQC, 7500 ng/ml	ULOQ, 10,000 ng/ml
1	262	765	2680	7990	10,300
	287	842	2940	8400	10,300
	273	795	2850	8280	9770
Intra-run, mean	274	801	2820	8220	10,100
Intra-run, SD	12.7	39.2	128	210	299
Intra-run, %CV	4.6	4.9	4.5	2.6	3.0
Intra-run, %RE	9.6	6.8	13.0	9.6	1.1
2	321‡	790	2610	7890	10,300
	271	757	2660	7950	10,500
	271	776	2560	7970	10,000
Intra-run, mean	288	774	2610	7940	10,300
Intra-run, SD	29.0	16.6	48.9	40.9	233
Intra-run, %CV	10.1	2.1	1.9	0.5	2.3
Intra-run, %RE	15.1	3.2	4.4	5.9	2.8
3	264	761	2540	7090	9780
	279	795	2620	6970	9200
	262	782	2470	7320	9060
Intra-run, mean	269	779	2540	7130	9350
Intra-run, SD	9.36	17.1	78.6	182	385
Intra-run, %CV	3.5	2.2	3.1	2.6	4.1
Intra-run, %RE	7.4	3.9	1.7	-5.0	-6.5
	LLOQ QC, 250 ng/ml	**LQC, 750 ng/ml**	**MQC, 2500 ng/ml**	**HQC, 7500 ng/ml**	**ULOQ, 10,000 ng/ml**
4	257	741	2130	5970†	8070
	244	734	2540	6070	7850
	239	733	2250	6210	6260‡
Intra-run, mean	247	736	2310	6080	7390
Intra-run, SD	9.75	4.05	209	122	987
Intra-run, %CV	4.0	0.6	9.1	2.0	13.3
Intra-run, %RE	-1.4	-1.9	-7.8	-18.9	-26.1
5	307	786	2550	8420	11,200
	308	815	2610	5760†	10,500
	312	810	2560	7110	10,100
Intra-run, mean	309	803	2570	7100	10,600
Intra-run, SD	2.70	15.6	34.3	1330	579
Intra-run, %CV	0.9	1.9	1.3	18.8	5.5
Intra-run, %RE	23.6	7.1	2.9	-5.3	6.0
6	270	815	1980†	6590	8860
	270	777	2790	7540	9550
	263	777	2820	10,400†	7870
Intra-run, mean	267	790	2530	8190	8760
Intra-run, SD	3.82	22.2	474	2000	843
Intra-run, %CV	1.4	2.8	18.7	24.5	9.6
Intra-run, %RE	7.0	5.3	1.1	9.2	-12.4
Inter-run, mean	276	781	2560	7440	9420
Inter-run, SD	23.1	29.6	243	1140	1240
Inter-run, %CV	8.4	3.8	9.5	15.4	13.2
Inter-run, %RE	10.2	4.1	2.6	-0.8	-5.8
Inter-run, %TE	18.6	7.9	12.1	16.2	19.0
n	18	18	18	18	18

†>20.0 %RE.
‡>25.0 %RE.
HQC: High-quality control; LLOQ: Lower limit of quantification; LQC: Low-quality control; MQC: Mid-quality control; %CV: Percent coefficient of variation; %RE: Percent relative error; %TE: Percent total error; QC: Quality control; SD: Standard deviation; ULOQ: Upper limit of quantification.

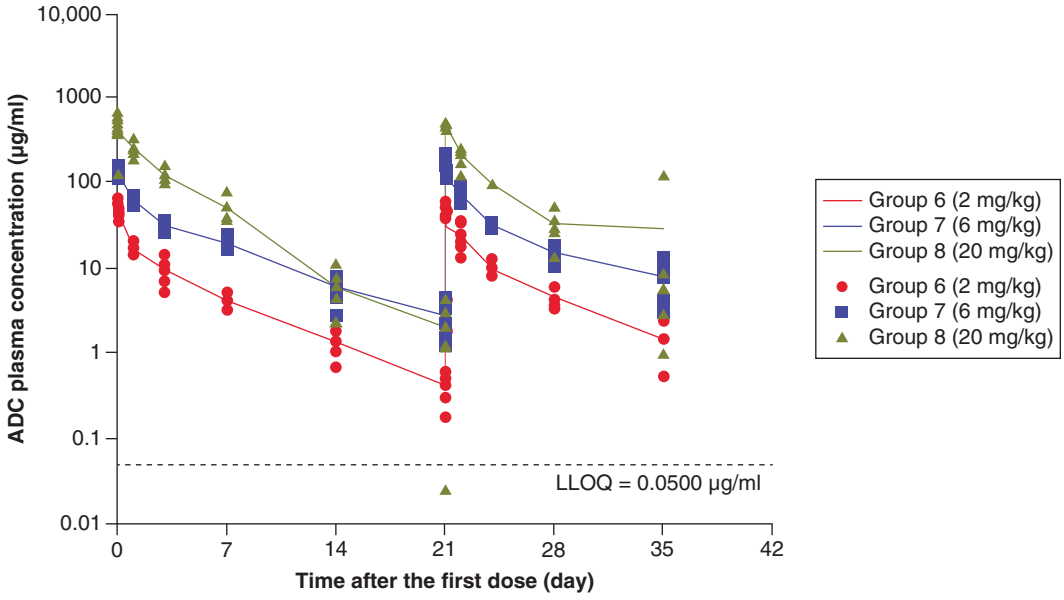

Figure 2. Mean and individual concentration–time profiles of PYX-201 by dose group following bolus intravenous administration of PYX-201 in rats. Values below the LLOQ (0.0500 µg/ml), as shown by dashed line, are plotted at half of LLOQ for illustrative purposes only. Solid lines represent mean values and symbols represent individual values. ADC: Antibody–drug conjugate; LLOQ: Lower limit of quantification.

Table 3. Summary of primary toxicokinetic parameters for PYX-201 by dose group in rats.

Group (dose)	Dose day	T_{max}, h	C_{max}, µg/ml	AUC_{τ}, h × µg/ml	AUC_{inf}, h × µg/ml	CL†, ml/h/kg	$t_{1/2}$, h
6 (2 mg/kg)	1	0.0830	49.6	2560	2620	0.763	101
	22	0.0830	51.1	2770	NA	0.721	97.6
7 (6 mg/kg)	1	0.0830	147	9770	10,300	0.585	119
	22	0.0830	171	10,000	NA	0.598	134
8 (20 mg/kg)	1	0.0830	547	29,700	30,000	0.668	66.7
	22	0.0830	520	29,900	NA	0.668	114

All toxicokinetic parameters are rounded to three significant figures.
†Clearance at steady state (CLss) is presented for day 22.
AUC_{inf}: Area under the curve from time zero to infinity; AUC_{τ}: Area under the curve from time zero to dosing interval τ; CL: Clearance; C_{max}: Maximum concentration; NA: Not applicable; T_{max}: Time to maximum concentration; $t_{1/2}$: Half-life.

Parallelism

Parallelism is a parameter that is used to demonstrate that assay integrity is not impacted by interference and is defined as the parallel relationship between serially diluted study samples and calibration curves [22]. Parallelism was not assessed in the authors' assay because of the limited preclinical sample volumes and the fact that lack of parallelism is a rare occurrence in pharmacokinetics assays.

Assay application

This validated assay was successfully applied in analyzing PYX-201 concentrations in rat and monkey K_2EDTA plasma samples in 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). Mean and individual concentration–time profiles of PYX-201 by dose group (2 mg/kg in group six, 6 mg/kg in group seven and 20 mg/kg in group eight with doses at days 1 and 22) in rat K_2EDTA plasma are depicted in Figure 2 and primary toxicokinetic parameters for PYX-201 by dose group in rat K_2EDTA plasma are summarized in Table 3. Mean plasma concentration–time profiles of PYX-201 after day 1 and 22 dosing by dose group (1 mg/kg in group two, 3 mg/kg in group three and 10 mg/kg in group four with doses at days 1 and 22) and sex (male and female) in monkey K_2EDTA plasma are shown in Figure 3 and summary statistics of primary toxicokinetic parameters by dose group and sex are provided

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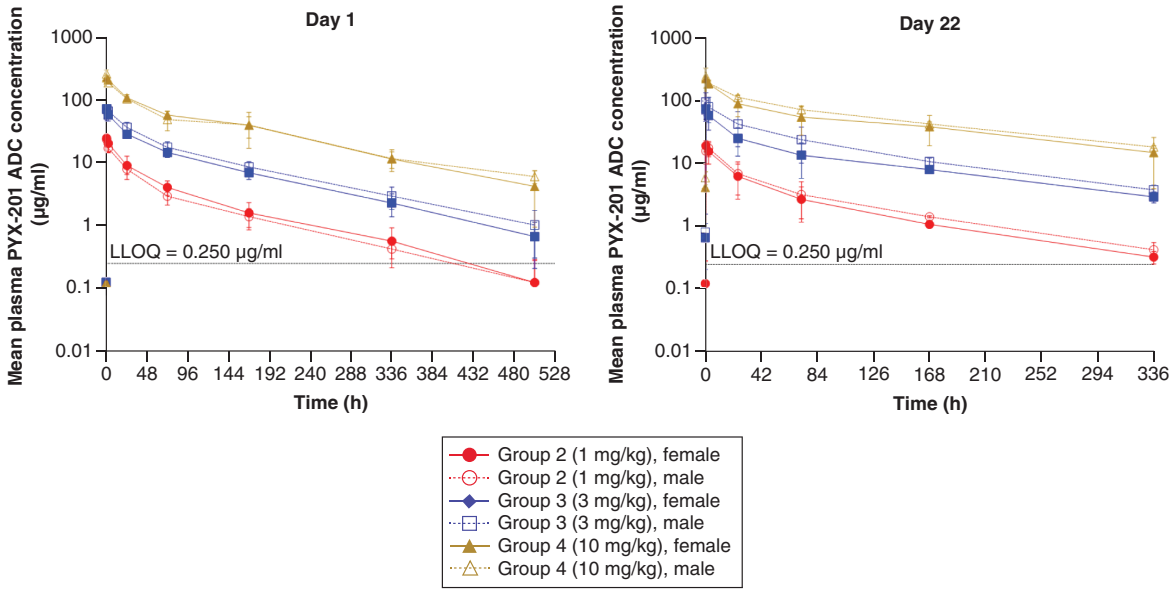

Figure 3. Mean plasma concentration–time profiles of PYX-201 after day 1 and 22 dosing by dose group and sex in monkeys. Values below the LLOQ (0.250 µg/ml), as shown by dashed lines, are plotted at half of LLOQ for illustrative purposes only.
ADC: Antibody–drug conjugate; LLOQ: Lower limit of quantification.

Table 4. Summary statistics of primary toxicokinetic parameters for PYX-201 by dose group and sex in monkeys.

Group (dose)	Sex	Dose day	T_{max}, h	C_{max}, µg/ml	AUC_{τ}, h × µg/ml	AUC_{inf}, h × µg/ml	CL†, ml/h/kg	$t_{1/2}$, h
2 (1 mg/kg)	F	1	1.30	24.3	1110	1140	0.969	92.4
	M	1	0.500	23.1	912	938	1.10	101
	F	22	0.500	19.1	804	NA	1.26	55.8
	M	22	1.70	19.7	1000	NA	1.00	88.0
3 (3 mg/kg)	F	1	0.500	70.0	3920	4040	0.766	103
	M	1	1.00	70.3	4800	4990	0.625	111
	F	22	0.500	72.9	3180	NA	2.24	63.8
	M	22	0.900	99.4	6370	NA	0.436	88.7
4 (10 mg/kg)	F	1	0.900	221	16,700	17,400	0.590	100
	M	1	0.500	252	15,900	18,200	0.554	118
	F	22	0.500	222	12,400	NA	1.25	74.2
	M	22	0.900	242	17,500	NA	0.486	122

All toxicokinetic parameters are presented with mean values and rounded to three significant figures.
†Clearance at steady state (CLss) is presented for day 22.
AUC_{inf}: Area under the curve from time zero to infinity; AUC_{τ}: Area under the curve from time zero to dosing interval τ; CL: Clearance; C_{max}: Maximum concentration; F: Female; M: Male; NA: Not applicable; T_{max}: Time to maximum concentration; $t_{1/2}$: Half-life.

in Table 4.

Conclusion

For the first time, a bioanalytical assay was validated in rat and monkey K_2EDTA plasma for the investigational ADC PYX-201 using an ELISA method. Selectivity, matrix interference, linearity, accuracy, precision, dilution, and stability were tested according to the 2018 US FDA guidance [21] and 2019 ICH M10 guideline [22]. A complex was formed by PYX-201, the capture antibody mouse monoclonal anti-monomethyl auristatin E and the detection antibody mouse or donkey anti-human IgG HRP and was measured by a SpectraMax™ i3 or i3× microplate reader. This assay was selective over the validated curve range of 50.0–10,000 ng/ml in rat K_2EDTA plasma and 250–10,000 ng/ml in monkey K_2EDTA plasma. Inter-day %RE was -5.9% to 5.0% with %CV ≤15.3% and %TE ≤21.3% for all QC samples in rat K_2EDTA plasma. Inter-day %RE was -5.8% to 10.2% with %CV ≤15.4% and

%TE ≤19.0% for all QC samples in monkey K_2EDTA plasma. Dilution linearity was proven to be valid using dilution factors between 1:125 and 1:10,000 in rat K_2EDTA plasma and 1:125 and 1:2500 in monkey K_2EDTA plasma. PYX-201 was stable after five freeze–thaw cycles, or being stored at room temperature for at least 22.5 h or at refrigerated temperatures for at least 120.5 h or being stored at -80°C for 99 days in rat K_2EDTA plasma. PYX-201 was stable after four freeze–thaw cycles, or being stored at room temperature or refrigerated temperatures for at least 20 h or being stored at -80°C for 86 days in monkey K_2EDTA plasma.

This validated assay was effectively applied in preclinical rat and monkey K_2EDTA plasma sample analysis, supporting the PYX-201 investigational new drug application filing and subsequent approval (161622). Method development and validation for PYX-201 in human plasma are currently ongoing, with the goal of supporting future PYX-201 clinical trials.

Summary points

- This is the first bioanalytical assay validation of the investigational antibody–drug conjugate PYX-201.
- An ELISA assay was successfully validated for the measurement of PYX-201 concentrations in rat and monkey K_2EDTA plasma.
- Selectivity, linearity, accuracy and precision were effectively demonstrated by this assay in accordance with 2018 US FDA bioanalytical method validation guidance and the 2019 European Medicines Agency ICH M10 guideline for bioanalytical method validation.
- Utilizing this assay, PYX-201 was proven stable after being tested various stability parameters (benchtop, refrigeration, freeze–thaw and long-term stability).
- This bioanalytical ELISA assay successfully supported the pharmacokinetics assays in preclinical studies.
- In addition to concentration–time profiles, primary toxicokinetic parameters were generated using concentration data via this validated ELISA assay.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.4155/bio-2022-0233

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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