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Bioanalysis of an antibody drug conjugate (ADC) PYX-201 in human plasma using a hybrid immunoaffinity LC–MS/MS approach

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A B S T R A C T

PYX-201 is an anti-extra domain B splice variant of fibronectin (EDB + FN) antibody drug conjugate (ADC) composed of a fully human IgG1 antibody, a cleavable mcValCitPABC linker, and four Auristatin 0101 (Aur0101, PF-06380101) payload molecules. To better understand the pharmacokinetic (PK) profile of PYX-201 after it is administered to cancer patients, the development of a reliable bioanalytical assay to accurately and precisely quantitate PYX-201 in human plasma is required. In this manuscript, we present a hybrid immunoaffinity LC-MS/MS assay used to successfully analyze PYX-201 in human plasma. PYX-201 was enriched by MABSelect beads coated with protein A in human plasma samples. The bound proteins were subjected to "on-bead" proteolysis with papain to release the payload Aur0101. The stable isotope labelled internal standard (SIL-IS) Aur0101-d_8 was added and the released Aur0101 was quantified as a surrogate for the total ADC concentration. The separation was performed on a UPLC C18 column coupled with tandem mass spectrometry. The LC-MS/MS assay was validated over the range 0.0250 to 25.0 μg/mL with excellent accuracy and precision. The overall accuracy (%RE) was between −3.8% and −0.1% and the inter-assay precision (%CV) was <5.8%. PYX-201 was found to be stable in human plasma for at least 24 h on ice, 15 days after being stored at –80 °C, as well as after five freeze/thaw cycles of being frozen at −25 °C or –80 °C and thawed on ice. The assay this paper reports on, has been successfully applied to human sample analysis to support clinical studies.

1. Introduction

Antibody-drug conjugate (ADC) research has attracted more and more attention from the pharmaceutical industry in recent years, and particularly since the first US Food and Drug Administration (FDA) approved ADC drug Mylotarg in 2000 [1–3]. ADCs are composed of an antigen-specific monoclonal antibody, a uniquely designed linker, and a cytotoxic payload. A carefully designed ADC makes the payload more efficiently delivered to the tumor target [4], resulting in improved efficacy, less toxicity or both. As of the time of this paper, more than a dozen ADC drugs have been approved globally, all in oncology and more than half of them in hematological malignancies [5–7]. The success of ADC drugs is greatly attributed to the distinct linker modification to balance the toxicity of the payload and the overall efficacy of the ADC drug [4,8–9].

PYX-201 (Fig. 1**A**) is an investigational ADC drug with a fully human IgG1 antibody, a cleavable mcValCitPABC linker, and four Auristatin 0101 (Aur0101) (Fig. 1**B**) payloads (Drug Antibody Ratio (DAR) = 4). This ADC targets the extra domain B splice variant of fibronectin (EDB + FN) [10]. EDB + FN is a promising oncology target due to the nature of it selectively accumulating in stroma around new blood vessels in tumors while being low in normal adult vasculature [11]. PYX-201 binds to the target, and its highly cell-permeable payload Aur0101 is enzymatically released to bind to microtubules and inhibit tumor cell proliferation. A fraction of PYX-201 may also be internalized by the cancer cells and the payload Aur0101 is released into cancer cell cytosol by lysosome proteolysis. After the release of the toxic payload, the original ADC drug turns into a complex mixture of ADC, total antibody, and free payload. PYX-201 is under investigation in a first-in-human (FIH) phase I clinical trial to evaluate the safety, tolerability, pharmacokinetics, pharmacodynamics (PDs), and preliminary efficacy in patients with advanced solid tumors, 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, thyroid cancer, pancreatic ductal adenocarcinoma (PDAC), soft tissue sarcoma (STS), hepatocellular carcinoma (HCC), and kidney cancer (phase I study: NCT05720117, https://www.clinicaltrials.ggov, EudraCT Number: 2022–002284-30). PK parameters are one of the secondary endpoints. Hence, the development and validation of a reliable bioanalytical assay to accurately and precisely analyze PYX-201 in

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human plasma is essential to support the ongoing clinical study and further clinical development.

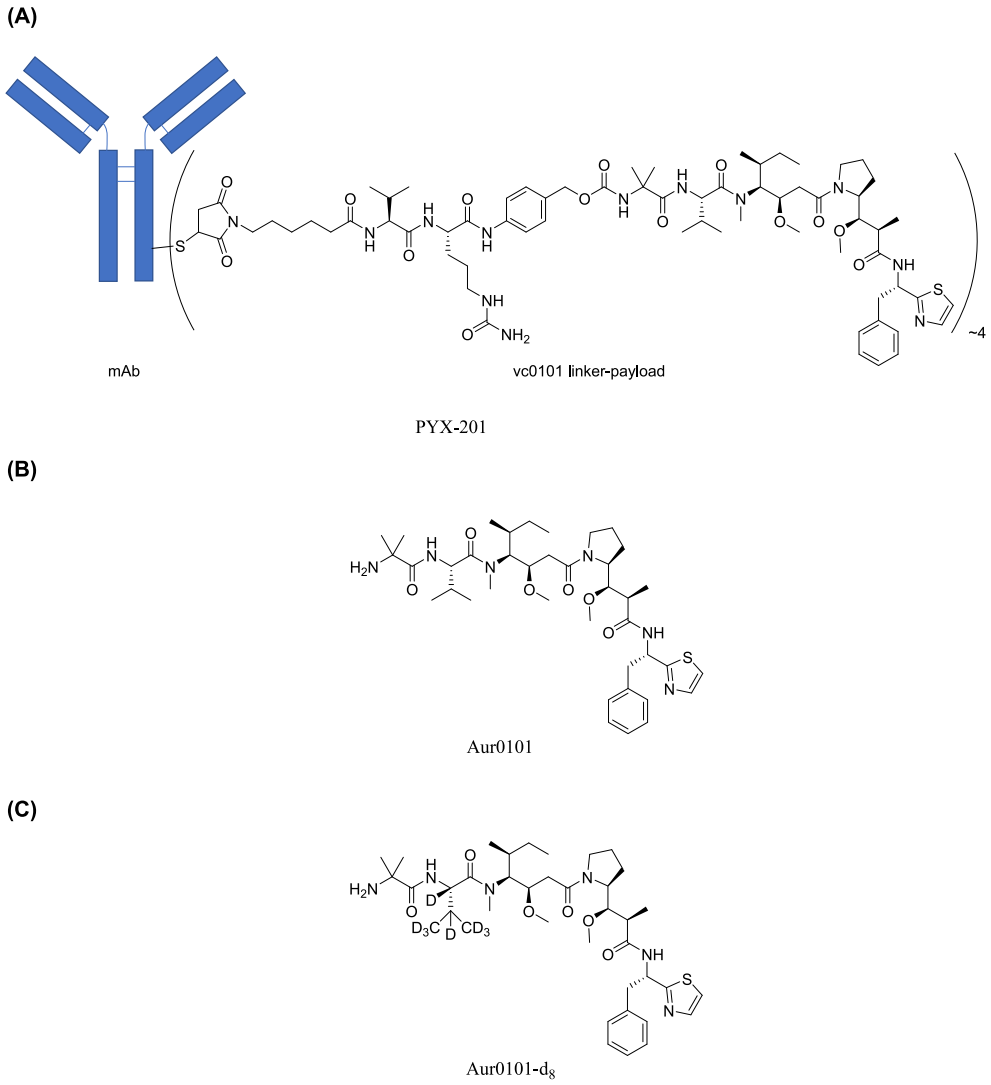
The complexity of these ADC systems necessitates the use of several pharmacokinetic (PK) studies to characterize the distribution of ADC components, namely total antibody (bound and unbound antibody), total conjugated ADC, and unconjugated payloads. Enzyme-linked immunosorbent assays (ELISAs) have traditionally served as the gold standard PK assay for total antibody and total ADC bioanalysis [12–18]. However, despite the improved stability of current generation of ADCs, in vivo biotransformation and degradation (e.g., hydrolysis) of ADCs can lead to substantial heterogeneity in the drug-to-antibody ratio (DAR) and DAR distribution. As such, many drug development teams have turned to liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) for DAR sensitive PK bioanalysis of ADCs. Within the oncology field, LC-MS/MS is a well-documented, robust tool for bioanalysis of small-molecule therapeutics [19–22]. Moreover, the specificity, selectivity, and sensitivity of LC–MS/MS platforms make them an appealing alternative for bioanalysis of mixed-molecule biologics, including ADCs [16–18, 23–30]. In general, the biologics samples are digested, and then a signature portion of the antibody or ADC is chosen to be analyzed on LC–MS/MS.

Recently, we reported the use of a bioanalytical ELISA assay to quantitate PYX-201 in rat and monkey plasma [31]. For the first time, our lab developed and validated a robust LC-MS/MS assay to quantitate PYX-201 in human plasma. This assay was fully validated under regulatory guidance [32–33] and has been successfully applied to human plasma sample analysis to support the clinical trial.

2. Experimental

2.1. Chemicals and reagents

PYX-201 was produced by WuXi Biologics (Shanghai, China). Stable isotope labelled internal standard Aur0101-d_8 (Fig. 1**C**) was manufactured at MedChemExpress (Monmouth, NJ, USA). Ammonium acetate, bovine serum albumin (BSA), L-cysteine, formic acid, HPLC grade acetonitrile, HPLC grade methanol, HPLC grade N,N-dimethylformamide (DMF), and phosphate buffered saline (PBS) were produced by Sigma-Aldrich (St. Louis, MO, USA). Water was purified in-house on Millipore Milli-Q IQ 7000 ultrapure lab waters system (Burlington, MA, USA). MabSelect beads were acquired from GE Healthcare (Chicago, IL, USA). Lyophilized papain from papaya latex (>15 units/mg protein) was obtained from Worthington Biochemical (Lakewood, NJ, USA). Dipotassium EDTA human plasma was purchased from BioIVT



mAb=monoclonal antibody; vc0101 (pelidotin) =valine-citrulline linker to Aur0101 payload.

Fig. 1. Structures of (A) PYX-201 drug substance, (B) PYX-201 payload Aur0101, and (C) internal standard Aur0101-d_8.

(Westbury, NY, USA).

2.2. Equipment and apparatus

This LC-MS/MS assay was performed on a Sciex API 6500 triple quadrupole mass spectrometer (Sciex, Framingham, MA, USA) coupled with Agilent 1260 binary pumps (Agilent technologies, Santa Clara, CA, USA) and CTC analytics PAL DLW autosampler (Leap technologies, Carrboro, NC, USA). Chromatographic separation was conducted on an Acquity UPLC BEH C18 130 A, 2.1 mm × 50 mm, 1.7 μm column (Waters, Milford, MA, USA).

2.3. Preparation of calibration standards and quality control (QC) samples

Stock solution of PYX-201 was provided at 14.8 mg/mL in 20 mM histidine with 6% (w/v) sucrose and 0.02% (w/v) polysorbate 80 (PS80) at pH 5.5. Calibration standards and QC samples were prepared by spiking PYX–201 stock solution into human K_2EDTA plasma. PYX-201 calibration standards were analyzed in duplicate over the nominal ADC concentration range of 0.0250 to 25.0 μg/mL. Calibration standards were made on ice in human K_2EDTA plasma at 0.0250, 0.0500, 0.100, 0.400, 1.50, 6.00, 20.0, and 25.0 μg/mL. All calibration standard curves were freshly prepared for each run in this assay validation. QC samples were prepared on ice in human K_2EDTA plasma at four concentrations: 0.0250 (LLOQ), 0.0750 (LQC), 10.0 (MQC), and 19.0 μg/mL (HQC). QC samples for one precision and accuracy run, and all matrix stability runs were freshly prepared. QC samples in the other runs were stored frozen until use. Accuracy and precision were evaluated on the above four QCs in human K_2EDTA plasma in six replicates.

2.4. Assay procedure

Human plasma samples were thawed on ice. MabSelect bead slurry in PBS (400 μL) was filtered in a 96-well filter plate and mixed well with 100 μL of 1% BSA in PBS and 25 μL of the thawed human plasma sample. The sample mixture was incubated for approximately 1 h with shaking at room temperature, filtered by centrifugation, then washed three times with 200 μL of 20 mM ammonium acetate. 200 μL of 2 mg/mL papain in 20 mM ammonium acetate and 2 mM L-cysteine solution was mixed and incubated at 37 °C for 15 min before adding into the sample. The sample was incubated at 25 °C for 1 h, then 25 μL of Aur0101-d_8 at 10 ng/mL was added in the sample mixture and mixed well. The sample mixture was filtered by centrifugation. 100 μL of ACN was added to each well of the filter plate and the sample mixture was filtered by centrifugation again. The eluants were combined with 500 μL of ACN. 200 μL of the supernatant was evaporated to dryness under nitrogen (N_2) and reconstituted in 120 μL of 35/65/0.1 ACN/H_2O/formic acid for LC-MS/MS analysis. Detailed LC-MS/MS conditions were summarized in Table 1.

2.5. Data acquisition and processing

ASSIST LIMS (Version 7, PPD Laboratories Services, Richmond, VA, USA), Analyst software (Version 1.6.3, Sciex, Framingham, MA, USA), and MultiQuant (Version 3.0.3, Sciex, Framingham, MA, USA) were used for data acquisition and processing. The calibration curves were evaluated employing a linear regression with $1/x^2$ weighting.

3. Results and discussion

3.1. Method development

In our previously published work, we validated an ELISA assay for ADC drug PYX-201 in rat and monkey plasma [31]. However, in our experience, most ELISAs lack the requisite sensitivity to reliably track and quantify time-dependent changes in DAR and DAR distribution. For example, in most ELISA-based systems samples with a preponderance of DAR 8 or DAR 1 ADCs might yield identical total ADC values while LC-MS/MS platform has the capability of differentiating ADC drugs with different DAR values because the payload is selected as the surrogate analyte. Accordingly, the objective of the work presented here describes the validation of a more robust and reliable LC-MS/MS assay for PYX-201 quantitation in human plasma. This validated assay is supporting the ongoing clinical trial. PYX-201 has a molecular weight of 152,213 Dalton, and is a too large molecule for practical direct quantitative analysis using LC-MS/MS technology. Commonly used LC-MS/MS based approaches for large molecule, ADC drug bioanalysis use enzymes to permit direct quantitation of the released payload or the peptide-linker-payload complex. For example, ADC drugs harboring relatively stable linkers undergo tryptic digestion to produce appropriately sized peptide-linker-payload complexes. Conversely, ADC drugs containing unstable, cleavable linkers (e.g., PYX-201) are subjected to papain or cathepsin B, and releases the cytotoxic payload [34–36]. In this assay validation study, we employed "on-bead" proteolysis with papain. Hydrolysis conditions optimization, including the amount of papain, proteolysis temperature and duration, etc. was the biggest challenge in the method development. Hydrolysis conditions were optimized in preliminary developmental studies to find the best analyte recovery and the lowest mass (MS) baselines.

High-performance liquid chromatography (HPLC) and mass spectrometry (MS) conditions were optimized using an Aur0101 solution in solvent, i.e., without mobile phases A and B. Optimized MS conditions are summarized in Table 1. A Waters Acquity UPLC BEH C18 column was used for chromatographic separation: mobile phases A and B consisted of 100:2:0.1 water:1M ammonium acetate:formic acid and methanol, respectively. Mobile phase gradient scheme, column temperature, and autosampler washes were key variables in ameliorating carryover issue and improving chromatographic peak morphology (Table 1). A relatively rapid HPLC gradient and high column

Table 1
LC-MS/MS assay settings.

Chromatography Settings							
Analytical column	Acquity UPLC BEH C18, 130 A, 2.1 mm × 50 mm, 1.7 μm, Waters						
Column temperature	65 °C						
Mobile phase A	100:2:0.1 Water:1M ammonium acetate:formic acid						
Mobile phase B	Methanol						
Autosampler wash 1	0.1% Formic acid in ACN						
Autosampler wash 2	0.1% Formic acid in 20/80 ACN/H_2O						
Program	Gradient						
Time (min)	0	0.5	1.5	1.51	3.5	3.6	5
%B	35	35	85	96	96	35	Stop
Flow rate	0.5 mL/min						
Auto-injector temperature	4℃						
Flow rate	0.5 mL/min						
Injection volume	10 μL						
Retention time	~2.1 min						
Mass Spectrometer Settings							
Mass Spectrometer	Sciex 6500, triple quadrupole LC-MS/MS						
Ionization Mode	ESI+, MRM						
MRM Mass Transitions	743.3 > 559.2 for Aur0101 as the surrogate analyte for ADC						
	751.3 > 205.2 for Aur0101-d_8						
Source Temperature (TEM)	500 ℃						
Collision Gas (CAD)	9 psig N_2						
Curtain Gas (CUR)	25 psig N_2						
Ion Source Gas 1 (GS1)	70 psig N_2						
Ion Source Gas 2 (GS2)	70 psig N_2						
Ion Spray Voltage (IS)	5500 V						
Entrance Potential (EP)	8 V						
Declustering Potential (DP)	110 V						
Collison Energy (CE)	40 V						
Cell Exit Potential (CXP)	17 V						

temperature at 65 °C were applied to achieve a sharp MS peak. Two different autosampler washes (0.1% formic acid in ACN and 0.1% formic acid in 20/80 ACN/H_2O) were employed to remove the carryover issue.

3.2. Assay selectivity and specificity

To ensure assay performance across a range for potential matrix-related backgrounds, six individual lots of blank human plasma were extracted and analyzed in singlet for PYX–201 total ADC and internal standard. The measured response ratio (interfering background peak response / internal standard peak response) in all six matrix lots was < 25% of the mean response ratio determined from the corresponding analyte in the acceptable LLOQ calibrator samples for each run. Across all six lots of blank human matrix, interfering background chromatographic peak responses at the expected retention time of an internal standard were < 5% of the mean chromatographic response determined for the internal standard in the specificity samples fortified with internal standard. Simply stated, there were no significant interfering chromatographic peaks detected at the mass transitions and expected retention times of the analyte or internal standard that would interfere with quantitation.

To evaluate potential matrix suppression or enhancement effects, additional fortified samples were prepared from the same matrix lots (n = 6). These samples were fortified with PYX-201 at the LLOQ level (0.0250 μg/mL) and analyzed in triplicate. Across all six lots of human plasma, fortified selectivity and specificity samples met the acceptance criteria, i.e., at least two-thirds of the replicates for a lot quantitated within ± 25.0% of the theoretical value for at least five out of six fortified sample lots (Table 2).

3.3. Linearity and analytical range

For each run, calibration standards were analyzed in duplicate over the nominal PYX-201 concentration range of 0.0250 to 25.0 μg/mL. A linear, 1/concentration^2 weighted, least-squares regression algorithm was used to plot the peak area ratio (i.e., analyte with respect to its internal standard) versus concentration. A representative chromatogram from a matrix blank spiked with the internal standard Aur0101-d_8 in human K_2EDTA plasma is presented in Fig. 2. Likewise, a representative chromatogram from an LLOQ sample was depicted in Fig. 3. The high signal to ratio (S/N) of the LLOQ chromatogram demonstrated adequate sensitivity to measure PYX-201 at 0.0250 μg/mL in human plasma. Seven runs were included in this assay validation with all regression equations and correlation coefficients (Rs) summarized in Table 3.

3.4. Accuracy and precision (A & P)

Accuracy and precision were evaluated by analyzing quality control (QC) pools at 0.0250 (LLOQ), 0.0750 (LQC), 10.0 (MQC), and 19.0 μg/mL (HQC) in six replicates. Accuracy was measured as the percent relative error (%RE) from theoretical, and precision was expressed as the percent coefficient of variation (%CV) of each QC pool. For all QC levels in human K_2EDTA plasma, the intra-run %RE ranged from −6.0% to 3.0%, with %CV between 1.3% and 5.3% (Table 3). Inter-run %RE was from −3.8% to −0.1%, with %CV between 2.1% and 5.8% (Table 4). The intra–run and inter-run accuracy and precision met regulatory guidance acceptance requirements [32–33].

3.5. Dilution linearity

The ability to dilute samples originally above the upper limit of the calibration range was validated by analyzing six replicate QCs, containing 100 μg/mL of PYX-201, as 10-fold dilutions. The ability to analyze samples with insufficient volume for a full aliquot was validated by analyzing six replicate QCs, containing 10.0 μg/mL of PYX-201, as 2-fold dilutions. Dilution QC results met the published regulatory guidance acceptance criteria (Table 5) [32–33]. Taken together, these findings indicated that human plasma samples with PYX-201 concentrations higher than ULOQ 25.0 μg/mL can be diluted 10 folds, and that human plasma samples with insufficient volumes can be diluted 2 folds in the same matrix and analyzed in this assay.

3.6. Stability assessment

As required by the regulatory guidance, bench-top stability, freeze/thaw stability, extract stability, long-term stability, and whole blood stability were assessed [32–33].

Bench-top stability was evaluated by allowing a set of frozen LQC and HQC samples to thaw and remain on ice for 24 h prior to extraction and analysis. Freeze/thaw stability was evaluated by subjecting two sets of LQC and HQC samples to five successive freeze/thaw cycles. Freeze/thaw stability samples were thawed on ice. For each freeze cycle, one set was frozen at –25 °C, and the other set was frozen at –80 °C. Post-preparative extract stability QC samples were extracted, injected, then stored at 4 °C for approximately 225 h prior to reanalysis with freshly prepared calibrators. Long-term stability QC samples were stored at –80 °C for 15 days. Stability acceptance criteria for these stability studies required that %CV at each QC level be ≤ 20.0% and that %RE at each QC level be within ± 20.0% from the nominal concentration. All LQC and HQC samples met the acceptance criteria.

The stability of PYX-201 in human whole blood samples was tested at approximate low and high concentrations. Prior to processing to plasma in either a 4 °C or room temperature centrifuge, the whole blood stability samples were held for two hours at room temperature or in an ice bath. A control sample was prepared by processing a whole blood sample immediately: centrifuged at 4 °C. All whole blood LQC and HQC samples met the acceptance criteria, i.e., %CV at each QC level and the %difference from the control QC samples were ≤ 20.0%.

3.7. Hemolysis and lipemia effects

The effect of hemolysis on PYX-201 quantitation was evaluated in blanks, with and without internal standard, and at the LQC and HQC levels. These samples were prepared in human plasma fortified with

Table 2
Fortified selectivity/specificity evaluation for PYX-201 in human K_2EDTA plasma.

	Lot 1 (μg/mL)	Lot 2 (μg/mL)	Lot 3 (μg/mL)	Lot 4 (μg/mL)	Lot 5 (μg/mL)	Lot 6 (μg/mL)
	0.0285	0.0262	0.0221	0.0253	0.0273	0.0257
	0.0234	0.0267	0.0261	0.0239	0.0264	0.0288
	0.0271	0.0258	0.0245	0.0261	0.0263	0.0251
N	3	3	3	3	3	3
Nominal concentration (μg/mL)	0.0250	0.0250	0.0250	0.0250	0.0250	0.0250
Low limit (μg/mL)	0.0188	0.0188	0.0188	0.0188	0.0188	0.0188
High limit (μg/mL)	0.0313	0.0313	0.0313	0.0313	0.0313	0.0313

Analyst Version: 1.6.3 MultiQuant Version: 3.0.3 File: 4RWWO2-A_SX109.wiff Printing Date: 7/19/2022 4:03 PM
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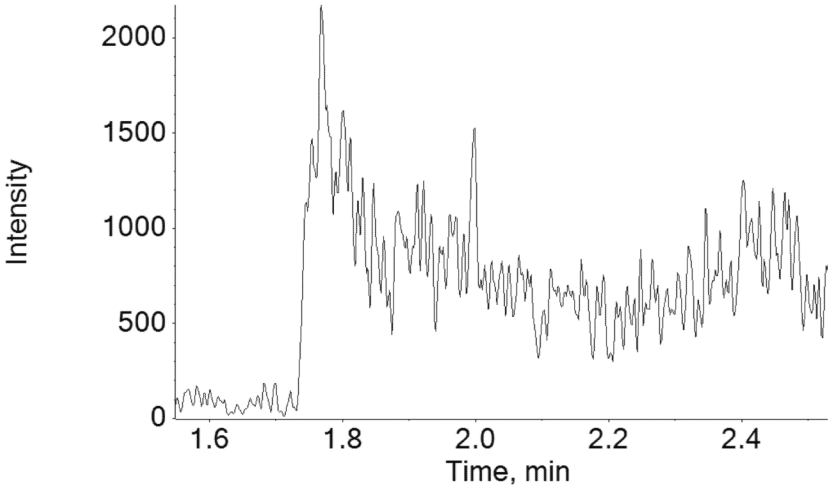
Sample Name: **MB/IS 1-1** **Sample ID**: **4RWWO2_002**
Acq. Date & Time: 7/13/2022 12:16:16 AM Modified: False

Peak Name: Total ADC from PYX-201

Mass(es): 743.3 / 559.2
Proc. Algorithm: MQ4
Gaussian Smooth Width: 1.0 points
Expected RT: 2.05 min
RT Half Window: 10.0 sec
Update Expected RT: DontUpdate
Report Largest Peak: Yes
Min. Peak Width: 3 points
Min. Peak Height: 1200.00
Noise Percentage: 95.0%
Baseline Sub. Window: 2.00 min
Peak Splitting Factor: 4 points
RT Window: 0.166666666666667
Retention Time: N/A
Int. Type: N/A
Area: N/A
Height: N/A
Start Time: N/A
End Time: N/A



Peak Name: PF-06380101-d8

Mass(es): 751.3 / 205.2
Proc. Algorithm: MQ4
Gaussian Smooth Width: 1.0 points
Expected RT: 2.04 min
RT Half Window: 5.0 sec
Update Expected RT: DontUpdate
Report Largest Peak: Yes
Min. Peak Width: 3 points
Min. Peak Height: 5000.00
Noise Percentage: 99.0%
Baseline Sub. Window: 2.00 min
Peak Splitting Factor: 2 points
RT Window: 0.0833333333333333
Retention Time: 2.05
Int. Type: Baseline
Area: 1879471.72
Height: 585584.81
Start Time: 2.01
End Time: 2.18

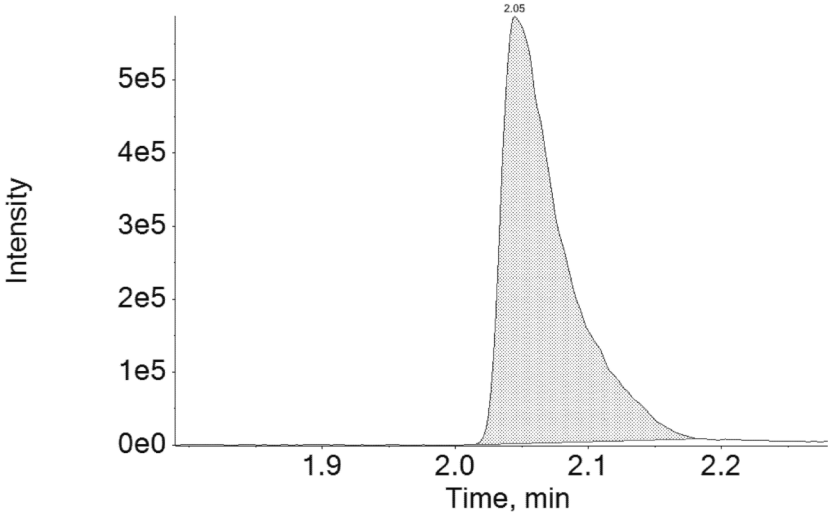


Fig. 2. Chromatograms of Aur0101 (top) as the surrogate analyte for PYX-201 and internal standard Aur0101–d_8 (bottom) from a blank human K_2EDTA plasma sample containing internal standard.

hemolyzed human whole blood such that the final matrix represented 5% hemolysis. The effect of lipemia on PYX-201 quantitation was evaluated in blanks, with and without internal standard, and at the LQC and HQC levels. These samples were prepared in lipemic human plasma with a triglyceride concentration > 300 mg/dL. There was no hemolysis effect or lipemia effect on PYX–201 quantitation. That is, there were no significant, interfering chromatographic peaks detected at the mass transitions and expected retention times of the analyte or internal standard.

3.8. Capture capacity evaluation

Over-the-curve dilution QC samples were analyzed without dilution. The measured concentration for all replicates was >20% of the ULOQ concentration, demonstrating adequate capture capacity for samples that are above the upper limit of quantitation.

Analyst Version: 1.6.3 MultiQuant Version: 3.0.3 File: 4RWWO2-A_SX109.wiff Printing Date: 7/19/2022 4:03 PM
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Sample Name: **CAL 1-1** **Sample ID**: **4RWWO2_003**
Acq. Date & Time: 7/13/2022 12:22:03 AM Modified: False

Peak Name: Total ADC from PYX-201

Mass(es): 743.3 / 559.2
Proc. Algorithm: MQ4
Gaussian Smooth Width: 1.0 points
Expected RT: 2.05 min
RT Half Window: 10.0 sec
Update Expected RT: DontUpdate
Report Largest Peak: Yes
Min. Peak Width: 3 points
Min. Peak Height: 1200.00
Noise Percentage: 95.0%
Baseline Sub. Window: 2.00 min
Peak Splitting Factor: 4 points
RT Window: 0.166666666666667
Retention Time: 2.05
Int. Type: Valley
Area: 36298.46
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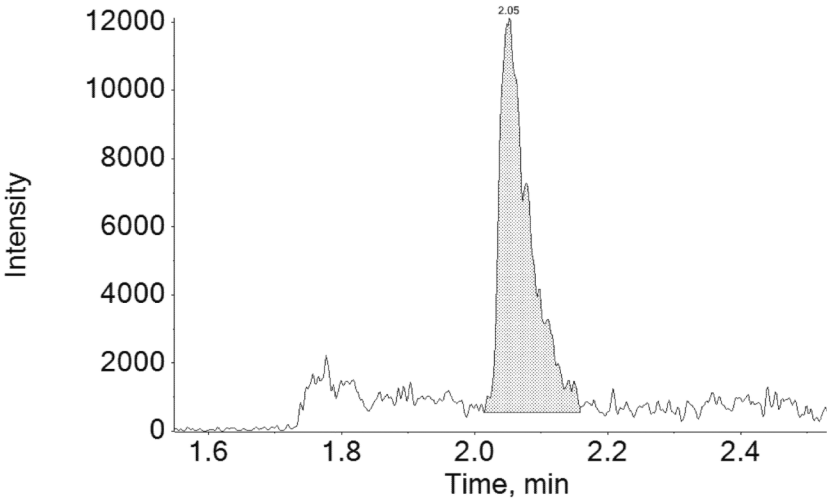
Start Time: 2.01
End Time: 2.16



Peak Name: PF-06380101-d8

Mass(es): 751.3 / 205.2
Proc. Algorithm: MQ4
Gaussian Smooth Width: 1.0 points
Expected RT: 2.04 min
RT Half Window: 5.0 sec
Update Expected RT: DontUpdate
Report Largest Peak: Yes
Min. Peak Width: 3 points
Min. Peak Height: 5000.00
Noise Percentage: 99.0%
Baseline Sub. Window: 2.00 min
Peak Splitting Factor: 2 points
RT Window: 0.0833333333333333
Retention Time: 2.05
Int. Type: Baseline
Area: 1914303.71
Height: 610938.04
Start Time: 2.01
End Time: 2.18

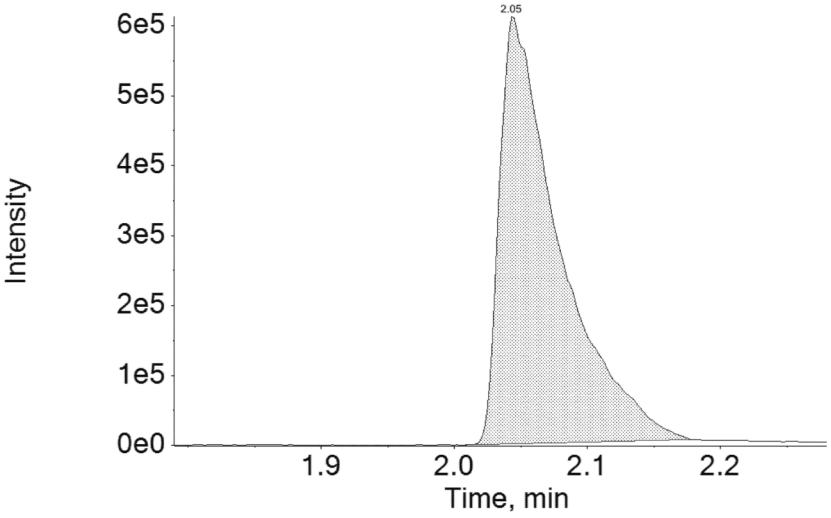


Fig. 3. Chromatograms of Aur0101 (top) as the surrogate analyte for PYX-201 and internal standard Aur0101–d_8 (bottom) from an LLOQ sample in human K_2EDTA plasma.

3.9. Recovery

The apparent recovery associated with the affinity capture was evaluated at LQC, MQC, and HQC levels. A set of unfortified blank human plasma samples were processed through the affinity capture assay steps. Immediately prior to the digestion step, the recovery samples were fortified with the same concentrations of PYX-201 and internal standard (Aur0101-d_8). Samples were then processed as usual. The peak area of the analyte and internal standard from normal LQC, MQC, and HQC samples were compared to these recovery QCs at the corresponding levels. Mean recovery of PYX-201 was 102% and mean internal standard recovery was 103%.

3.10. Matrix effect

This total ADC assay prescribes an enzymatic digestion step. In turn,

Table 3
Regression equations and correlation coefficients of PYX-201 assay validation in human K_2EDTA plasma.

Run number	Regression equation	Correlation coefficient (R)
1	Y = 1.110907E-03 + 7.194752E-01 * X	0.9996
2	Y = 2.824244E-04 + 7.301821E-01 * X	0.9993
3	Y = -7.919913E-05 + 7.138628E-01 * X	0.9979
4	Y = 4.719534E-04 + 7.345952E-01 * X	0.9978
5	Y = 7.773697E-04 + 7.084603E-01 * X	0.9986
6	Y = 1.262827E-03 + 6.765855E-01 * X	0.9992
7	Y = 1.271677E-03 + 6.659165E-01 * X	0.9994

Regression method: linear regression, Y = mX + b, weighted ($1/concentration^2$).

this digestion step precluded traditional matrix factors studies that require post-spiking matrix extracts with PYX–201 reference material. Therefore, a modified matrix factor experiment to evaluate the consistency of analyte ionization via the detection and quantitation of matrix components in the same extracts was conducted. LQC and HQC samples were prepared in four normal human plasma lots, two 5% hemolyzed human plasma lots, and two lipemic human plasma lots with > 300 mg/dL triglyceride. Peak area ratios of the analyte and internal standard were compared. For all lots and at both QC levels, the %CV of the peak area ratios was < 20%, indicating matrix effects were consistent across the tested lots.

3.11. Reinjection reproducibility

Calibration standards (n = 2 per calibrator level) and run acceptance QC samples (n = 2 per QC level) were injected in one run, stored at the autosampler temperature (4 °C), then re-injected in a separate run. All standards and run acceptance QC samples met the acceptance criteria for reinjection reproducibility, i.e., within ± 20% difference from theoretical.

3.12. Run length evaluation

Run length was evaluated to simulate the expected size and duration of a full analytical run containing 96 injections. Additional human plasma blanks were extracted and analyzed in a run with calibration curves and run acceptance QC samples. There was no evidence of system performance degradation observed over the course of an analytical run.

3.13. Assay application

This validated assay has been successfully applied in analyzing PYX-201 concentrations in human K_2EDTA plasma samples in clinical trial PYX-201–101 (a first-in-human, open-label, multicenter, phase 1 clinical study to evaluate the safety, tolerability, pharmacokinetics, pharmacodynamics, and preliminary efficacy of PYX-201 in participants with advanced solid tumors) (phase I study: NCT05720117, https://www.clinicaltrials.gov, EudraCT Number: 2022–002284-30). Clinical data and PK profiles will be reported in a separate, forthcoming manuscript.

4. Conclusions

A hybrid immunoaffinity LC-MS/MS assay for the quantitation of the ADC drug PYX-201 from 25 μL of human plasma was developed and validated. PYX-201 was enriched by MABSelect beads coated with protein A, then was subjected to "on-bead" proteolysis with papain to release the payload, Aur0101. The SIL-IS Aur0101-d_8 was added, and the released Aur0101 was quantified as a surrogate analyte for the ADC concentration. LC-MS/MS analysis was conducted on a Waters Acquity UPLC BEH C18 column (130 A, 2.1 mm × 50 mm, 1.7 μm) coupled with Sciex 6500 triple quadrupole mass spectrometer. This assay is accurate and precise over the range 0.0250 to 250 μg/mL in human plasma. The intra-run %RE ranged from −6.0% to 3.0% with %CV between 1.3% and 5.3% and the inter-run %RE was from −3.8% to −0.1% with %CV between 2.1% and 5.8% for all QC levels in human plasma. PYX-201 was found to be stable in human plasma for at least 24 h on ice, after five freeze (–25 °C or –80 °C)/thaw (on ice) cycles, and after 15 days when stored at –80 °C. PYX-201 post-preparative extract was stable for at least 225 h when stored at 4 °C and PYX-201 was stable in human whole blood for at least two hours stored at room temperature or being kept in

Table 4
Accuracy and precision for PYX-201 in human K_2EDTA plasma.

Run Number	LLOQ 0.0250 μg/mL	LQC 0.0750 μg/mL	MQC 10.0 μg/mL	HQC 19.0 μg/mL
1	0.0241	0.0729	9.94	18.4
	0.0237	0.0756	9.67	18.3
	0.0215	0.0744	10.0	18.5
	0.0226	0.0713	9.90	18.1
	0.0243	0.0719	9.96	18.3
	0.0248	0.0726	10.0	17.7
Intra-run Mean	0.0235	0.0731	9.92	18.2
Intra-run S. D.	0.00124	0.00161	0.129	0.270
Intra-run % CV	5.3	2.2	1.3	1.5
Intra-run % RE	−6.0	−2.5	−0.8	−4.1
n	6	6	6	6
2	0.0251	0.0744	*	18.1
	0.0253	0.0745	9.98	18.7
	0.0262	0.0774	9.73	18.1
	0.0249	0.0705	10.5	17.7
	0.0269	0.0743	9.85	17.9
	0.0255	0.0752	10.1	17.8
Intra-run Mean	0.0256	0.0744	10.0	18.0
Intra-run S. D.	0.000760	0.00223	0.281	0.335
Intra-run % CV	3.0	3.0	2.8	1.9
Intra-run % RE	2.6	−0.8	0.2	−5.0
n	6	6	5	6
3	0.0252	0.0758	10.1	18.9
	0.0248	0.0777	9.58	17.9
	0.0245	0.0763	9.77	18.5
	0.0269	0.0771	9.40	18.5
	0.0271	0.0744	10.1	18.8
	0.0262	0.0772	9.48	18.9
Intra-run Mean	0.0258	0.0764	9.74	18.6
Intra-run S. D.	0.00111	0.00119	0.307	0.393
Intra-run % CV	4.3	1.6	3.2	2.1
Intra-run % RE	3.0	1.9	−2.6	−2.3
n	6	6	6	6
Inter-run Mean	0.0250	0.0746	9.89	18.3
Inter-run S. D.	0.00146	0.00214	0.261	0.385
Inter-run % CV	5.8	2.9	2.6	2.1
Inter-run % RE	−0.1	−0.5	−1.1	−3.8
n	18	18	17	18

*Excluded from calculation due to no peaks detected.
%CV, percent coefficient of variation; %RE, percent relative error; n, number; S. D., standard deviation.

Table 5
Dilutional linearity of PYX-201 in human K_2EDTA plasma.

	QC Dil 10-fold (100 μg/mL)	QC Dil 2-fold (10.0 μg/mL)
	101	9.84
	95.2	9.93
	96.5	10.5
	102	9.84
	99.4	9.73
	98.0	10.2
Mean	98.7	10.0
S.D.	2.64	0.302
%CV	2.7	3.0
%RE	−1.3	0.1
n	6	6

%CV, percent coefficient of variation; %RE, percent relative error; n, number; S.D., standard deviation.

an ice bath.

This robust and rugged validated assay has been successfully applied in human plasma sample analysis to support the clinical study. PYX-201 is composed of a fully human IgG1 antibody, a cleavable mcValCitPABC linker, and four auristatin analog (Aur0101) payloads. Since fully human IgG1 antibodies, mcValCitPABC linkers, and auristatin analogs are all commonly used ADC components, our assay will greatly benefit bioanalytical scientists who are working on ADC quantitation.

CRediT authorship contribution statement

Feng Yin: Methodology, Investigation, Validation, Writing – original draft. **Diana Adhikari:** Methodology, Investigation, Validation, Writing – review & editing. **Minghao Sun:** Methodology, Investigation, Validation, Writing – review & editing. **M. Shane Woolf:** Visualization, Writing – review & editing. **Eric Ma:** Methodology, Investigation, Validation, Project administration, Supervision, Writing – review & editing. **William Mylott:** Resources, Methodology, Investigation, Validation, Project administration, Supervision, Writing – review & editing. **Elizabeth Shaheen:** Resources, Writing – review & editing. **Shawn Harriman:** Resources, Investigation, Validation, Writing – review & editing. **Jan Pinkas:** Resources, Methodology, Validation, 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.

Data availability

No data was used for the research described in the article.

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