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Development and validation of a hybrid immunoaffinity LC–MS/MS assay for quantitation of total antibody (TAb) from an antibody drug conjugate (ADC) PYX-201 in human plasma

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ABSTRACT

A hybrid immunoaffinity LC-MS/MS assay was developed and validated for the quantitation of total antibody (TAb) from an antibody drug conjugate (ADC) PYX-201 in human plasma. PYX–201 was proteolyzed using trypsin, and a characteristic peptide fragment PYX-201 P1 with ten amino acids IPPTFGQGTK from the complementarity-determining regions (CDRs) was used as a surrogate for the quantitation of the TAb from PYX-201. Stable isotope labelled (SIL) peptide I($^{13}C_6$, ^{15}N)PPTFG($^{13}C_9$, ^{15}N)QGTK was used as the internal standard (IS). We performed chromatographic analysis using a Waters Acquity BEH Phenyl column (2.1 mm × 50 mm, 1.7 μm). Quantification of PYX-201 TAb was carried out on a Sciex triple quadrupole mass spectrometer API 6500 using multiple reaction monitoring (MRM) mode with positive electrospray ionization. To validate PYX–201 TAb, a concentration range of 0.0500 μg/mL to 20.0 μg/mL was used, yielding a correlation coefficient (r) of ≥ 0.9947. For intra-assay measurements, the percent relative error (%RE) ranged from –23.2% to 1.0%, with a coefficient of variation (%CV) of ≤ 14.2%. In terms of inter–assay measurements, the %RE was between −10.5% and −5.7%, with a %CV of ≤ 12.7%. The average recovery of the analyte was determined to be 81.4%, while the average recovery of the internal standard (IS) was 97.2%. Furthermore, PYX–201 TAb demonstrated stability in human plasma and human whole blood under various tested conditions. This assay has been successfully applied to human sample analysis to support a clinical study.

1. Introduction

The intellectual underpinnings of modern chemotherapeutics were proposed >100 years ago by Nobel Prize winner Paul Ehrlich [1]. The evolution of molecular biology, genetics, and bioanalytics continues to drive the field of chemotherapeutics ever closer toward the realization of Ehrlich's vision of "magic bullets", i.e., compounds that can selectively target and cure diseases. After more than a century, in the year 2000, the U.S. Food and Drug Administration (FDA) approved the first "magic bullets" – an ADC drug Gemtuzumab ozogamicin (marketed as Mylotarg) for treatment of acute myeloid leukemia [2–4]. This approval heralded the beginning of the ADC era of cancer research and therapeutics. To date, 13 ADCs have been approved by U.S. FDA for solid tumors and hematological malignancies and over 100 ADC candidates have been investigated in clinical stages [3,5–8]. ADCs like Mylotarg are composed of three primary parts: an antigen-specific monoclonal antibody (mAb), a cytotoxic small-molecule drug (payload or warhead), and a uniquely designed linker molecule that covalently connects the mAb and warhead and balances the toxicity, stability, and the overall efficacy of the ADC drugs [9–14]. That is, an ideal ADC effectively delivers the warhead to targeted tumor cells [10], resulting in improved efficacy with less toxicity for normal, non-target cells. In vivo, ADCs attach to cellular surface antigens (e.g., overexpressed proteins, like human epidermal growth factor receptor 2 (HER2) or nectin4), where they may

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be internalized by the target cell. Upon release, the cytotoxic warhead is free to bind molecular targets, e.g., tubulin or DNA. For non-internalizing ADCs, the mechanism of cell death has been linked to the bystander effect [6,15–16].

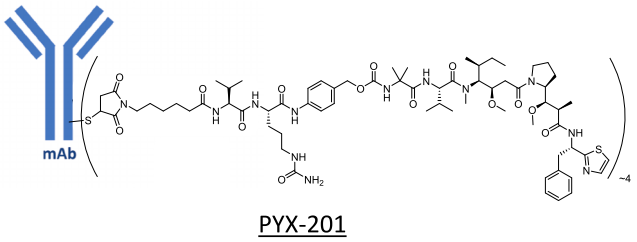
As mentioned, ADCs consist of several molecular components, which can have different pharmacokinetic (PK) profiles impacting the overall safety and efficacy of the biotherapeutic, hence the reason for multiple PK assays that monitor major ADC components: total ADC, free payload, and total antibody (TAb). Explicitly, the TAb assessment is performed to quantify the amount of antibody present in the sample, providing crucial information about the conjugation efficiency, stability, and dosage accuracy of the ADC formulation. Comparative evaluation of TAb and total ADC PK profiles provides additional critical information on the rate of in vivo ADC drug loss, i.e. greater congruence between TAb and total ADC PK curves would suggest good linker stability and minimal in vivo loss of payload from the ADC [17–18]. Ultimately, this TAb assay helps ensure that the desired amount of antibody is present to effectively target the intended antigen.

PYX-201 (Fig. 1) is an investigational ADC, composed of a fully human IgG1 antibody (Table 1), a cleavable linker mcValCitPABC, and toxic auristatin payloads Aur0101 with drug antibody ratio (DAR) of approximately 4. The backbone antibody of PYX-201 specifically targets the extra domain B splice variant of fibronectin (EDB + FN), making PYX-201 an attractive oncology drug candidate since target EDB + FN expresses low in normal adult vasculature while specifically accumulates in new blood vessel stroma in solid tumors [19]. PYX-201 is currently under a first-in-human (FIH) phase I clinical trial for patients with advanced solid tumors (NCT05720117, https://www.clinicaltrials.gov, EudraCT Number: 2022–002284-30). To fully characterize the distribution of PYX-201 ADC components, PK assessments of total ADC, free payload, and TAb were conducted. In previous publications, we discussed the validation of a hybrid immunoaffinity LC-MS/MS assay for PYX-201 total ADC quantitation in human plasma [20], the validation of an LC-MS/MS assay for the quantitation of free payload from PYX-201 in human plasma [21], as well as the validation of a bioanalytical enzyme–linked immunosorbent assay (ELISA) for PYX-201 TAb quantitation in rat and monkey plasma [22]. Here, we report a bioanalytical assay development and validation for quantitation of TAb from PYX-201 in human plasma using a hybrid immunoaffinity LC–MS/MS assay. A characteristic peptide fragment originating from the complementarity-determining regions (CDRs) was used as a surrogate for the quantitation of TAb from PYX-201 in this assay. This assay was validated under regulatory guidance [23–24] and has been successfully applied in clinical sample analysis.

2. Experimental

2.1. Chemicals and reagents

PYX-201 and a recombinant form of EDB + FN, human FN-7-EDB-89 were produced by WuXi Biologics (Shanghai, China). Surrogate analyte peptide PYX–201 P1 (HPLC purity > 95%) with amino acid sequence IPPTFGQGTK and the stable isotope labelled internal standard (SIL-IS) I



PYX-201

Fig. 1. Structure of PYX-201 drug substance.

($^{13}C_6$, ^{15}N)PPTFG($^{13}C_9$, ^{15}N)QGTK (HPLC purity > 95%) were manufactured at Elim Biopharmaceuticals (Hayward, CA, USA). HPLC grade water, HPLC grade acetonitrile, HPLC grade methanol, DL-dithiothreitol, and iodoacetamide were produced by Sigma-Aldrich (St. Louis, MO, USA). Streptavidin Mag Sepharose beads were acquired from Cytiva (Marlborough, MA, USA). Biotin antigen (human EDB + FN) bound streptavidin magnetic beads were produced in PPD Laboratories Services (Henrico, VA, USA). Mass spectrometry (MS) grade Pierce trypsin protease was obtained from ThermoFisher Scientific (Waltham, MA, USA). Ammonium bicarbonate and hydrochloric acid were ordered from VWR international (Radnor, PA, USA). RapiGest SF surfactant was bought from Waters (Milford, MA, USA). Tris buffered saline (TBS)-wash buffer (20 ×, pH 7.4) and TBS–tween 20 (20 ×, pH 7.4) were supplied by Boston BioProducts (Milford, MA, USA). Dipotassium EDTA human plasma was purchased from BioIVT (Westbury, NY, USA).

2.2. LC-MS/MS system

A Sciex API 6500 triple quadrupole mass spectrometer (Sciex, Framingham, MA, USA) coupled with Agilent 1200 or 1100 binary pumps (Agilent technologies, Santa Clara, CA, USA) and CTC analytics PAL DLW autosampler (Leap technologies, Carrboro, NC, USA) were applied in this LC-MS/MS assay validation. A Waters Acquity BEH Phenyl, 2.1 mm × 50 mm, 1.7 μm column (Waters, Milford, MA, USA) was utilized for the chromatographic separation.

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

Stock solution of PYX-201 at 15.2 mg/mL was supplied in 20 mM histidine with 6% (w/v) sucrose and 0.02% (w/v) PS80 at pH 5.5. PYX-201 stock solution was spiked into human K_2EDTA plasma to produce calibration standard at nominal PYX-201 concentrations of 0.0500, 0.100, 0.160, 0.600, 2.00, 6.50, 16.0, and 20.0 μg/mL and QC samples at nominal PYX-201 concentrations of 0.0500 (LLOQ), 0.150 (LQC), 10.0 (MQC), and 15.0 μg/mL (HQC). Pools were prepared using protein low-binding containers. Calibration standards, QC samples from one accuracy and precision run, and QC samples from all matrix stability runs were freshly prepared on the day of use during this assay validation. All other QC samples were stored frozen until use. Calibration standards were analyzed at the beginning and the end of each run and QC samples were analyzed in four runs for accuracy and precision evaluation.

2.4. Sample processing

Human plasma samples were thawed on ice. 300 μL of loading buffer (TBS-tween 20 diluted 1:20 in water) was mixed well with 10 μL of the thawed human plasma sample in a 96–well protein LoBind plate. 25 μL of washed biotin antigen (human EDB + FN) bound streptavidin magnetic beads were added and the sample mixture was vortexed overnight at 4 °C. PYX-201 TAb analytes absorbed on magnetic beads were washed three times with 300 μL of the washing buffer (TBS–wash buffer 1:20 diluted in water), then dissolved in a well preloaded with 50 μL of RapiGest solution (0.05/37.5/10 RapiGest/50 mM ammonium bicarbonate/ACN), 40 μL of 50 mM ammonium bicarbonate, 10 μL of 0.1 M dithiothreitol solution, and 10 μL of the IS working solution (10 μL of methanol in blank samples without IS), and vortexed at 90 °C for approximately 30 min with shaking. Under yellow light, 25 μL of 0.1 M iodoacetamide in 50 mM ammonium bicarbonate was added in each well, and the sample mixture was incubated at room temperature for approximately another 30 min. 10 μL of 0.250 mg/mL trypsin solution was added in each sample and the sample plate was incubated at 37 °C for approximate 120 min with shaking. 15 μL of 2 N HCl was added to each well and the sample plate was vortexed for 5 min to end the digestion. All samples were filtered using a Multiscreen high throughput

Table 1
Amino acid sequence of TAb from PYX-201.

TAb from PYX-201 – light chain
EIVLTQSPGTLSLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRATGIPDRFSGSGSGTD
FTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYP
REAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSCADYEKHKVYACEVTHQGLSSPVTKSFNRG
EC
CDRs underlined, engineered cysteine for site-specific conjugation: K183**C** (Kabat and EU numbering).
TAb from PYX-201 – heavy chain
EVQLLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSISGSSGTTYYADSVKGRFTIS
RDNSKNTLYLQMNSLRAEDTAVYYCARPFPYFDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALG
CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKK
VEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH
NAKTCPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRE
EMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSV
MHEALHNHYTQKSLSLSPG
CDRs underlined, K(H94)**R** putative V_H glycation sequence liability removed, engineered cysteine for site-specific conjugation: K290**C** (EU numbering) or K307C (Kabat numbering).

screening (HTS) filter plate by centrifuging at 4 °C for 5 min and submitted for LC-MS/MS. LC-MS/MS conditions were optimized and summarized in Table 2.

3. Results and discussion

3.1. Method development

The TAb concentration is the sum of the naked and conjugated antibody concentrations. At its core, the TAb PK assay is an evaluation of ADC stability. By the comparison with total ADC concentration, the TAb PK profile can be used to evaluate the linker and payload stability. This assay uses bead-based immunocapture (streptavidin magnetic beads with biotinylated antigen) to pulldown and enrich the target PYX-201 mAbs. Consequently, this assay will not capture target PYX-201 mAbs if both antibody arms (Fab regions) are already antigen bound. As such, we define the term TAb to describe and encompass antibodies with at least one unbound Fab region, i.e. free and partially-free mAbs.

Table 2
LC-MS/MS assay conditions for TAb from PYX-201.

Chromatography Settings							
Analytical column	Acquity BEH Phenyl, 2.1 mm × 50 mm, 1.7 μm, Waters						
Column temperature	60 °C						
Mobile phase A	100:0.1 Water:formic acid						
Mobile phase B	100:0.1 ACN:formic acid						
Autosampler wash 1	100:0.1 ACN:formic acid						
Autosampler wash 2	100:0.1 Water:formic acid						
Program	Gradient						
Time (min)	0	0.5	3.0	3.1	4.1	5.2	7
%B	5	5	40	95	95	5	Stop
Flow rate	0.3 mL/min						
Auto-injector temperature	4 °C						
Injection volume	25 μL						
Retention time	~2.3 min						
Mass Spectrometer Settings							
Mass Spectrometer	Sciex 6500, triple quadrupole LC-MS/MS						
Ionization Mode	ESI+, MRM						
MRM Mass Transitions	523.5 → 835.6 for IPPTFGQGTK as the surrogate analyte for TAb from PYX-201 532.0 → 845.7 for I($^{13}C_6$, ^{15}N)PPTFG($^{13}C_9$, ^{15}N)QGTK						
Source Temperature (TEM)	650 °C						
Collision Gas (CAD)	9 psig N_2						
Curtain Gas (CUR)	30 psig N_2						
Ion Source Gas 1 (GS1)	90 psig N_2						
Ion Source Gas 2 (GS2)	90 psig N_2						
Ion Spray Voltage (IS)	5000 V						
Entrance Potential (EP)	8 V						
Declustering Potential (DP)	50 V						
Collison Energy (CE)	29 V						
Cell Exit Potential (CXP)	20 V						

Owing to the inherent selectivity, specificity, and sensitivity of the platform, LC-MS/MS has been widely used in bioanalysis in drug research for both small molecules [25–28] and large molecule biologics [17,29–36]. LC–MS/MS platform was employed to analyze TAb from PYX-201 in this assay validation. An immunoaffinity approach was applied to enrich TAb from PYX-201 from human plasma using Streptavidin Sepharose magnetic beads and biotinylated EDB + FN. Because TAb from PYX-201 is too large with a molecular weight approximately 150 k Dalton for a practical direct quantitative analysis using LC-MS/MS technology, the bound proteins were subjected to "on-bead" proteolysis using trypsin, following standard protein denaturation, reduction, and alkylation processing steps. As a result of the digestion, a characteristic peptide originating from the CDRs (Table 1) was used as a surrogate for the quantitation of the TAb from PYX-201. We screened numerous candidate CDR fragments and ultimately chose two peptides for further evaluation: PYX-201 P1 with ten amino acids IPPTFGQGTK and PYX-201 P2 with nine amino acids LLIYYASSR. PYX-201 P1 was eventually determined to be the final surrogate analyte due to the better accuracy and precision and low background in the LC-MS/MS chromatogram. PYX–201 P2 (MRM mass transitions 543.5 → 746.5) was still monitored in the assay validation, only for the purpose of assay monitoring and troubleshooting.

PYX-201 P1, PYX-201 P2, and their corresponding SIL-ISs were weighed and dissolved in solvent. These reference solvent standards were directly tuned into the mass spectrometry for optimized MS conditions, and injected into the high performance liquid chromatography (HPLC) system to adjust the retention time and potential carryover issues. A Waters Acquity BEH Phenyl column was used for chromatographic separation and Sciex triple quadrupole 6500 mass spectrometer was employed for the MS detection. Optimized HPLC and MS conditions are summarized in Table 2.

3.2. LC-MS/MS conditions

HPLC conditions were summarized in Table 2. A quadratic regression with $1/x^2$ weighting factor was employed in the calibration curve regression. The mass spectrometer was operated on Sciex triple quad 6500 mass spectrometer using electrospray ionization (ESI) in the positive ion mode. Data were acquired and processed on 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). Multiple ions were detected for the surrogate analyte IPPTFGQGTK and the IS I($^{13}C_6$, ^{15}N)PPTFG($^{13}C_9$, ^{15}N)QGTK in Q1 scan and product ion scan. MRM mass transitions 523.5 → 835.6 and 532.0 → 845.7 were eventually selected based on the MS signal to noise ratio (S/N) for the surrogate analyte and the IS, respectively. Detailed MS conditions were summarized in Table 2.

3.3. Acceptance criteria

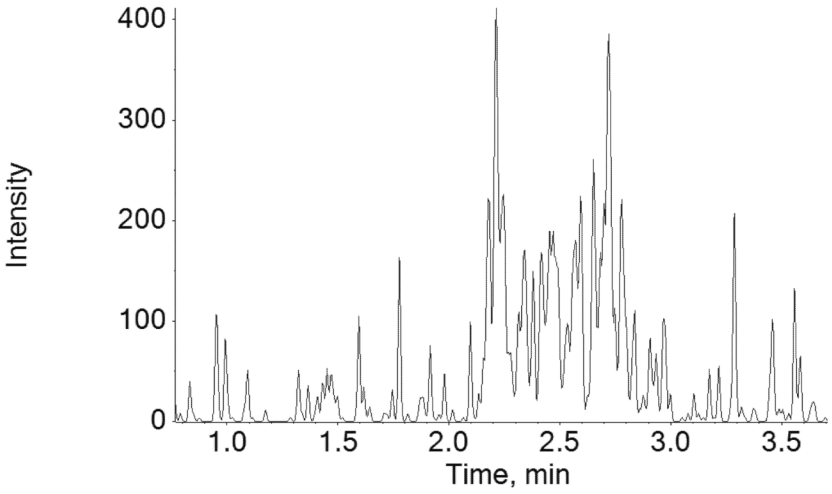
This LC-MS/MS assay was validated under regulatory guidance [23–24] in terms of selectivity, linearity, accuracy and precision, dilution integrity, stability, recovery, matrix effect, hemolysis effect, lipemia effect, reinjection reproducibility, and run length evaluation, etc. There are three steps in this hybrid assay: immunoaffinity of TAb from PYX-201 to magnetic beads, digestion of TAb from PYX-201 to generate a signature peptide, and LC-MS/MS of the signature peptide as a surrogate analyte [37–40]. Due to the involvement of an immunoaffinity enrichment in the assay, acceptance criteria of %CV $\leq$ 25% and %RE within $\pm$ 25% at the LLOQ level, and %CV $\leq$ 20% and %RE within $\pm$ 20% at the other calibration standards or QC levels were pre-set at the beginning of the assay validation.

Analyst Version: 1.6.3 MultiQuant Version: 3.0.3 File: SX87_1RWWN2-A.wiff Printing Date: 12/30/2022 2:38 PM
Page 6 of 100

Sample Name: **MB/IS 1-1** **Sample ID**: **1RWWN-A_006**
Acq. Date & Time: 12/29/2022 7:25:06 PM Modified: False

Peak Name: Total Antibody

Mass(es): 523.5 / 835.6
Proc. Algorithm: MQ4
Gaussian Smooth Width: 1.0 points
Expected RT: 2.27 min
RT Half Window: 30.0 sec
Update Expected RT: DontUpdate
Report Largest Peak: Yes
Min. Peak Width: 3 points
Min. Peak Height: 800.00
Noise Percentage: 95.0%
Baseline Sub. Window: 2.00 min
Peak Splitting Factor: 2 points
RT Window: 0.5
Retention Time: N/A
Int. Type: N/A
Area: N/A
Height: N/A
Start Time: N/A
End Time: N/A



Peak Name: PYX-201 SILIS P1

Mass(es): 532.0 / 845.7
Proc. Algorithm: MQ4
Gaussian Smooth Width: 1.0 points
Expected RT: 2.29 min
RT Half Window: 30.0 sec
Update Expected RT: DontUpdate
Report Largest Peak: No
Min. Peak Width: 3 points
Min. Peak Height: 1000.00
Noise Percentage: 90.0%
Baseline Sub. Window: 2.00 min
Peak Splitting Factor: 2 points
RT Window: 0.5
Retention Time: 2.29
Int. Type: Valley
Area: 527905.50
Height: 101870.61
Start Time: 2.19
End Time: 2.51

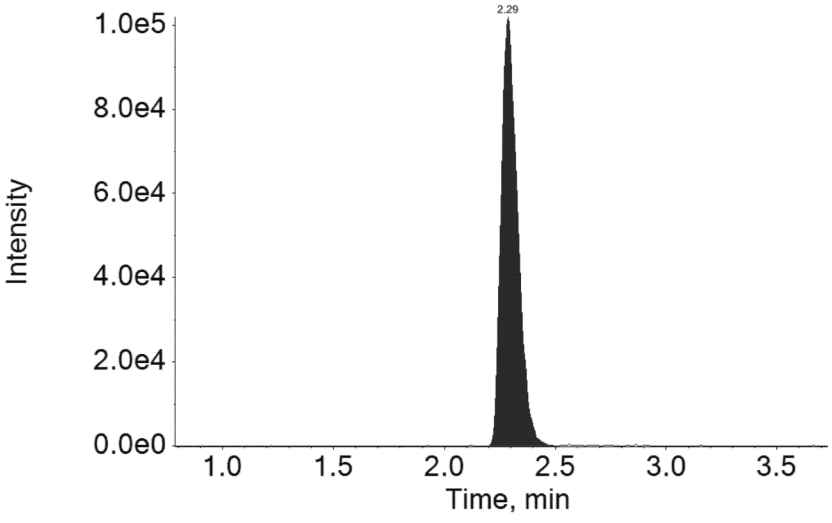


Fig. 2. Chromatograms of PYX-201 P1 IPPTFGQGTK (top) as the surrogate analyte for TAb from PYX-201 and IS I($^{13}C_6$, ^{15}N)PPTFG($^{13}C_9$, ^{15}N)QGTK (bottom) from a blank human K_2EDTA plasma sample containing IS.

3.4. Range and sensitivity

Calibration standards were prepared by fortifying blank matrix pools with the appropriate amount of standard solution to obtain the desired concentration or by diluting higher concentration matrix pools with additional blank matrix. Non-matrix components (solvent, buffers, etc.) added to the matrix during pool preparation comprised $\leq$ 5% of the final pool volume. Calibration standards were freshly prepared at 0.0500, 0.100, 0.160, 0.600, 2.00, 6.50, 16.0, and 20.0 μg/mL for each run. The LLOQ in this assay was determined to be 0.0500 μg/mL for TAb from PYX-201 in human plasma. Calibration standards were analyzed in duplicate over the nominal TAb from PYX-201 concentration range of 0.0500 to 20.0 μg/mL in seven separate runs. The correlation coefficient (R) was $\geq$ 0.9947. A quadratic, 1/concentration^2 weighted, least-squares regression algorithm was used to plot the peak area ratio of the analyte to its IS versus concentration. A representative

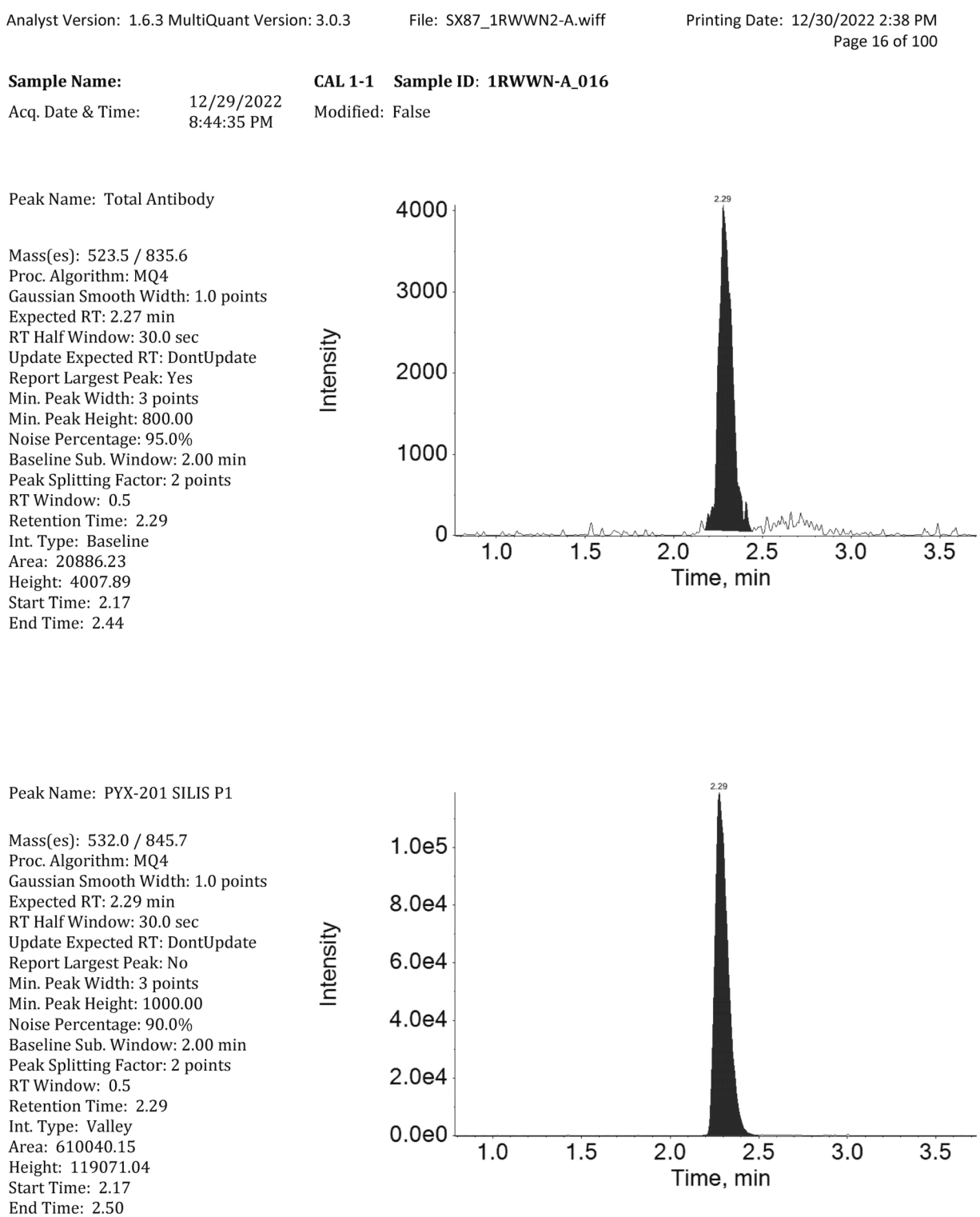


Fig. 3. Chromatograms of PYX-201 P1 IPPTFGQGTK (top) as the surrogate analyte for TAb from PYX-201 and IS I($^{13}C_6$, ^{15}N)PPTFG($^{13}C_9$, ^{15}N)QGTK (bottom) from an LLOQ sample in human K_2EDTA plasma.

chromatogram from a blank matrix spiked with IS and a representative chromatogram from an LLOQ sample in human K_2EDTA plasma are presented in Fig. 2 and Fig. 3, respectively. The obvious high signal to noise ratio (S/N) in the LLOQ chromatogram indicates good sensitivity at the LLOQ 0.0500 μg/mL.

3.5. Accuracy and precision (A & P)

Quality control (QC) samples were prepared by fortifying human plasma pools with PYX-201, and QC samples were employed to assess the assay accuracy and precision. QC samples were prepared at LLOQ (0.0500 μg/mL), LQC (0.150 μg/mL), MQC (10.0 μg/mL), and HQC (15.0 μg/mL), and six replicates of each QC level in four runs were analyzed to calculate the intra- and inter-run accuracy and precision. Accuracy and precision were evaluated in four QC levels (LLOQ, LQC, MQC, and HQC) in six replicates from four separate runs. Accuracy was expressed in percent relative error (%RE) to the nominal concentrations and precision was measured as percent coefficient of variation (%CV) of each QC pool. Accuracy and precision data are summarized in Table 4. For all QC levels, the intra-run %RE ranged from –23.2% to 1.0%, with %CV between 2.4% and 14.2%, and the inter-run %RE was from –10.5% to −5.7%, with %CV between 7.2% and 12.7%. Typically, accuracy and precision were evaluated across three runs. However, during the second run, the observed %RE at the HQC level was slightly outside acceptance limits (%RE = –20.1%). Accordingly, accuracy and precision were evaluated with another set of QCs in a fourth run. The %CV and %RE values for all QCs in the fourth run met the acceptance criteria. Thus, assay accuracy and precision were deemed acceptable (Table 4).

Due to the complexity of the assay, a slightly low intra-run %RE at −20.1% was observed on HQC in run 2, however, the integrity of the assay was not impacted.

3.6. Selectivity

Multiple independent sources of control matrix were evaluated to ensure performance of the assay is not compromised by variations in matrix-related background. Human plasma from six individual lots were extracted and analyzed (n = 1) for TAb from PYX-201 and the IS in blank samples and blank with the IS samples. Additional samples, fortified PYX-201 in order to yield TAb at 0.0500 μg/mL were prepared from the same six individual human plasma lots and analyzed (n = 3) to evaluate potential matrix suppression or enhancement effects.

As is depicted in a typical blank sample in Fig. 2, the response of an interfering chromatographic background peak present at the expected retention time of the IS was < 5% of the mean chromatographic response determined for the IS in the specificity samples fortified with IS. As is observed in a typical blank sample spiked with IS in Fig. 3, the response ratio (interfering background peak response / IS peak response) measured in all blank six matrix lots spiked with IS was < 20% of the mean response ratio determined from the corresponding analyte in the acceptable LLOQ calibrator samples for each run. There were no significant interfering chromatographic peaks that would interfere with quantitation. Selectivity data at LLOQ level are displayed in Table 3. There were no significant matrix suppression or enhancement effects based on the observation that at least two-thirds of replicates for each of the six lots quantitated within ± 25.0% of the nominal value.

3.7. Dilution linearity

The ability of this assay to dilute samples originally above the upper limit of the calibration range was validated by analyzing six replicate QCs, containing 100 μg/mL TAb as 10-fold dilutions. As is shown in Table 5, dilution QC results met the acceptance criteria required in the regulatory guidance [23–24], effectively demonstrating that human plasma samples with concentrations of TAb from PYX-201 higher than ULOQ 20.0 μg/mL can be diluted 10-fold with no negative impact on assay performance or analyte quantitation.

3.8. Stability assessment

LQC and HQC samples were stored at different conditions for stability test. Bench top stability, freeze/thaw stability, long-term stability, extract stability, and whole blood stability were assessed in this assay validation. Bench-top stability was assessed on a set of frozen LQC and HQC samples that were thawed and remained on ice for 25.6 h prior to extraction and analysis. Freeze/thaw stability was evaluated on LQC and HQC samples that have endured five freeze (–25 °C or –80 °C)/thaw (on ice) cycles. Long-term stability was appraised on two sets of LQC and HQC samples that have been stored at –25 °C or –80 °C for 25 days. Extract stability was tested on LQC and HQC samples that have been analyzed and stored at 4 °C for approximately 432.4 h prior to reanalysis with freshly prepared calibrators. Whole blood stability was assessed at approximate low and high concentrations by the comparison of the peak area ratios of the tested LQC and HQC samples after being stored at room temperature or at 4 °C for up to two hours to those of the control LQC and HQC samples that were processed immediately in a centrifuge set at 4 °C.

The acceptance criteria for bench top stability, freeze/thaw stability, long-term stability, and extract stability are %CV at each QC level be ≤ 20.0% and that %RE at each QC level be within ± 20.0% from the nominal concentration. All stability QC samples met the acceptance criteria. TAb from PYX-201 was stable for at least 25.6 h on ice, after at least five cycles of freeze (–25 °C or –80 °C)/thaw (on ice), for at least 25 days after being stored at –25 °C or –80 °C. TAb from PYX–201 extract was stable for at least 432.4 h after being stored at 4 °C. The acceptance criteria for whole blood stability are the %difference from control QC samples be ≤ 20.0% with %CV at each QC level within 20%. All whole blood stability QC samples met the acceptance criteria. TAb from PYX-201 is stable for at least two hours after being stored at room temperature or in an ice bath then processed to plasma in a centrifuge set at room temperature or 4 °C.

3.9. Recovery

Recovery was evaluated on the immunoaffinity capture efficiency of the analyte at LQC, MQC, and HQC levels and on the non-specific binding of the IS PYX-201 SIL-IS P1 at the working level from human plasma by comparing the analyte or IS responses of pre–capture fortified

Table 3
Fortified selectivity evaluation for TAb from 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)
Replicate 1	0.0502	0.0502	0.0628*	0.0547	0.0509	0.0493
Replicate 2	0.0499	0.0528	0.0560	0.0698*	0.0559	0.0504
Replicate 3	0.0522	0.0515	0.0601	0.0545	0.0548	0.0532
n	3	3	3	3	3	3
Nominal concentration (μg/mL)	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500
Low limit (μg/mL)	0.0375	0.0375	0.0375	0.0375	0.0375	0.0375
High limit (μg/mL)	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625

* Outside specified limits; n: number.

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

Run Number	LLOQ (0.0500 μg/mL)	LQC (0.150 μg/mL)	MQC (10.0 μg/mL)	HQC (15.0 μg/mL)
1	0.0495	0.150	8.44	14.1
	0.0473	0.129	9.02	12.9
	0.0511	0.138	8.22	14.0
	0.0478	0.133	8.42	13.5
	0.0497	0.148	8.32	14.4
	0.0416	0.128	8.20	14.6
Intra-run Mean	0.0478	0.138	8.44	13.9
Intra-run S. D.	0.00337	0.00933	0.300	0.631
Intra-run % CV	7.0	6.8	3.6	4.5
Intra-run % RE	−4.3	−8.3	−15.6	−7.3
n	6	6	6	6
2	0.0415	0.134	8.87	12.8
	0.0454	0.145	8.89	9.78
	0.0393	0.139	8.58	12.4
	0.0395	0.133	8.54	11.4
	0.0347	0.132	9.10	12.5
	0.0298	0.127	8.80	13.0
Intra-run Mean	0.0384	0.135	8.79	12.0
Intra-run S. D.	0.00545	0.00630	0.210	1.21
Intra-run % CV	14.2	4.7	2.4	10.1
Intra-run % RE	–23.2	−10.0	−12.1	−20.1
n	6	6	6	6
3	0.0453	0.130	7.80	12.3
	0.0485	0.150	9.08	13.5
	0.0498	0.137	9.00	13.7
	0.0475	0.142	9.22	14.1
	0.0478	0.145	9.87	14.6
	0.0507	0.147	9.93	13.9
Intra-run Mean	0.0482	0.142	9.15	13.7
Intra-run S. D.	0.00190	0.00714	0.774	0.788
Intra-run % CV	3.9	5.0	8.5	5.8
Intra-run % RE	−3.5	−5.5	−8.5	−8.8
n	6	6	6	6

Accuracy and precision for TAb from PYX-201 in human K_2EDTA plasma (continued)

Run Number	LLOQ(0.0500 μg/mL)	LQC(0.150 μg/mL)	MQC(10.0 μg/mL)	HQC(15.0 μg/mL)
4	0.0531	0.164	8.02	13.1
	0.0447	0.141	9.84	14.9
	0.0492	0.181	9.36	14.5
	0.0507	0.145	9.53	13.3
	0.0544	0.154	9.86	15.1
	0.0496	0.124	9.96	15.6
Intra-run Mean	0.0503	0.152	9.43	14.4
Intra-run S. D.	0.00340	0.0197	0.727	0.992
Intra-run % CV	6.8	13.0	7.7	6.9
Intra-run % RE	0.6	1.0	−5.7	−4.0
n	6	6	6	6
Inter-run Mean	0.0462	0.141	8.95	13.5
Inter-run S. D.	0.00586	0.0128	0.648	1.27
Inter-run % CV	12.7	9.1	7.2	9.4
Inter-run % RE	−7.6	−5.7	−10.5	−10.0
n	18	18	18	18

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

Table 5
Accuracy and precision of TAb from PYX-201 in human K_2EDTA plasma for dilution QCs.

RunNumber	10-Fold dilution QC100 μg/mL
5	108
	102
	93.6
	91.6
	79.0
	102
Mean	96.1
S.D.	10.4
%CV	10.8
%RE	−3.9
n	6

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

samples to those of post-capture fortified samples representing 100% capture efficiency. The apparent recovery associated with the digestion was not evaluated due to experimental design challenges that often give erroneous digestion efficiency results. Mean recovery of TAb from PYX-201 was 81.4% with a range of 79.1% to 84.4% for different QC levels and mean IS recovery was 97.2%.

3.10. Matrix effect

PYX-201 requires an enzymatic digestion, and post spiking matrix extracts using PYX-201 reference material was not feasible. Therefore, a modified matrix effect experiment to evaluate the consistency of ionization of analytes by the presence of matrix components in the sample extracts was conducted. Matrix effect samples from four normal human plasma lots, two hemolyzed lots (5% hemolysis), and two lipemic lots (>300 mg/dL triglyceride) were fortified pre-extraction to the approximate LQC and HQC levels. The analyte peak area ratios were compared. The %CV of the peak area ratios of the analyte response to the IS response was < 20% at LQC and HQC in all the eight lots tested in matrix effect experiment. There is no matrix effect that would impact this assay.

3.11. Hemolysis effect

The effect of hemolysis on the quantitation of TAb from PYX-201 was evaluated by analyzing blanks, blanks with IS, LQC, and HQC in human plasma fortified with 5% hemolyzed human whole blood. There were no significant chromatographic peaks detected at the mass transitions and expected retention times of the analyte in blank with and without IS samples. There were no significant chromatographic peaks detected at the mass transitions and expected retention times of the IS in blank without IS samples. %CV at LQC and HQC levels was ≤ 20.0% and %RE at each QC level was within ± 20.0% from the nominal concentration. There was no hemolysis effect on the quantitation of TAb from PYX-201.

3.12. Lipemia effect

The effect of lipemia on the quantitation of TAb from PYX-201 was evaluated by analyzing blanks, blanks with IS, LQC, and HQC in lipemic human plasma with a triglyceride concentration of > 300 mg/dL. There were no significant chromatographic peaks detected at the mass transitions and expected retention times of the analyte in blank with and without IS samples. There were no significant chromatographic peaks detected at the mass transitions and expected retention times of the IS in blank without IS samples. %CV at LQC and HQC levels was ≤ 20.0% and %RE at each QC level was within ± 20.0% from the nominal

concentration. There was no lipemia effect on the quantitation of TAb from PYX-201.

3.13. Capture capacity

To demonstrate a lack of impact on samples that are above the upper limit of quantitation, the over-the-curve dilution QC was analyzed without dilution. The percent difference between analyte to IS peak area ratios for all replicates of the undiluted over-the-curve quality control, and analyte to IS peak area ratios of ULOQ calibration standards was > 20% (Table 6). There is no impact on samples that are above the upper limit of quantitation in this assay.

3.14. Reinjection reproducibility

To evaluate an analytical run's reinjection reproducibility, calibration standards and run acceptance QC samples originally injected and passed the acceptance criteria were stored at 4 °C and reinjected into the LC-MS/MS system. All standard calibrators and run acceptance QC samples were within ± 20% difference from theoretical for reinjection reproducibility. The integrity of this assay is not impacted after samples are stored at 4 °C and reinjected.

3.15. Run length evaluation

Multiple blank human plasma samples were extracted and injected within a run containing calibration standards and run acceptance QC samples to mimic the expected maximum runs. There was no evidence of system performance degradation observed over the course of an analytical run containing a total of 96 injections.

3.16. Pharmacokinetic application

A phase I 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" is ongoing. PYX-201 was administered to patients from 0.3 mg/kg to 8 mg/kg as an intravenous (IV) infusion every 3 weeks (Q3W). Up to 45 patients are being enrolled to the dose escalation cohorts. This validated assay has been successfully applied in analyzing TAb from PYX-201 concentrations in human K_2EDTA plasma samples in the clinical trial. Clinical data and PK profiles will be reported in a separate manuscript.

4. Conclusions

A hybrid immunoaffinity LC-MS/MS assay was developed and validated for the quantitation of TAb from an ADC PYX-201 in human plasma. TAb from PYX-201 was enriched by human FN-7-EDB-89, then was hydrolyzed with trypsin to release a characteristic peptide fragment PYX-201 P1 IPPTFGQGTK originating from the CDRs as the surrogate analyte. PYX-201 P1 was quantitated on an LC-MS/MS system with the SIL-IS of PYX-201 P1 I($^{13}C_6$, ^{15}N)PPTFG($^{13}C_9$, ^{15}N)QGTK. The LC-MS/MS system was composed of a Waters Acquity BEH Phenyl column (2.1 mm × 50 mm, 1.7 μm) coupled with Sciex 6500 triple quadrupole mass spectrometer. This assay was validated over the calibration range 0.0500 to 20.0 μg/mL and a quadratic calibration curve with 1/concentration^2 weight was used in the standard curve regression. The intra-run %RE ranged from –23.2% to 1.0% with %CV between 2.4% and 14.2% and the inter-run %RE was from −10.5% to −5.7% with %CV between 7.2% and 12.7% for all QC levels in human plasma. TAb from PYX–201 was found to be stable in human plasma for at least 25.6 h on ice, after five freeze (–25 °C or –80 °C)/thaw (on ice) cycles, and after 25 days when stored at –25 °C or –80 °C. TAb from PYX-201 post-preparative extract was stable for at least 432.4 h when stored at 4 °C and TAb from PYX-201 was stable in human whole blood for at least two hours stored at room temperature or in an ice bath. This validated assay has been successfully applied in human plasma sample analysis to support an ongoing clinical trial.

Table 6
Capture capacity of assay of TAb from PYX-201 in human K_2EDTA plasma.

	Peak Area Ratio(ULOQ)	Peak Area Ratio(undiluted over-the-curve QC)	% Difference from ULOQ
Replicate-1	13.7	43.3	216
Replicate-2	13.8	48.5	254
Replicate-3	NA	46.9	242
Replicate-4	NA	46.7	241
Replicate-5	NA	45.0	229
Replicate-6	NA	47.2	245
Mean	13.7		

NA, not applicable.

CRediT authorship contribution statement

Feng Yin: Methodology, Investigation, Validation, Writing – original draft. **Diana Adhikari:** Methodology, Investigation, Validation, Writing – review & editing. **Marlking Peay:** Methodology, Investigation, Validation, Writing – review & editing. **Diego Cortes:** Methodology, Investigation, Validation, Writing – review & editing. **Mohammed Garada:** Methodology, Investigation, Validation, Writing – review & editing. **M. Shane Woolf:** Visualization, Writing – original draft. **Eric Ma:** Methodology, Investigation, Validation, Project administration, Supervision, Writing – review & editing. **Diane Lebarbenchon:** Writing – review & editing. **William Mylott:** Resources, Methodology, Investigation, Validation, Project administration, Supervision, Writing – review & editing. **Mike Dyszel:** 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

Data will be made available on request.

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