Preliminary Communication

For reprint orders, please contact: reprints@future-science.com

Bioanalysis

A sensitive LC–MS/MS assay to quantitate free payload Aur0101 from ADC PYX-201 in rat and monkey plasma

Feng Yin[1], Farah Ahsan[2], Jan Pinkas*[,1], Biplab Das[1], Frank Wang[1], Nancy Zheng[3], David Hahn[3], Aniruddha Amrite[1], Jianwen Feng[1], Diana Adhikari[1], Jack Sikora[1], Elizabeth Shaheen[1] & Shawn Harriman**[,1]

[1]Pyxis Oncology, Inc., 321 Harrison Avenue, Suite 1, Boston, MA 02118, USA

[2]Q^2 Solutions BioSciences LLC, 19 Brown Road, Ithaca, NY 14850, USA

[3]Amador Bioscience, Inc., 4695 Chabot Drive, Pleasanton, CA 94588, USA

*Author for correspondence: jpinkas@pyxisoncology.com

**Author for correspondence: sharriman@pyxisoncology.com

Aim: Aur0101 is a cytotoxic and small-molecule microtubule depolymerizing agent, and is the payload conjugated to antibody–drug conjugate PYX-201. Developing and validating a sensitive bioanalytical method to quantitate Aur0101 was novel and crucial in preclinical PYX-201 studies. **Materials & methods:** Reference standard Aur0101 and its stable isotope labelled internal standard Aur0101-d_8 were used in this LC–MS/MS method. **Results:** This sensitive assay was validated at a lower limit of quantitation of 15 pg/ml and successfully applied to support preclinical rat and monkey toxicology studies. Preclinical plasma toxicokinetic parameters were presented. **Conclusion:** A sensitive and robust LC–MS/MS assay was validated for Aur0101 in rat and monkey plasma.

First draft submitted: 27 March 2023; Accepted for publication: 1 June 2023; Published online: 16 August 2023

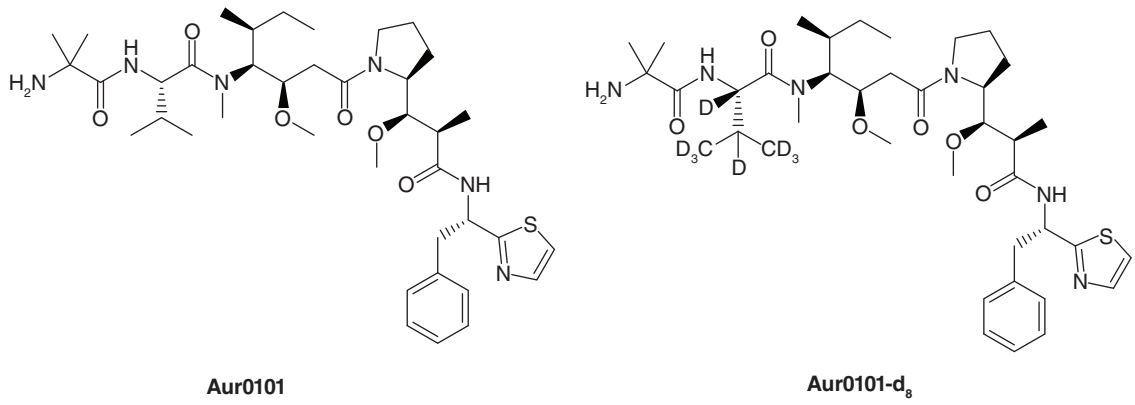
Keywords: ADC • Aur0101 • LC–MS/MS • microtubule inhibitor • oncology • preclinical • small molecule • toxicokinetic • validation

Antibody–drug conjugates (ADCs) have been widely researched in oncology in the last decade [1]. Since the first approved ADC drug Mylotarg™ by the US FDA in 2000, more than a dozen ADC drugs have been approved globally to treat different types of cancers [2–6]. Compared with traditional systemic chemotherapy, approved ADCs have demonstrated better clinical responses than their unconjugated counterparts. Generally, an ADC has three components: a monoclonal antibody (mAb), a linker and payload(s) (toxin or warhead) [7]. The conjugated mAb helps deliver the ADC drugs to the targeted tumor by recognizing the specific target antigen, the linker enables the ADC drugs to carry the cytotoxic payload directly to the cancer cells, and when the linker is cleaved in the location of the tumor, the payload finally is released and kills the tumor cells [8–10].

PYX-201 is an ADC currently under investigation in clinical trials to treat patients with advanced solid tumors (NCT no. NCT05720117; EudraCT no. 2022-002284-30), including non-small-cell lung cancer (NSCLC), hormone receptor-positive (HR+) breast cancer, triple-negative breast cancer (TNBC), head and neck squamous cell carcinoma (HNSCC), ovarian cancer, pancreatic cancer, and so on. PYX-201 targets the extra domain B splice variant of fibronectin (EDB + FN) [11], which can be found in a large number of human solid tumor tissues while being significantly low in normal adult human tissues [12–16]. PYX-201 is composed of a fully human IgG1 antibody, a linker maleimidocaproyl-valine-citrulline-*p*-aminobenzyloxycarbonyl (mcValCitPABC) and an auristatin payload Aur0101 (Figure 1). Auristatins are a family of analogs to a natural product dolastatin 10 and are microtubule inhibitors or microtubule depolymerizing compounds [17,18]. There are more than 30 ADCs with auristatin payloads currently in clinical development. Aur0101 is a potent antimitotic and cytotoxic agent, and has been used as the payload in a few ADC drugs [19–25].

A number of LC–MS/MS methods have been published recently for the quantitation of small-molecule antitumor drugs or ADC payloads and drug metabolites, as well as biomarkers [26–32]. An enzyme-linked immunosorbent

newlands press



Aur0101 **Aur0101-d_8**

Figure 1. PYX-201 payload Aur0101 and the IS Aur0101-d_8 structures.

assay (ELISA) was validated recently in our laboratory to quantitate PYX-201 ADCs in rat and monkey plasma [33]. This manuscript described a sensitive reversed-phase LC–MS/MS assay developed and validated to quantitate free payload Aur0101 from ADC drug PYX-201 in rat and monkey K_2EDTA plasma in order to support investigational new drug (IND) approval (IND 161622) for the first-in-human clinical trial to treat patients with advanced solid tumors (NCT no. NCT05720117; EudraCT no. 2022-002284-30). Regulatory guidance was followed in this assay validation [34,35]. Inter-assay accuracy and precision (A&P) were not in scope for this assay validation study, as a full assay validation of Aur0101 has been validated in a different study. In this partial assay validation, a Shimadzu (MD, USA) ultra-performance liquid chromatography (UPLC) system was utilized to replace the Waters (MA, USA) Acquity UPLC system. This Aur0101 assay was successfully employed in rat and monkey good laboratory practice (GLP) toxicology studies. As a result, plasma concentration–time profiles and toxicokinetic (TK) parameters for PYX-201 free payload were presented.

Experimental

Chemicals & reagents

Acetone (HPLC grade), methanol (HPLC grade), acetonitrile (HPLC grade) and 50/50 methanol/water (HPLC grade) were purchased from Honeywell Burdick & Jackson (MI, USA). Acetic acid (ACS grade), 2-proponal (HPLC grade) and sodium acetate (ACS grade) were provided by JT Baker (NJ, USA). Water was purified in-house using a Millipore Milli-Q IQ 7000 ultrapure lab water system (MA, USA). Ammonium acetate (≥99.99% trace metals basis) was obtained from Sigma-Aldrich (MO, USA). K_2EDTA rat and monkey plasma were acquired from BioIVT (NY, USA). Aur0101 (PF-06380101, purity 98.89%) was supplied by Carbogen Amcis (Bubendorf, Switzerland). PF-06691147 (Aur0101-d_8, purity 99.17%) was received from MedChemExpress (NJ, USA).

LC–MS/MS system

The LC–MS/MS system includes an API-5000 triple quadrupole mass spectrometer (Sciex, MA, USA) and a Shimadzu UPLC system (CBM-20A for HPLC controller, LC-30AD for HPLC pumps, SIL-30AC for HPLC autosampler). Data were acquired and processed using Analyst® 1.6.2 software (Sciex) and the Watson laboratory information management system (LIMS) v7.4.2 software (Thermo Fisher Scientific, MA, USA). An Acquity UHPLC BEH C_4 analytical column (Waters; 2.1 × 100 mm, 1.7 µm) was applied in method validation as well as preclinical sample analysis.

Calibration standard & quality control sample preparation

Aur0101 dry chemical was weighed on a microbalance, corrected for potency, and dissolved in 50/50 acetonitrile (ACN)/H_2O to give a stock solution and a working solution at 100 and 0.500 µg/ml, respectively. The stock solution and working solution were stored in a refrigerator kept at 4°C when not in use. Aur0101 stock solution and working solution have been demonstrated to be stable at 4°C for at least 9 and 6 months, respectively, during assay validation. This assay had a range from 15.0 to 15,000 pg/ml, and the calibration standards were made in rat or monkey K_2EDTA plasma at 15.0, 30.0, 150, 600, 1500, 7500, 12,000 and 15,000 pg/ml. Calibration standard

Table 1. Intra-run accuracy and precision for Aur0101 in rat and monkey K_2EDTA plasma.

Run number	LLOQ 15.0 pg/ml	LQC 45.0 pg/ml	MQC 4500 pg/ml	HQC 11,300 pg/ml
1	13.3	42.5	4400	11,000
(rat K_2EDTA plasma)	13.8	43.2	4470	10,900
	12.9	42.9	4470	10,900
	13.6	43.6	4600	10,700
	13.5	43.2	4400	10,800
	13.7	43.1	4430	11,500
Intra-run mean	13.5	43.1	4460	11,000
Intra-run SD	0.327	0.366	74.7	280
Intra-run %CV	2.4	0.8	1.7	2.5
Intra-run %RE	-10.0	-4.2	-0.9	-2.7
n	6	6	6	6
Run number	**LLOQ 15.0 pg/ml**	**LQC 45.0 pg/ml**	**MQC 4500 pg/ml**	**HQC 11,300 pg/ml**
1	14.5	43.6	4300	10,900
(monkey K_2EDTA plasma)	13.5	43.6	4320	10,800
	14.2	41.5	4170	11,300
	13.1	44.4	4380	10,700
	13.7	41.7	4400	10,600
	14.2	43.8	4420	10,700
Intra-run mean	13.9	43.1	4330	10,800
Intra-run SD	0.524	1.20	91.7	250
Intra-run %CV	3.8	2.8	2.1	2.3
Intra-run %RE	-7.3	-4.2	-3.8	-4.4
n	6	6	6	6

%CV: Percent of coefficient of variation; %RE: Percent relative error; HQC: High-quality control; LQC: Low-quality control; LLOQ: Lower limit of quantitation; MQC: Medium-quality control; SD: Standard deviation.

curves were prepared on a daily basis in the assay validation. This assay required 25-µl rat or monkey K_2EDTA plasma.

Quality control (QC) samples were prepared by spiking the analyte (Aur0101) into rat or monkey K_2EDTA plasma at four concentrations: 15.0 pg/ml (lower limit of quantitation [LLOQ]), 45.0 pg/ml (low-quality control [LQC]), 4500 pg/ml (medium-quality control [MQC]), and 11,300 pg/ml (high-quality control [HQC]). QC samples were analyzed in six replicates to evaluate intra-day A&P, results shown in Table 1. In addition, LQC and HQC samples were prepared and kept at -20°C and -70°C for stability testing.

Aur0101-d_8 was used as the stable isotope-labelled (SIL) internal standard (IS). An IS stock solution was prepared in 50/50 ACN/H_2O at 100 µg/ml, and an IS working solution was prepared in ACN at 300 pg/ml by diluting the IS stock solution. The IS stock solution and working solution were kept at 4°C when not in use. Equivalent stock solution stability was assigned to the IS as that of the analyte because the IS was an SIL IS of the analyte, was prepared in the same solvent, and was stored in the same condition as that of the analyte.

Sample extraction

Standards, QCs or study samples were thawed in an ice bath. Protein precipitation followed by a solid-phase extraction (SPE) procedure was employed to extract the analyte and the IS. A total of 25 µl of rat or monkey K_2EDTA plasma was aliquoted in a 1-ml LoBind 96-well block and fortified with 50 µl of 300 pg/ml Aur0101-d_8 IS working solution, vortex mixed and centrifuged at 4°C. All samples were added in 225 µl of 0.14 M sodium acetate in 0.03% acetic acid solution, vortex mixed, centrifuged at 4°C and transferred to an Oasis® HLB µElution SPE plate (Waters), which had already been conditioned by methanol then equilibrated by water. The SPE plate was washed with water and 2/8 methanol/water, and then eluted with 3 × 30 µl of methanol. The eluent was collected and mixed with 40 µl of water, and the samples were centrifuged at 4°C. A total of 10 µl of the supernatant was injected into the LC–MS/MS system.

Software for data acquisition & processing

LC–MS/MS data were acquired and processed on Analyst 1.6.2 and Watson LIMS 7.4.2. A weighted ($1/x^2$) linear regression and all statistics (e.g., mean, standard deviation [SD], percent relative error [%RE], percent coefficient of variation [%CV]) were performed and calculated by Watson LIMS 7.4.2. A Phoenix® WinNonlin® v8.3.4 (Certara, NJ, USA) was used for TK noncompartmental analysis.

Results & discussion

LC–MS/MS conditions

An Acquity UHPLC BEH C4 column (2.1 × 100 mm, 1.7 µM) was used for chromatographic separation. A 10 mM ammonium acetate in water solution and ACN were optimized as mobile phases A and B, respectively. A high column temperature of 80°C was applied to obtain the sharp and symmetric peak shapes and the flow rate was set up at 350 µl/min. A gradient was chosen as follows: 0.0 min (40% B), 0.6 min (40% B), 3.0 min (95% B), 5.0 min (95% B), 5.1 min (40% B) and 6.5 min (40% B). The total run time was 6.5 min and the autosampler temperature was set at 4°C.

Multiple reaction monitoring mode with MS transitions m/z 743.5 > 188.1 for Aur0101 and m/z 751.6 > 188.1 for Aur0101-d_8 were analyzed in this assay on a Sciex API 5000 mass spectrometer using turbo ion spray as the ion source in positive ion mode. Unit resolution was used for both Q1 and Q3 resolutions. The source temperature was set at 650°C, ion spray voltage was kept at 4500 V, curtain gas was maintained at 35, collision gas was held at 6, GS1 and GS2 were optimized at 35 and 50, respectively, declustering potential was selected at 55, and collision energy was chosen to be 65. The structures of Aur0101 and AUR0101-d_8 are shown in Figure 1.

Assay range & sensitivity

A nominal concentration range of 15.0–15,000 pg/ml for Aur0101 was validated in this assay and was adequate to quantitate Aur0101 in rat and monkey K_2EDTA plasma. Representative chromatograms from a matrix blank spiked with the IS for Aur0101 and Aur0101-d_8 in rat and monkey K_2EDTA plasma are shown in Supplementary Figures 1 & 2, respectively. Figures 2 & 3 show representative chromatograms of Aur0101 and Aur0101-d_8 from LLOQ samples in rat and monkey K_2EDTA plasma, demonstrating adequate sensitivity to measure Aur0101 at 15.0 pg/ml in matrix.

Assay selectivity

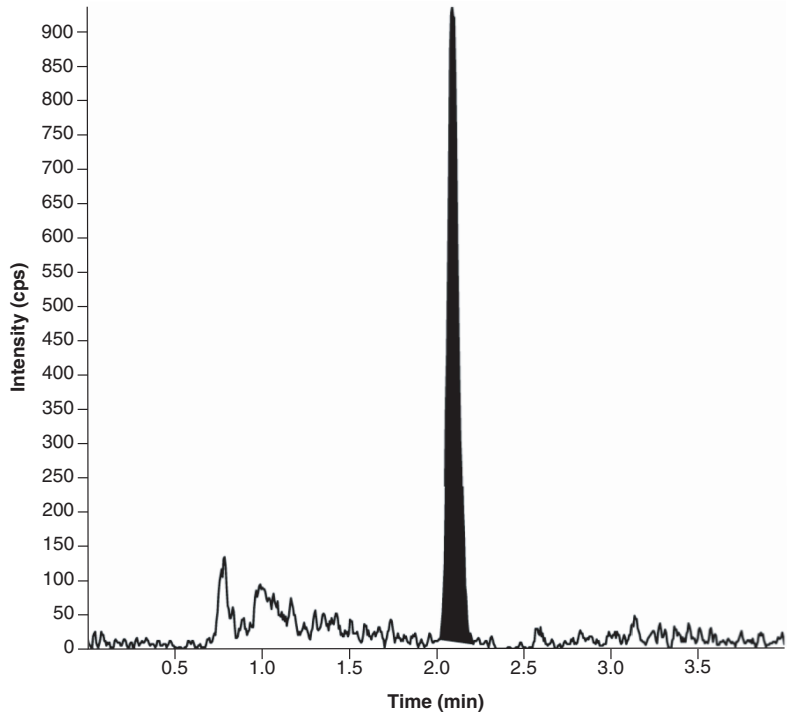
Assay selectivity was assessed to ensure differentiation and quantification of the analyte in the presence of potential interfering substances in the blank biological matrix [34,35]. Six normal individual lots and one hemolyzed lot of rat K_2EDTA plasma, as well as six normal individual lots and one hemolyzed lot of monkey K_2EDTA plasma, were evaluated in this assay with and without IS to assess assay selectivity. The analyte responses in all the blank with IS samples were < 20% of the responses in the corresponding LLOQ samples, and the IS responses in all the blank samples were < 5% of the average IS responses in the calibrators and QC samples, indicating this assay was selective for Aur0101 analysis in rat and monkey plasma.

Accuracy & precision

One A&P run was analyzed for Aur0101 QC samples prepared in K_2EDTA plasma for each species (rat and monkey). QC samples were prepared by fortifying the pools of blank matrix with Aur0101, and A&P was calculated on these QC sample results. A&P was presented as the percent relative error (%RE) and the percent coefficient of variation (%CV), respectively, with accuracy expressing the percentage of measured concentration compared with nominal concentration, and precision displaying the closeness of replicate analyses. Six replicates for each QC level at four QC levels (LLOQ, LQC, MQC and HQC) were assessed for intra-assay A&P. Inter-assay A&P was not evaluated in this assay validation study as full-assay validation of Aur0101 had been validated in a different study. QC data met the acceptance criteria for intra-assay validation according to regulatory guidance [34,35]; the statistics are shown in Table 1 for the rat and monkey plasma methods. The intra-run %RE ranged from -10.0 to -0.9%, with %CV between 0.8 and 2.5% for all QC levels in rat K_2EDTA plasma. The intra-run %RE ranged from -7.3 to -3.8%, with %CV between 2.1 and 3.8% for all QC levels in monkey K_2EDTA plasma.

Sample name: "1 005 PYCM201 PVRPL STD1 1 1" Sample ID: "5" File: "PYCM201_PVRPL_01.wiff"
Peak name: "PF-06380101" Mass(es): "743.500/188.100 Da"
Comment: "none" Annotation: ""

Sample index:	7
Sample type:	Standard
Concentration:	15.0
Calculated conc:	No intercept
Acq. date:	20 May 2022
Acq. time:	3:52:53 PM
Modified:	No
Proc. algorithm:	specify parameters – MQ III
Noise percentage:	50
Base. sub. window:	1.00 min
Peak-split. factor:	2
Report largest peak:	Yes
Min. peak height:	55.00 cps
Min. peak width:	0.00 sec
Smoothing width:	3 points
RT window:	10.0 sec
Expected RT:	2.12 min
Use relative RT:	No
Int. type:	Base to base
Retention time:	2.09 min
Area:	3891 counts
Height:	925 cps
Start time:	2.02 min
End time:	2.21 min



Sample name: "1 005 PYCM201 PVRPL STD1 1 1" Sample ID: "5" File: "PYCM201_PVRPL_01.wiff"
Peak name: "PF-06691147(IS)" Mass(es): "751.600/188.100 Da"
Comment: "none" Annotation: ""

Sample index:	7
Sample type:	Standard
Concentration:	1.00
Calculated conc:	N/A
Acq. date:	20 May 2022
Acq. time:	3:52:53 PM
Modified:	No
Proc. algorithm:	specify parameters – MQ III
Noise percentage:	50
Base. sub. window:	1.00 min
Peak-split. factor:	2
Report largest peak:	Yes
Min. peak height:	500.00 cps
Min. peak width:	0.00 sec
Smoothing width:	3 points
RT window:	10.0 sec
Expected RT:	2.10 min
Use relative RT:	No
Int. type:	Base to base
Retention time:	2.08 min
Area:	135,006 counts
Height:	33,079 cps
Start time:	1.93 min
End time:	2.28 min

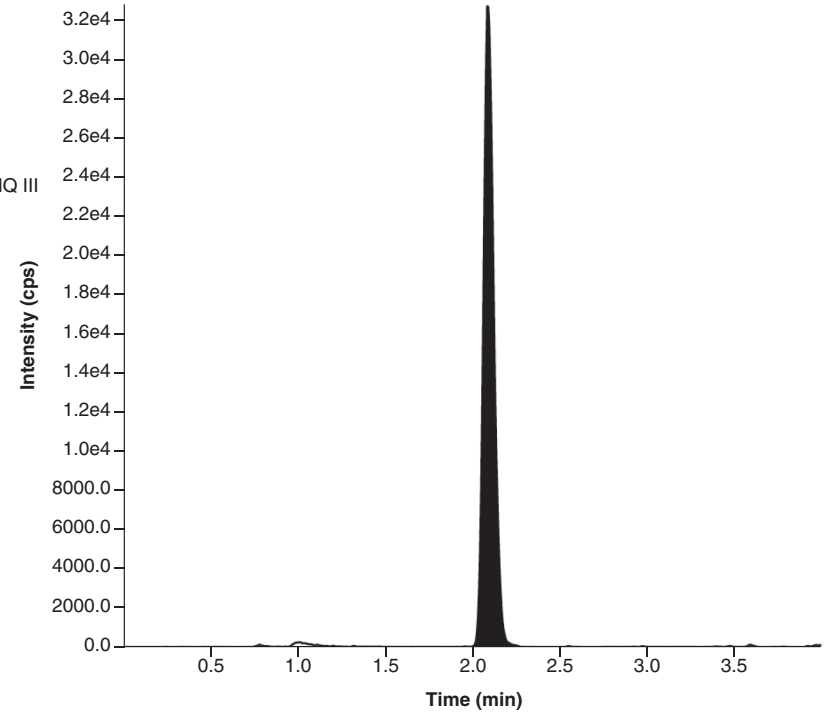
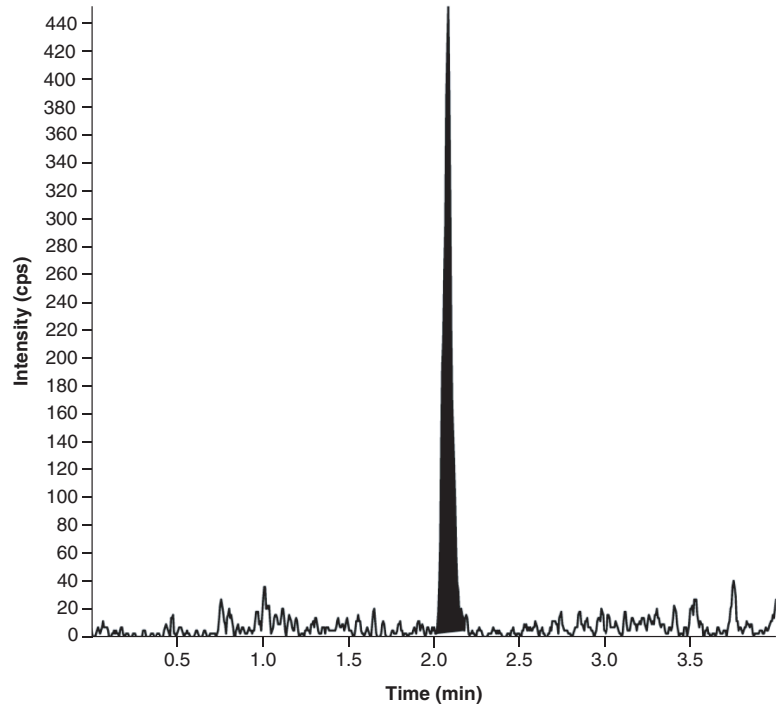


Figure 2. Chromatograms of Aur0101 (top) and internal standard Aur0101-d_8 (bottom) from a lower limit of quantitation sample in rat K_2EDTA plasma.
RT: Retention time.

Sample name: "2 005 PYCM201 PVMPL STD1 1 1" Sample ID: "5" File: "PYCM201_PVMPL_02.wiff"
Peak name: "PF-06380101" Mass(es): "743.500/188.100 Da"
Comment: "none" Annotation: ""

Sample index:	18
Sample type:	Standard
Concentration:	15.0
Calculated conc:	32.5
Acq. date:	27 May 2022
Acq. time:	4:52:54 PM
Modified:	No
Proc. algorithm:	specify parameters – MQ III
Noise percentage:	75
Base. sub. window:	1.00min
Peak-split. factor:	4
Report largest peak:	No
Min. peak height:	45.00 cps
Min. peak width:	0.00 sec
Smoothing width:	3 points
RT window:	10.0sec
Expected RT:	2.06 min
Use relative RT:	Yes
Int. type:	Base to base
Retention time:	2.08 min
Area:	1426 counts
Height:	449 cps
Start time:	2.01 min
End time:	2.18 min



Sample name: "2 005 PYCM201 PVMPL STD1 1 1" Sample ID: "5" File: "PYCM201_PVMPL_02.wiff"
Peak name: "PF-06691147(IS)" Mass(es): "751.600/188.100 Da"
Comment: "none" Annotation: ""

Sample index:	18
Sample type:	Standard
Concentration:	1.00
Calculated conc:	N/A
Acq. date:	27 May 2022
Acq. time:	4:52:54 PM
Modified:	No
Proc. algorithm:	specify parameters – MQ III
Noise percentage:	50
Base. sub. window:	0.25 min
Peak-split. factor:	2
Report largest peak:	Yes
Min. peak height:	250.00 cps
Min. peak width:	0.00 sec
Smoothing width:	3 points
RT window:	5.00 sec
Expected RT:	2.05 min
Use relative RT:	No
Int. type:	Base to base
Retention time:	2.06 min
Area:	48,989 counts
Height:	13,533 cps
Start time:	1.98 min
End time:	2.23 min

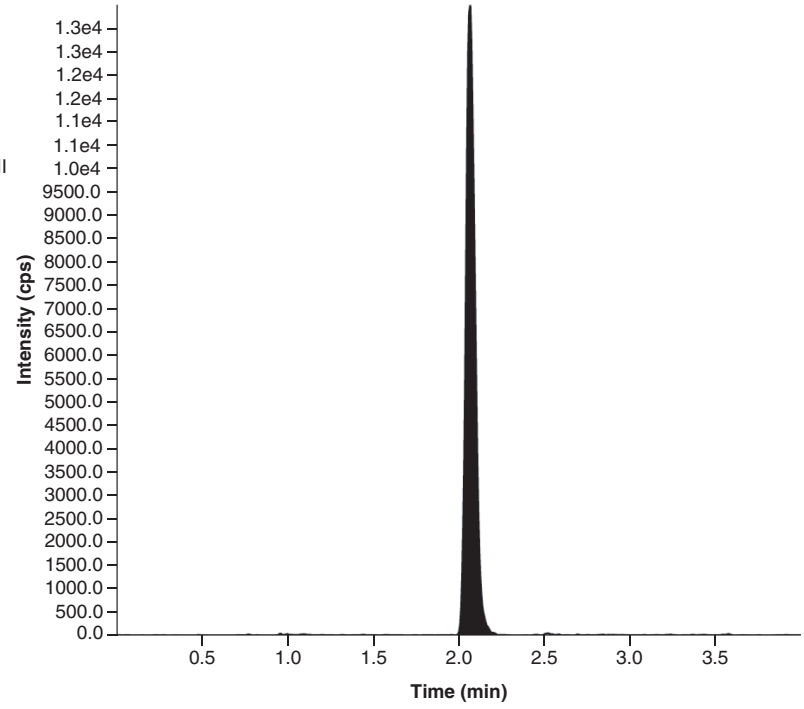


Figure 3. Chromatograms of Aur0101 (top) and internal standard Aur0101-d_8 (bottom) from a lower limit of quantitation sample in monkey K_2EDTA plasma.
RT: Retention time.

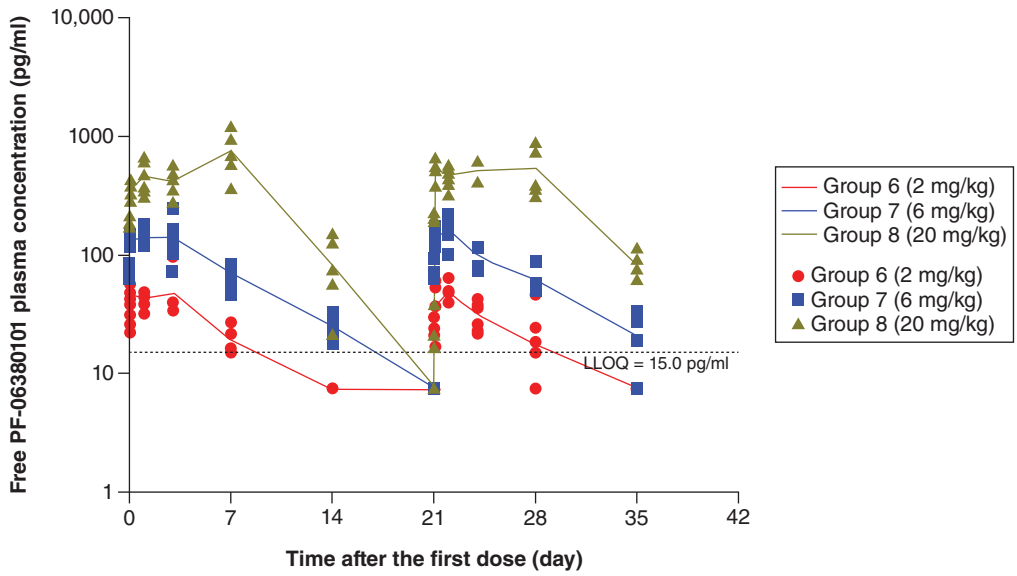


Figure 4. Mean and individual concentration–time profiles of free payload Aur0101 (PF-06380101) by dose group following bolus intravenous administration of PYX-201 in rats.
LLOQ: Lower limit of quantitation.

Table 2. Summary of toxicokinetic parameters for PYX-201 free payload Aur0101 by dose group in rat plasma.

Group (dose)	Dose day	T_{max} (h)	C_{max} (pg/ml)	AUC_{tlast} (h*pg/ml)	$t_{1/2}$† (h)
Group 6	1	72.0	47.5	6230	NC
(2 mg/kg)	22	24.0	48.6	5710	NC
Group 7	1	72.0	142	26,900	115
(6 mg/kg)	22	24.0	165	23,800	111
Group 8	1	168	760	146,000	77.3
(20 mg/kg)	22	168	538	126,000	96.0

All toxicokinetic parameters are rounded to three significant figures.
† $t_{1/2}$ was calculated from female rats only.
AUC: Area under the curve; AUC_{tlast}: AUC from Time 0 to last observed quantifiable concentration; C_{max}: Maximum observed concentration measured after dosing; NC: Not calculable; $t_{1/2}$: Terminal half-life; T_{max}: Time of maximum observed concentration after dosing.

Dilution integrity

Dilution integrity was proven in this assay by diluting a QC sample that had a concentration higher than the upper limit of quantitation level to extend the application of this assay. A rat or monkey plasma sample at a nominal concentration of 100,000 pg/ml was diluted tenfold in the same matrix and analyzed against the freshly prepared standard curve. The results are shown in Supplementary Tables 1 & 2. The intra-run %RE was -1.8%, with a %CV of 2.5% in the rat K_2EDTA plasma method. The intra-run %RE was -3.9%, with a %CV of 1.2% in the monkey K_2EDTA plasma method. The dilution QC data met the acceptance criteria based on the regulatory guidance requirement [34,35] and demonstrated that rat or monkey plasma samples with Aur0101 concentrations higher than the upper limit of quantitation of 15,000 pg/ml could be diluted tenfold in the same matrix and analyzed in this assay.

Assessment of stability

Bench-top stability, freeze/thaw stability, long-term stability and whole-blood stability were assessed in this assay, as recommended by regulatory guidance [34,35].

Aliquots of LQC and HQC samples from rat and monkey plasma were left in an ice bath for a short period of time and analyzed with a set of calibrators and freshly prepared QC samples. Aur0101 was stable for at least 24 h in an ice bath in rat or monkey K_2EDTA plasma.

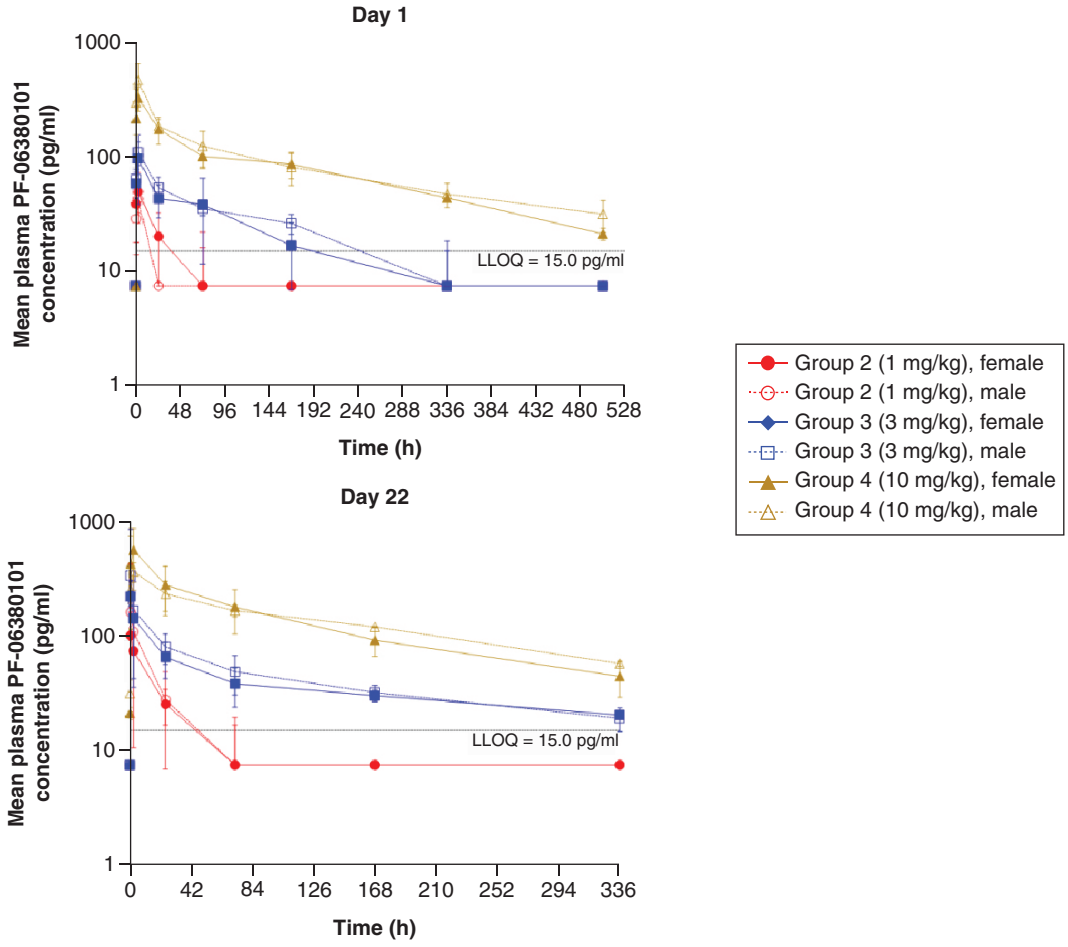


Figure 5. Mean (+ standard deviation) plasma concentration–time profiles of free payload Aur0101 (PF-06380101) after day 1 and day 22 dosing by dose group and sex in monkeys.
LLOQ: Lower limit of quantitation.

Table 3. Summary statistics of toxicokinetic parameters for PYX-201 free payload Aur0101 by dose group and sex in monkey plasma.

Group (dose)	Sex	Dose day	T_{max} (h)	C_{max} (pg/ml)	AUC_{tlast} (h*pg/ml)	$t_{1/2}$ (h)
Group 2	F	1	2.00	58.6	1350	NC
(1 mg/kg)	M	1	2.50	42.9	898	NC
	F	22	2.10	112	1570	NC
	M	22	2.10	172	2230	NC
Group 3	F	1	16.4	99.4	6520	NC
(3 mg/kg)	M	1	2.50	109	7760	NC
	F	22	2.10	244	8120	17.2
	M	22	1.30	359	9470	73.0
Group 4	F	1	2.50	325	36,800	171
(10 mg/kg)	M	1	2.50	463	41,300	205
	F	22	2.50	568	30,000	135
	M	22	2.50	369	28,000	164

All toxicokinetic parameters are rounded to three significant figures.
AUC: Area under the curve; AUC_{tlast}: AUC from Time 0 to last observed quantifiable concentration; C_{max}: Maximum observed concentration measured after dosing; F: Female; M: Male; NC: Not calculable; $t_{1/2}$: Terminal half-life; T_{max}: Time of maximum observed concentration after dosing.

Aliquots of LQC and HQC samples from rat and monkey plasma were frozen at -20 or -70°C and then thawed at room temperature to experience one cycle of freeze/thaw. These LQC and HQC samples were exposed to four more freeze/thaw cycles and then analyzed against a set of calibrator and freshly prepared QC samples. Aur0101 was stable after at least five freeze (-20 or -70°C)/thaw (room temperature) cycles in rat or monkey K_2EDTA plasma.

Aliquots of LQC and HQC samples from rat and monkey plasma were frozen at -20 or -70°C for a long period of time and analyzed with a set of calibrators and freshly prepared QC samples. Aur0101 was stable for at least 3 months in a -20 or -70°C freezer in rat or monkey K_2EDTA plasma.

Aliquots of QC samples at LQC and HQC levels from rat and monkey whole blood were left at room temperature or in an ice bath for 0 h (baseline) and 1 h, and then centrifuged to harvest plasma samples to be analyzed with a set of calibrators and freshly prepared plasma LQC and HQC samples. The calculated concentrations of 1-h whole-blood samples were compared to the baselines of the whole-blood samples. Aur0101 was stable for at least 1 h at room temperature or in an ice bath in rat or monkey K_2EDTA blood.

Assay application

This assay smoothly supported free Aur0101 bioanalysis in rat and monkey plasma samples in two GLP toxicology 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"). The TK noncompartmental analysis was performed on the concentration–time data from PYX-201 treated animals using Phoenix® WinNonlin® v 8.3.4. Mean and individual concentration–time profiles of free payload Aur0101 by dose group following bolus intravenous administration of PYX-201 in rats were illustrated in Figure 4, and TK parameters for PYX-201 free payload Aur0101 by dose group in rat plasma is summarized in Table 2. Mean (+ SD) plasma concentration–time profiles of free payload Aur0101 after day 1 and 22 dosing by dose group and sex in monkeys are depicted in Figure 5, and summary statistics of TK parameters for Aur0101 by dose group and sex in monkey plasma are presented in Table 3.

Conclusion

A sensitive and selective bioanalytical LC–MS/MS assay was validated to quantitate Aur0101 in rat and monkey plasma. The SIL-IS, Aur0101-d_8, was spiked in rat or monkey plasma and the analyte and IS extracted by protein precipitation followed by SPE, then analyzed in multiple reaction monitoring mode on an LC–MS/MS system. A linear calibration curve with $1/x^2$ weighted regression was applied in this assay validation. The intra-run %RE ranged from -10.0 to -0.9% with a %CV of $\leq$ 2.5% for all QC levels in rat K_2EDTA plasma. The intra-run %RE ranged from -7.3 to -3.8%, with %CV $\leq$ 3.8% for all QC levels in monkey K_2EDTA plasma. Aur0101 was capable of being diluted ten-fold in rat or monkey plasma and analyzed in this assay. Aur0101 was proven to be stable in rat and monkey plasma for at least 24 h in an ice bath, 3 months when stored at -20 or -70°C, and after five freeze/thaw cycles. Aur0101 was stable for up to 1 h in rat or monkey whole blood at room temperature or in an ice bath. Two preclinical GLP toxicology studies in rats and monkeys have been supported by this validated assay, and TK parameters have been calculated and analyzed accordingly.

Summary points

- An LC–MS/MS method was validated to measure Aur0101 concentrations in rat and monkey K_2EDTA plasma.
- Stable isotope labelled compound Aur0101-d_8 was used as the internal standard.
- Selectivity, accuracy, precision and dilution integrity were proven according to US FDA guidance for bioanalytical method validation.
- Aur0101 was stable under tested conditions (whole-blood stability, freeze/thaw stability, benchtop stability, long-term stability, etc.).
- Preclinical toxicokinetic (TK) sample analysis was successfully supported by this LC–MS/MS assay for investigational new drug approval.
- Mean and individual plasma concentration–time profiles and TK parameters of Aur0101 by dose group were generated in a rat good laboratory practice toxicology study.
- Mean plasma concentration–time profiles and TK parameters of Aur0101 by dose group and sex were generated in a monkey good laboratory practice toxicology study.
- A bioanalytical assay for Aur0101 concentration in human plasma is being developed and validated based on this 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-2023-0056

Acknowledgments

The authors greatly thank Dr R Criste from Amador Bioscience for his contribution to toxicokinetic analysis in support of this manuscript.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

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.

References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Wang AJ, Gao Y, Shi YY, Dai MY, Cai HB. A review of recent advances on single use of antibody-drug conjugates or combination with tumor immunology therapy for gynecologic cancer. *Front. Pharmacol.* 13, DOI: 10.3389/fphar.2022.1093666 (2022).
2. Abdollahpour-Alitappeh M, Lotfinia M, Gharibi T *et al.* Antibody–drug conjugates (ADCs) for cancer therapy: strategies, challenges, and successes. *J. Cell. Physiol.* 234(5), 5628–5642 (2019).
3. Beck A, Goetsch L, Dumontet C, Corvaia N. Strategies and challenges for the next generation of antibody–drug conjugates. *Nat. Rev. Drug Discov.* 16(5), 315–337 (2017).
4. Pandit A. Antibody–drug conjugates in oncology: key considerations and future trends (2022). Premier-research.com/blog-antibody-drug-conjugates
5. Axispharm. Antibody–drug conjugates (ADCs) list approved by FDA (2000–2022) (2022). Axispharm.com/antibody-drug-conjugatesadcs-list-approved-by-fda2000-2022
6. Pazo C, Nawaz K, Webster RM. The oncology market for antibody-drug conjugates. *Nat. Rev. Drug Discov.* 20(8), 583–584 (2021).
7. Ritchie M, Tchistiakova L, Scott N. Implications of receptor-mediated endocytosis and intracellular trafficking dynamics in the development of antibody drug conjugates. *mAbs* 5(1), 13–21 (2013).
8. Tarcsa E, Guffroy MR, Falahatpisheh H, Phipps C, Kalvass JC. Antibody–drug conjugates as targeted therapies: are we there yet? A critical review of the current clinical landscape. *Drug Discov. Today Technol.* 37, 13–22 (2020).
9. Jain N, Smith SW, Ghone S, Tomczuk B. Current ADC linker chemistry. *Pharm. Res.* 32(11), 3526–3540 (2015).
10. Chen H, Lin Z, Arnst KE, Miller DD, Li W. Tubulin inhibitor-based antibody–drug conjugates for cancer therapy. *Molecules* 22(8), 1281 (2017).
11. Sauer S, Erba PA, Petrini M *et al.* Expression of the oncofetal ED-B-containing fibronectin isoform in hematologic tumors enables ED-B-targeted ^{131}I-L19SIP radioimmunotherapy in Hodgkin lymphoma patients. *Blood* 113(10), 2265–2274 (2009).
12. Menrad A, Menssen HD. ED-B fibronectin as a target for antibody-based cancer treatments. *Expert Opin. Ther. Targets* 9(3), 491–500 (2005).
13. Khan ZA, Caurtero J, Barbin YP, Chan BM, Uniyal S, Chakrabarti S. ED-B fibronectin in non-small cell lung carcinoma. *Exp. Lung Res.* 31(7), 701–711 (2005).
14. Balza E, Sassi F, Ventura E *et al.* A novel human fibronectin cryptic sequence unmasked by the insertion of the angiogenesis-associated extra type III domain B. *Int. J. Cancer* 125(4), 751–758 (2009).
15. Saw PE, Xu X, Kang BP *et al.* Extra-domain B of fibronectin as an alternative target for drug delivery and a cancer diagnostic and prognostic biomarker for malignant glioma. *Theranostics* 11(2), 941–957 (2021).
16. Qiao PL, Gargesha M, Liu Y *et al.* Magnetic resonance molecular imaging of extradomain B fibronectin enables detection of pancreatic ductal adenocarcinoma metastasis. *Magn. Reson. Imaging* 86, 37–45 (2022).
17. Graziani E, Sung M, Ma D *et al.* 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), 2068–2078 (2020).

18. Gebleux R, Stringhini M, Casanova R, Soltermann A, Neri D. Non-internalizing antibody–drug conjugates display potent anti-cancer activity upon proteolytic release of monomethyl auristatin E in the subendothelial extracellular matrix. *Int. J. Cancer* 140(7), 1670–1679 (2017).
19. Hooper AT, Marquette K, Chang C-PB *et al.* Anti-extra domain B splice variant of fibronectin antibody–drug conjugate eliminates tumors with enhanced efficacy when combined with checkpoint blockade. *Mol. Cancer Ther.* 21(9), 1462–1472 (2022).
20. Damelin M, Bankovich A, Bernstein J *et al.* A PTK7-targeted antibody–drug conjugate reduces tumor-initiating cells and induces sustained tumor regressions. *Sci. Transl. Med.* 9(372), DOI: 10.1126/scitranslmed.aag2611 (2017).
21. Maderna A, Doroski M, Subramanyam C *et al.* Discovery of cytotoxic dolastatin 10 analogues with N-terminal modifications. *J. Med. Chem.* 57(24), 10527–10543 (2014).
22. Rosen LS, Wesolowski R, Baffa R *et al.* A phase I, dose–escalation study of PF-06650808, an anti-Notch3 antibody–drug conjugate, in patients with breast cancer and other advanced solid tumors. *Invest. New Drug.* 38(1), 120–130 (2020).
23. Meric-Bernstam F, Calvo E, Moreno V *et al.* A phase I dose escalation study evaluating the safety and tolerability of a novel anti-HER2 antibody-drug conjugate (PF-06804103) in patients with HER2-positive solid tumors. *J. Clin. Oncol.* 38(Suppl. 15), 1039 (2020).
24. Maitland ML, Sachdev JC, Sharma MR *et al.* First-in-human study of PF-06647020 (cofetuzumab pelidotin), an antibody–drug conjugate targeting protein tyrosine kinase 7, in advanced solid tumors. *Clin. Cancer Res.* 27(16), 4511–4520 (2021).
25. King GT, Eaton KD, Beagle BR *et al.* A phase 1, dose–escalation study of PF-06664178, an anti-Trop 2/Aur0101 antibody–drug conjugate in patients with advanced or metastatic solid tumors. *Invest. New Drug.* 36(5), 836–847 (2018).
26. Dong L, Li C, Locuson C, Chen S, Gian MG. A two-step immunocapture LC/MS/MS assay for plasma stability and payload migration assessment of cysteine-maleimide-based antibody drug conjugates. *Anal. Chem.* 90(10), 5989–5994 (2018).
27. Park MH, Lee BI, Byeon JJ *et al.* Pharmacokinetic and metabolism studies of monomethyl auristatin F via liquid chromatography-quadrupole-time-of-flight mass spectrometry. *Molecules* 24(15), 2754 (2019).
28. Yin F, Keller J, Kraus D, Mangus H, Li F, Liu G. 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, DOI: 10.1016/j.jpba.2019.112916 (2020).
29. Yin F, Ling Y, Keller J *et al.* Quantitation of 2-hydroxyglutarate in human plasma via LC–MS/MS using a surrogate analyte approach. *Bioanalysis* 12(16), 1149–1159 (2020).
30. Lee BI, Park MH, Choi J *et al.* Liquid chromatography-high resolution mass spectrometric method for the quantitation of monomethyl auristatin E (MMAE) and its preclinical pharmacokinetics. *Biomed. Chromatogr.* 34(8), e4855 (2020).
31. Yin F, Ling Y, Martin J *et al.* Quantitation of uridine and L-dihydroorotic acid in human plasma by LC–MS/MS using a surrogate matrix approach. *J. Pharm. Biomed. Anal.* 192, DOI: 10.1016/j.jpba.2020.113669 (2021).
32. Yin F, Yu S, Narayanaswamy R, Mangus H, McCourt E, Liu G. Quantitation of ivosidenib in human plasma via LC–MS/MS and its application in clinical trials. *Bioanalysis* 13(11), 875–889 (2021).
33. Yin F, DeCiantis C, Pinkas J *et al.* Quantitation of an ADC drug PYX-201 in rat and monkey plasma via ELISA and its application in preclinical studies. *Bioanalysis* 15(1), 43–52 (2023).

• **First publication of PYX-201 bioanalytical method validation in any matrix.**

34. US FDA. Guidance for industry, bioanalytical method validation (2018). https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf

•• **Guidance from the US FDA for bioanalytical method validation.**

35. USDepartment of Health and Human Services FDA, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER). Guidance for Industry, M10 bioanalytical method validation and study sample analysis (2022). https://www.fda.gov/media/162903/download

•• **Guidance from the FDA for bioanalytical method validation and sample analysis.**