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A sensitive and rapid LC-MS/MS assay for quantitation of free payload Aur0101 from antibody drug conjugate (ADC) PYX-201 in human plasma

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ABSTRACT

PYX-201 is an investigational antibody drug conjugate (ADC) with an engineered, fully human IgG1 antibody, a cleavable chemical linker, and a toxin (Aur0101) with an average drug-antibody ratio (DAR) of ~ 4. A sensitive and rapid liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed and fully validated to determine the presence in human plasma, of free payload Aur0101 from PYX-201 to assess drug safety and efficacy. Aur0101 and its deuterated internal standard (IS), Aur0101_d_8, were extracted from 25 µL of human plasma using a solid liquid extraction (SLE) method. Chromatographic analysis was carried out on a Waters Acquity UPLC BEH C18 (2.1 mm × 50 mm, 1.7 µm, 130 A) column. Quantitation of free Aur0101 was conducted on a Sciex triple quadrupole mass spectrometer API 6500 + using multiple reaction monitoring (MRM) mode via positive electrospray ionization. The calibration curve was linear over the concentration range of 25.0 to 12,500 pg/mL with correlation coefficient, $r^2 \geq 0.9988$. The intra-assay %RE was between −4.3% to 14.3% with % CV was ≤ 6.2%. The inter–assay %RE was between −0.2% to 9.5% with % CV was ≤ 6.1%. The average analyte recovery was 89.7% and the average IS recovery was 88.7%. Aur0101 was found to be stable in human plasma and human whole blood under various tested conditions with and without the presence of PYX-201. To our knowledge, this is the first published fully validated assay for free, unconjugated Aur0101 in any matrix, from any species. This assay has been successfully applied to clinical sample analysis to support clinical studies.

1. Introduction

Antibody drug conjugates (ADCs) are site-specific therapeutics that are designed to target cancerous cells and tissues. ADC drugs have three principal components: a monoclonal antibody (mAb), a chemical linker, and cytotoxic payloads (warheads). The mAb moiety gives the ADC its tumor targeting capability; an ideal mAb is specific to the targeted cancer cells, with high binding affinity for the intended extracellular (surface) antigen [1–2]. The chemical linker, which can be cleavable or non-cleavable, connects the mAb and the hydrophobic payload via covalent chemical bonds. For linkers that release warheads extracellularly, cell death occurs via either transmembrane cellular diffusion of the payload or bystander effect [1–4]. However, intracellular payload release is more often preferable. An ideal cleavable linker permits stable peripheral circulation of the ADC with rapid payload separation upon ADC delivery to cancer cells, at which point, the cytotoxic warhead kills the earmarked tumor cells [1–4]. This combination of highly specific, targeted ADC delivery and ADC stability during circulation minimizes non-specific drug exposure, reduces off-target tissue toxicity, and increases the efficacy and safety of the drug [1–2,5–6].

ADC drug research began in earnest during the early 1980 s and has attracted increased pharmaceutical investment since the first ADC drug (Mylotarg) was approved by the US Food and Drug Administration (FDA) in 2000 [7–9]. To date, more than a dozen ADC drugs have been globally approved, and more than half of the approved ADC drugs are for the cure of hematological malignancies [2,10–11]. PYX-201 is an investigational ADC, which is currently in phase I clinical trial for patients with advanced solid tumors (NCT05720117, https://www.

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clinicaltrials.gov, EudraCT Number: 2022–002284-30). PYX-201 (intact ADC) is an anti–extra domain B splice variant of fibronectin (EDB + FN) fully human IgG1 antibody engineered with four reactive cysteines to enable site-specific conjugation (kC183 + C290) to four Aur0101 (Fig. 1**A**) molecules using a cleavable mcValCitPABC linker. EDB + FN is an ideal oncology drug target since it is expressed in various human solid tumors (e.g. breast cancer) at moderate or high level, while its expression is low in normal adult tissues [12–19].

Recently, Pyxis Oncology reported bioanalytical enzyme-linked immunosorbent assays (ELISAs) for PYX-201 ADC quantitation in rat and monkey plasma [20] as well as a hybrid immunoaffinity LC-MS/MS assay for PYX-201 ADC quantitation in human plasma [21]. To support the ongoing PYX-201 clinical trial, reliable bioanalytical methods were required to underpin key pharmacokinetic (PK) studies assessing the distributions of total antibody (unbound mAb and payload bound mAb), total conjugated ADC [21], and unconjugated warheads. Due to high selectivity, specificity, and sensitivity, LC-MS/MS has been widely used in oncology bioanalysis for small-molecule drugs [22–25]. Here we report a fully validated, sensitive, and rapid bioanalytical assay for the quantitation of the PYX–201 free payload (unconjugated Aur0101 warhead) in human plasma. The free payload assay reported here was validated in compliance with current regulatory guidance [26–27], including standard validation studies (e.g. accuracy, precision, selectivity/specificity, analyte stability, etc.). Additionally, we present and discuss findings that highlight the importance of evaluating the impact of ADC presence on unconjugated payload quantitation. Explicitly, we performed an impact assessment to determine the effect of potential preexisting payload in ADC reference material on the quantitation of free payload. To our knowledge, this is the first publication of a fully validated assay for free Aur0101 in any matrix, from any species. This assay has been smoothly applied to clinical sample analysis.

2. Experimental

2.1. Chemicals and reagents

Reference standard Aur0101 (PF–06380101) was provided by Carbogen Amcis (Bubendorf, Switzerland), and the stable isotope labelled internal standard (SIL-IS) Aur0101-d_8 (Fig. 1**B**) was obtained from MedChemExpress (Monmouth Junction, NJ, USA). Ammonium acetate, formic acid, HPLC grade acetonitrile, HPLC grade methanol, HPLC grade ethyl acetate, and HPLC grade N,N-dimethylformamide (DMF) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Water was purified in-house using the Millipore Milli-Q IQ 7000 ultrapure lab waters system (Burlington, MA, USA). Human dipotassium ethylenediaminetetraacetic acid (K_2EDTA) plasma was purchased from BioIVT (Westbury, NY, USA).

2.2. LC-MS/MS system

The LC-MS/MS system was composed of an API-6500 + triple quadrupole mass spectrometer (Sciex, Framingham, MA, USA), an LCPAL DLW autosampler (CTC Analytics, Zwingen, Switzerland), and Agilent 1260 binary pumps (Agilent Technologies, Santa Clara, CA, USA). Analyst® v1.6.3 and MultiQuant v3.0.3 softwares (Sciex, Framingham, MA, USA) and Assist laboratory information management system (LIMS) v7 software (PPD, Wilmington, NC, USA) were used for data acquisition and processing. Chromatographic separation was conducted on an Acquity UPLC BEH C18 analytical column (2.1 × 50 mm, 1.7 μm, 130 A) (Waters, Milford, MA, USA).

2.3. Preparation of calibration standards and quality control samples

Calibrators (CALs) and quality controls (QCs) were prepared in human K_2EDTA plasma. 1 mg of Aur0101 reference standard was weighed on a microbalance and dissolved in 5 mL of DMF to yield a 200 μg/mL stock solution. The stock solution was stored in LoBind polypropylene tubes (Eppendorf, Hamburg, Germany) and kept at –80 °C when not in use. Serial dilution of the stock solution in human K_2EDTA plasma produced CALs at 25.0, 50.0, 90.0, 300, 1000, 3500, 10500, and 12500 pg/mL. CALs were prepared fresh each day.

Four QC levels were prepared by spiking the analyte (Aur0101) into human K_2EDTA plasma: lower limit of quantitation (LLOQ) = 25.0 pg/mL, low-QC (LQC) = 70.0 pg/mL, mid-QC (MQC) = 5,000 pg/mL, and high-QC (HQC) = 10,000 pg/mL. Replicate QC samples (n = 6 at each QC level) were used to evaluate intra– and inter-run accuracy and precision (Table 1). For stability tests, extra LQC and HQC samples were prepared and stored at −25 °C and −80 °C.

Stable isotope-labelled compound Aur0101-d_8 was used as the internal standard (IS). To make an IS stock solution at 200 μg/mL, 1 mg of the IS reference standard was weighed and dissolved in 5 mL of DMF in a LoBind polypropylene tube. To prepare an IS intermediate solution at 20 ng/mL, a 20 μL volume of the IS stock solution was diluted with 50/50 acetonitrile (ACN)/methanol (MeOH) to a final volume of 200 mL. A working IS solution (WIS) at 1.5 ng/mL was prepared by diluting a 0.225-mL volume of the IS intermediate solution with 50/50 human plasma/water (final volume = 3 mL). The IS stock solution and intermediate solution were stored at −80 °C when not in use. The WIS solution was prepared fresh daily and discarded after use.

2.4. Sample processing

Human plasma samples were extracted using an SLE procedure. Frozen study samples, CALs, and QCs were thawed on ice and vortexed. A 25 μL aliquot of human K_2EDTA plasma was transferred to a 96-well LoBind polypropylene plate, combined with 25 μL of 1.5 ng/mL WIS solution, and vortexed. A 150 μL volume of 0.1 M ammonium acetate solution was added in all samples which were then vortexed and centrifuged. Samples were transferred to an SLE + plate (Biotage, Uppsala, Sweden), where positive pressure was applied to push the sample through the SLE + plate. Samples were eluted using 0.6 mL × 2 of ethyl acetate. The eluant was evaporated to dryness under a nitrogen stream at 50 °C. Samples were reconstituted with 100 μL of 30:70:0.1 methanol/water/formic acid for LC-MS/MS analysis.

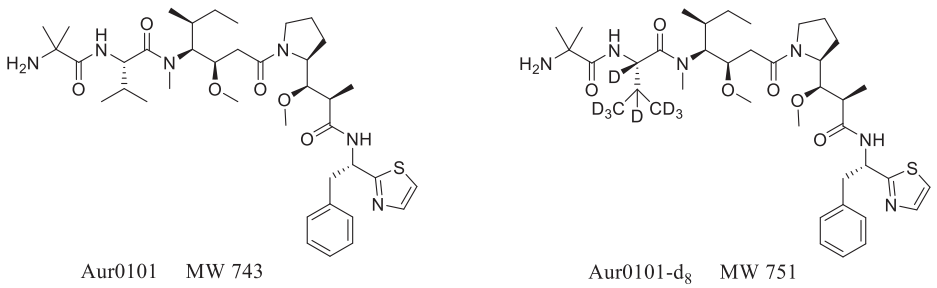

Fig. 1. Aur0101 and the IS Aur0101-d_8 structures.

Table 1
Intra- and inter-run accuracy and precision for Aur0101 in human K_2EDTA plasma.

Run Number	LLOQ 25.0 pg/mL	LQC 70.0 pg/mL	MQC 5,000 pg/mL	HQC 10,000 pg/mL
1	24.1	74.3	4,910	10,000
	25.7	75.6	5,170	10,400
	25.4	77.5	4,710	10,400
	24.0	75.9	5,110	9,990
	25.6	77.3	4,850	9,930
	24.0	75.0	5,050	10,000
Intra-run Mean	24.8	76.0	4,970	10,100
Intra-run S. D.	0.840	1.25	173	196
Intra-run % CV	3.4	1.7	3.5	1.9
Intra-run % RE	−0.9	8.5	−0.7	1.1
n	6	6	6	6
2	25.5	76.1	4,960	10,400
	22.1	72.2	5,010	9,830
	23.0	69.4	5,070	10,100
	23.6	76.6	4,910	9,610
	25.0	74.0	5,130	10,100
	24.4	76.0	4,820	10,600
Intra-run Mean	23.9	74.0	4,980	10,100
Intra-run S. D.	1.28	2.80	112	364
Intra-run % CV	5.4	3.8	2.3	3.6
Intra-run % RE	−4.3	5.8	−0.4	1.1
n	6	6	6	6
3	28.9	78.7	5,490	11,000
	25.8	82.9	5,250	10,800
	24.6	79.1	5,380	11,100
	25.3	77.3	5,480	10,800
	25.0	78.9	5,450	11,300
	27.0	83.2	5,380	10,700
Intra-run Mean	26.1	80.0	5,400	11,000
Intra-run S. D.	1.61	2.44	88.3	199
Intra-run % CV	6.2	3.1	1.6	1.8
Intra-run % RE	4.4	14.3	8.1	9.5
n	6	6	6	6
Inter-run Mean	24.9	76.7	5,120	10,400
Inter-run S. D.	1.52	3.33	242	478
Inter-run % CV	6.1	4.3	4.7	4.6
Inter-run % RE	−0.2	9.5	2.4	3.9
n	18	18	18	18

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

3. Results and discussion

3.1. Method development

Recently, bioanalytical ELISA and hybrid immunoaffinity LC-MS/MS assays were developed and validated for PYX-201 quantitation in rat, monkey, and human plasma in our lab [20–21]. So far, there has been no reported full assay validation on Aur0101 in any matrix from any species. Aur0101 has a molecular weight 743, and LC-MS/MS was naturally chosen to be the analytical method platform due to its high specificity, selectivity, and sensitivity properties. An Aur0101 solvent solution was used as the starting point to optimize the MS conditions for analyte sensitivity as well as the HPLC conditions, e. g. mobile phases and gradient for a decent peak shape and retention time. An Acquity UPLC BEH C18 column was eventually chosen along with mobile phase A (100/2/0.1 water/1 M ammonium acetate/formic acid) and mobile phase B (methanol) for the HPLC conditions. Different sample extraction procedures, e.g., protein precipitation, liquid–liquid extraction (LLE), and solid phase extraction (SPE), were tried in the method development. LLE was found to be able to give the cleanest sample extract. After the method extraction recovery, as well as the interference to the analyte and the IS were compared using different organic solvent, ethyl acetate was finally determined to be utilized in this assay. To make the sample extraction easier to operate, SLE was eventually optimized to replace LLE.

Stabilities (e.g. benchtop stability, freeze/thaw stability, short-term stability, and whole blood stability, etc.) were tested to be acceptable during the method development. Since the purpose of this assay is to measure free Aur0101 concentrations after the administration of ADC drug PYX–201, and the ADC drug concentration was expected to be much higher than the free payload in the clinical plasma samples, the impact of the presence of PYX-201 to the quantitation of Aur0101 was also investigated in the method development because of this relationship. Due to the endogenous level of Aur0101 from PYX-201, LQC and HQC nominal concentrations were adjusted accordingly in the stability assessment in the later method validation.

3.2. LC-MS/MS conditions

Chromatographic separation was conducted on an Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 μM, 130 A) and the HPLC column was kept at temperature 65 °C. Mobile phases A consisted of 100/2/0.1 water/1 M ammonium acetate/formic acid and methanol was used as mobile phase B. The mobile phase flow rate was 500 μL/min, and the following gradient was applied: 0.0 min (35% B), 0.5 min (35% B), 1.5 min (85% B), 1.51 min (96% B), 3.5 min (96% B), 3.6 min (35% B), and 5.0 min (35% B). A make-up pump was used to infuse 80%B into the MS system to increase the sensitivity. The autosampler temperature was maintained at 4 °C. The total run time was 5 min.

Multiple reaction monitoring (MRM) mode was used for quantitation. The mass transitions for Aur0101 and Aur0101-d_8 were m/z 743.3 → 559.2, and m/z 751.3 → 205.2, respectively. Turbo ion spray was utilized as the ion source in positive ion mode with unit resolution applied for both Q1 and Q3 resolutions. Mass conditions were as follows: ion source temperature (TEM) = 500 °C, ion spray voltage = 5500 V, curtain gas (CUR) = 25 psig N_2, collision gas (CAD) = 9 psig N_2, nebulizer gas (GS1) and turbo ion spray gas (GS2) = 70 psig N_2, declustering potential (DP) = 110 V, collision energy (CE) = 40 V, and cell exit potential (CXP) = 17 V.

3.3. Acceptance criteria

Statistics were calculated in Assist LIMS v7. Percent relative error was calculated as (%RE = [(μ/A) − 1] × 100), where μ and A represented the mean calculated concentration and theoretical concentration, respectively. Percent coefficient of variation was calculated as (%CV = (σ/μ) × 100), where σ and μ represented the observed standard deviation and mean calculated concentration, respectively. Acceptance criteria for the LLOQ level were %CV ≤ 20% and %RE within ± 20%, while acceptance criteria for low, mid, and high QC levels were %CV ≤ 15% and %RE within ± 15%. These acceptance criteria are consistent with current regulatory guidance [26–27].

3.4. Range and sensitivity

In this assay, Aur0101 CALs were analyzed in duplicate over the nominal concentration range of 25.0 to 12,500 pg/mL in human K_2EDTA plasma. A linear, 1/concentration^2 weighed, least–square regression algorithm was used to plot the peak area ratio (PAR) of the

analyte to its IS versus concentration. Fig. 2 displays a representative chromatogram from a matrix blank spiked with IS for Aur0101 and Aur0101–d_8 in human K_2EDTA plasma. A representative chromatogram of Aur0101 and Aur0101–d_8 from LLOQ samples in human K_2EDTA plasma is shown in Fig. 3, indicating sufficient sensitivity to measure Aur0101 at 25.0 pg/mL in matrix.

3.5. Accuracy and precision

To evaluate assay intra- and inter-run accuracy and precision, four QCs (LLOQ 25.0 pg/mL, LQC 70.0 pg/mL, MQC 5,000 pg/mL, and HQC 10,000 pg/mL) were analyzed in replicate (n = 6 per QC level) in three runs. Accuracy and precision are expressed as %RE and %CV, respectively (Table 1). For all QC levels in human K_2EDTA plasma, the intra-run accuracy and precision met acceptance criteria, i.e., %RE ranged from −4.3% to 14.3% with %CV between 1.6% and 6.2%. The inter-run accuracy and precision across the three runs also met acceptance criteria with %RE ranging from −0.2% to 9.5% and %CV between 4.3% and 6.1%.

3.6. Run acceptance QCs for supplemental validation runs

Three QCs (LQC, MQC, and HQC) were analyzed in duplicate in each supplemental (non–accuracy and precision) validation run (n = 2 per level, per plate). All run QCs met acceptance criteria, i.e., at least one replicate per QC level, and two thirds of all run acceptance QCs, quantitated within ± 15% for %RE and ≤ 15 % for %CV (Table 2).

3.7. Selectivity and specificity

To ensure the assay performance was not compromised by variations in matrix-related background, samples from six different individual lots of human plasma were extracted and analyzed in singlicate for Aur0101 and the IS Aur0101-d_8, with and without the addition of IS. Selectivity and specificity were successfully demonstrated. Explicitly, to assess experimental acceptability, we considered two peak response ratios. For unfortified samples, the peak area for a background peak present at the expected retention time of an IS were less than 5% of the mean IS peak area in the specificity samples fortified with the internal standard. When comparing IS fortified specificity samples against the associated LLOQ samples (in the same run), the IS fortified specificity response ratios (interfering background peak response / internal standard peak response) were less than 20% of the mean LLOQ sample response ratio.

To further demonstrate assay selectivity/specificity, the same six lots

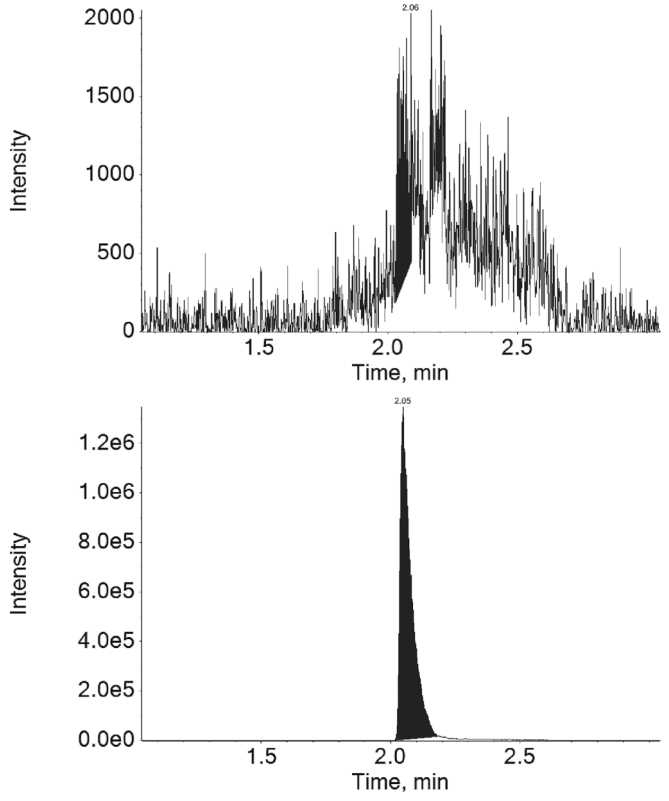

Fig. 2. Representative chromatograms of Aur0101 (top) and the internal standard, Aur0101-d_8 (bottom) from a matrix blank with internal standard in human K_2EDTA plasma sample.

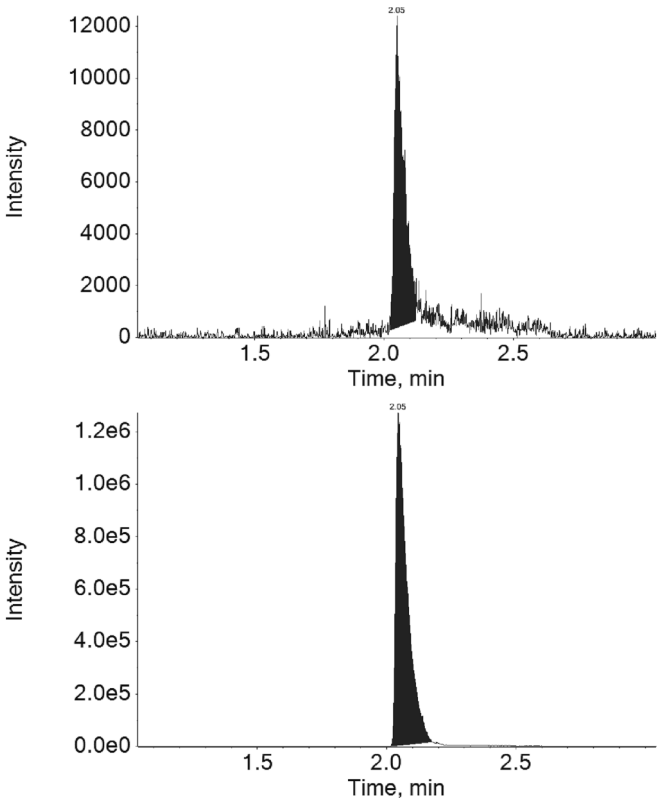

Fig. 3. Representative chromatograms of Aur0101 (top) and IS Aur0101-d_8 (bottom) from an LLOQ sample at 25.0 pg/mL in human K_2EDTA plasma.

Table 2
Run acceptance QCs for supplemental validation runs for Aur0101 in human K_2EDTA plasma.

Run Number	LQC (70.0 pg/mL)		MQC (5,000 pg/mL)		HQC (10,000 pg/mL)	
	pg/mL	%RE	pg/mL	%RE	pg/mL	%RE
4	79.1	13.0	5,500	9.9	11,100	10.5
	76.6	9.5	5,430	8.6	10,800	8.1
5	70.5	0.8	5,100	2.0	10,000	0.5
	74.8	6.9	4,910	−1.9	9,980	−0.2
6	76.1	8.7	5,000	0.0	10,100	0.8
	74.9	7.0	5,020	0.4	9,690	−3.1
7	72.2	3.2	4,910	−1.8	9,760	−2.4
	72.5	3.6	4,780	−4.4	9,700	−3.0
Mean	74.6		5,080		10,100	
S.D.	2.75		255		516	
%CV	3.7		5.0		5.1	
%RE	6.6		1.6		1.4	
n	8		8		8	

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

of human plasma were fortified with Aur0101 at the LLOQ (25.0 pg/mL) level and analyzed in triplicate (n = 3). These samples met selectivity acceptance criteria, i.e., for at least five out of six lots, at least two-thirds of the replicates quantitated within ± 20% of the theoretical value (25.0 pg/mL) (Table 3).

3.8. Dilution integrity

To assess assay performance when processing samples of insufficient volume for a full aliquot, six replicate MQCs were evaluated as 2.5-fold dilutions (10 μL aliquot + 15 μL matrix). To assess assay performance when processing samples that were originally above the upper limit of quantitation (ULOQ), six replicate QCs containing 25,000 pg/mL Aur0101 were evaluated as 10-fold dilutions. These QCs were deemed acceptable, i.e., intra-run accuracy and precision values met run acceptance criteria (Table 4). When taken together, these findings indicate robust assay performance when processing human plasma samples of insufficient volumes or with Aur0101 concentrations higher than ULOQ.

3.9. Analyte stability

To assess Aur0101 stability in human K_2EDTA plasma, we analyzed stability QCs at the LQC (70.0 pg/mL) and HQC (10,000 pg/mL) levels against freshly prepared CALs and run acceptance QCs. Notably, the linker utilized in PYX-201 is susceptible to cleavage due to hydrolysis. While payload release due to linker hydrolysis is expected to be significantly low, the impact of this preexisting payload on assay quantitation performance can be quite profound [28]. Accordingly, prior to analyte stability studies, we conducted an evaluation to determine the amount of preexisting, free Aur0101 in the ADC reference material. The concentration of free payload in the control human plasma with only PYX-201 ADC spiked at the maximum expected plasma concentration (C_{max}) 400 μg/mL was determined to be 101 pg/ml. Therefore, the new nominal LQC concentration was adjusted to be 171 pg/mL (101 pg/mL + 70 pg/mL (LQC in the absence of PYX–201)) and the new nominal HQC concentration was adjusted to be 10,100 pg/mL (101 pg/mL + 10,000 pg/mL (HQC in the absence of PYX-201)). This novel strategy may serve as a paradigm for stability test of free payloads from all the other ADCs.

3.9.1. Benchtop stability

Aur0101 benchtop stability was evaluated by allowing a set of LQCs and HQCs to thaw and remain on ice. Aur0101 was stable on ice for at least 26.7 h with no presence of PYX-201 and at least 27.5 h with the presence of PYX-201 (Table 5).

3.9.2. Freeze/thaw stability

Aur0101 freeze/thaw stability was evaluated by subjecting two sets of LQCs and HQCs to five freeze/thaw cycles. For each freeze cycle, one set was frozen at −25 °C, and the other set was frozen at −80 °C. Samples were thawed on ice. Aur0101 was stable after five freeze (-25 °C or –80 °C)/thaw (on ice) cycles (Table 6).

Table 3
Fortified selectivity/specificity evaluation for Aur0101 in human K_2EDTA plasma.

	Lot 1 (pg/mL)	Lot 2 (pg/mL)	Lot 3 (pg/mL)	Lot 4 (pg/mL)	Lot 5 (pg/mL)	Lot 6 (pg/mL)
Replicate 1	21.3	20.8	22.6	23.5	21.3	21.3
Replicate 2	22.8	24.7	20.9	21.2	20.7	21.6
Replicate 3	21.9	22.4	21.8	21.8	19.9	20.7
Nominal concentration (pg/mL)	25.0	25.0	25.0	25.0	25.0	25.0
Low limit (pg/mL)	20.0	20.0	20.0	20.0	20.0	20.0
High limit (pg/mL)	30.0	30.0	30.0	30.0	30.0	30.0
n	3	3	3	3	3	3

n, number.

Table 4
Accuracy and precision of Aur0101 in human K_2EDTA plasma for dilution QCs.

Run Number	2.5-Fold dilution QC 5,000 pg/mL	10-Fold dilution QC 25,000 pg/mL
4	5,650	26,200
	5,460	26,600
	5,630	27,300
	5,810	26,300
	5,680	28,000
	5,400	27,200
Mean	5,610	26,900
S.D.	152	696
%CV	2.7	2.6
%RE	12.1	7.7
n	6	6

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

Table 5
Aur0101 benchtop stability in human K_2EDTA plasma with and without the presence of PYX–201.

	With no presence of PYX-201 (On ice for 26.7 h)		With the presence of PYX-201 (On ice for 27.5 h)	
	LQC 70.0 pg/mL	HQC 10,000 pg/mL	LQC 171 pg/mL	HQC 10,100 pg/mL
Replicate 1	70.9	9,070	179	9,840
Replicate 2	85.1	9,230	179	9,990
Replicate 3	71.3	9,430	178	9,950
Replicate 4	75.1	9,080	184	10,100
Replicate 5	72.1	8,660	189	9,700
Replicate 6	79.8	8,840	190	9,920
Mean	75.7	9,050	183	9,920
S.D.	5.68	272	5.17	139
%CV	7.5	3.0	2.8	1.4
%RE	8.2	−9.5	7.1	−1.8
n	6	6	6	6

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

3.9.3. Long-term stability

Aur0101 long-term stability was evaluated by analyzing a set of LQCs and HQCs that had been stored at −25 °C or −80 °C with and without the presence of PYX-201. Aur0101 was stable for at least 144 days at −25 °C or −80 °C with no presence of PYX-201 and at least 7 days with the presence of PYX-201 (Table 7). Extra LQC and HQC samples are still being stored in freezers at both −25 °C and −80 °C for future long-term stability test to cover the clinical sample storage.

3.9.4. Autosampler stability

Aur0101 autosampler stability was evaluated by analyzing LQCs and HQCs that were extracted and injected, then stored at 4 °C prior to reanalysis with freshly prepared CALs and QCs with and without the presence of PYX-201. Aur0101 was stable for at least 181.4 h with no presence of PYX-201 and at least 147.6 h with the presence of PYX-201 (Table 8) in the sample extract.

3.9.5. Reinjection reproducibility

Aur0101 reinjection reproducibility was evaluated by re-injection of one set of CALs and run acceptance QCs that were originally injected

Table 6
Aur0101 stability in human K_2EDTA plasma with and without the presence of PYX–201 after five freeze/thaw cycles.

	With no presence of PYX-201		With the presence of PYX-201	
	LQC 70.0 pg/mL	HQC 10,000 pg/mL	LQC 171 pg/mL	HQC 10,100 pg/mL
Freeze at –25 °C and thaw on ice	69.9	9,870	175	9,970
	70.6	9,820	178	10,400
	72.3	9,810	169	9,920
	67.2	9,740	179	10,600
	68.5	10,500	177	10,200
	71.9	9,300	186	10,000
Mean	70.1	9,840	177	10,200
S.D.	1.98	387	5.69	277
%CV	2.8	3.9	3.2	2.7
%RE	0.1	−1.6	3.8	0.9
n	6	6	6	6
Freeze at –80 °C and thaw on ice	70.6	10,100	178	10,100
	70.3	10,100	175	10,500
	66.8	9,420	188	10,200
	73.8	9,750	186	10,200
	65.5	9,800	*	10,400
	68.2	9,950	186	10,900
Mean	69.2	9,860	183	10,400
S.D.	2.99	261	5.87	317
%CV	4.3	2.6	3.2	3.1
%RE	−1.1	−1.4	6.9	2.6
n	6	6	5	6

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

Table 7
Aur0101 long-term stability in human K_2EDTA plasma with and without the presence of PYX–201.

	With no presence of PYX-201		With the presence of PYX-201	
	LQC 70.0 pg/mL(144 days)	HQC 10,000 pg/mL(144 days)	LQC 171 pg/mL(7 days)	HQC 10,100 pg/mL(7 days)
Stored at –25 °C	72.7	9,560	156	9,570
	80.1	10,200	161	9,630
	76.7	10,700	169	9,570
	68.0	10,500	162	9,940
	74.2	10,400	156	9,580
	72.3	10,200	160	10,000
Mean	74.0	10,200	161	9,710
S.D.	4.12	389	4.65	200
%CV	5.6	3.8	2.9	2.1
%RE	5.7	2.4	−6.0	−3.8
n	6	6	6	6
	LQC 70.0 pg/mL(144 days)	HQC 10,000 pg/mL(144 days)	LQC 171 pg/mL(7 days)	HQC 10,100 pg/mL(7 days)
Stored at –80 °C	62.2	9,400	157	9,460
	65.2	9,340	166	9,870
	67.7	9,610	166	9,470
	66.9	9,530	164	9,590
	67.9	9,060	164	9,740
	68.7	9,080	163	10,000
Mean	66.4	9,340	163	9,690
S.D.	2.39	225	3.31	226
%CV	3.6	2.4	2.0	2.3
%RE	−5.1	−6.6	−4.5	−4.1
n	6	6	6	6

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

Table 8
Aur0101 autosampler stability at 4 °C with and without the presence of PYX–201.

	With no presence of PYX-201 (4 °C, 181.4 h)		With the presence of PYX-201 (4 °C, 147.6 h)	
Stored at 4 °C	LQC 70.0 pg/mL	HQC 10,000 pg/mL	LQC 171 pg/mL	HQC 10,100 pg/mL
	78.8	10,400	189	10,200
	79.7	10,100	*	10,600
	78.9	10,300	184	10,100
	78.3	10,100	178	10,600
	75.4	10,300	183	9,660
	77.1	10,700	188	10,600
Mean	78.0	10,300	184	10,300
S.D.	1.56	200	4.45	378
%CV	2.0	1.9	2.4	3.7
%RE	11.5	3.2	7.7	1.8
n	6	6	5	6

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

then stored at 4 °C. The reinjection reproducibility was demonstrated with and without the presence of PYX-201 by the observation of CALs and run acceptance QCs meeting the acceptance criteria according to regulatory guidance [26–27].

3.9.6. *Whole blood stability*

Aur0101 stability in human whole blood was evaluated at approximately low and high concentrations. Whole blood stability control samples were processed to plasma immediately. Non-control samples were held at room temperature (RT) or on ice prior to processing to plasma. Analyte stability was deemed stable if %CV was ≤ 15% and the %difference between the mean PARs and the corresponding control samples was with ± 15%. Whole blood stability samples with no presence of PYX-201 met acceptance criteria and Aur0101 was deemed stable in human whole blood for up to two hours at RT or on ice prior to processing to plasma in either a 2 to 8 °C or RT centrifuge. Aur0101 was deemed stable in human whole blood with the presence of PYX-201 for up to one hour at RT or for up to two hours on ice prior to processing to plasma in either a 2 to 8 °C or RT centrifuge.

3.10. *Hemolysis effect*

To evaluate the impact of hemolysis on Aur0101 quantitation, LQC and HQC samples were spiked with hemolyzed whole human blood to yield QCs representing 5% hemolysis and analyzed in replicate (n = 6). Matrix blanks, with and without IS, were also evaluated. There were no significant chromatographic peaks detected at the mass transitions and expected retention times of the analyte or the IS that would interfere with quantitation. Hemolysis didn't interfere with Aur0101 quantitation.

3.11. *Lipemic effect*

To evaluate the impact of lipemia on Aur0101 quantitation, LQCs and HQCs were prepared in human plasma with triglycerides concentration of > 300 mg/dL and were analyzed in replicate (n = 6). Matrix blanks, with and without IS, were also evaluated. There were no significant chromatographic peaks detected at the mass transitions and expected retention times of the analyte or the IS that would interfere with quantitation. Lipemia didn't interfere with Aur0101 quantitation.

3.12. *Recovery*

To evaluate the recovery of the analyte and the IS, the analyte responses of pre-extraction fortified samples (n = 3 per level) were

compared against post-extraction fortified samples representing 100% recovery (n = 3 per level). The overall recovery was 89.7% for Aur0101 and 88.7% for the IS Aur0101-d_8.

3.13. Matrix effect

Endogenous matrix components in sample extracts can cause MS signal suppression or enhancement of analyte ionization. Accordingly, we prepared samples to assess matrix factor (MF) using eight different lots of human plasma; these matrix lots included four normal lots, two hemolyzed lots (representative of 5% hemolysis), and two lipemic lots (with > 300 mg/dL triglyceride). Each MF lot was fortified post-extraction to the approximate LQC and HQC levels. Matrix factor results were calculated using peak response ratios of analyte peak area versus the IS peak area. The %CV of the peak response ratios met the criteria of ≤ 15% at each LQC and HQC level, indicating matrix effects were consistent across all the eight tested lots.

3.14. Carryover

The potential for carryover from a sample containing a high concentration of analyte Aur0101 to the following sample in an injection sequence was evaluated by injecting an extracted matrix blank immediately after the ULOQ calibration standards in each validation run. There were no contributions from chromatographic peaks, at the expected retention time of the analyte in the blank sample, >20% of the mean analyte response for the LLOQ calibration standards in the validation run.

3.15. Run length evaluation

To mimic the length and duration of a typical analytical run, additional matrix blanks were extracted and analyzed in a single analytical run containing 95 injections. Each set of CALs was placed at the beginning and the end of the run sequence, while QCs were distributed throughout the injection sequence. Data obtained from this study showed no evidence of system performance degradation over the course of the run containing a total of 95 injections.

3.16. Assay application

This fully validated assay for the quantitation of free payload (Aur0101) in human plasma is being used to support an ongoing, 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) (NCT05720117, www.clinicaltrials.gov, EudraCT Number: 2022–002284-30). Aur0101 clinical data and PK parameters will be reported in a separate manuscript in the future.

4. Conclusion

Under FDA guidance, a sensitive and rapid bioanalytical LC-MS/MS assay was validated to quantitate Aur0101 in human plasma with a stable isotope labelled compound Aur0101-d_8 as the internal standard. The analyte and the IS were extracted from human plasma using SLE as the extraction procedure, then analyzed in MRM mode on a Sciex API6500 + MS system. This assay validation applied a linear calibration curve with $1/x^2$ weighted regression. The intra-run %RE ranged from −4.3% to 14.3% with %CV ≤ 6.2% and the inter-run %RE ranged from −0.2% to 9.5% with %CV ≤ 6.1% for all QC levels in human K_2EDTA plasma. Aur0101 was capable of being diluted 10-fold in human plasma and analyzed in this assay. Aur0101 was demonstrated to be stable in human plasma on ice for at least 26.7 h with no presence of PYX-201 and at least 27.5 h with the presence of PYX-201. Aur0101 was stable in human plasma after five freeze (–25 °C or –80 °C)/thaw (on ice) cycles with or without the presence of PYX-201. Aur0101 was stable for at least 144 days after being stored at −25 °C or −80 °C with no presence of PYX-201 and at least 7 days with the presence of PYX-201. Aur0101 was stable for at least 181.4 h with no presence of PYX-201 and at least 147.6 h with the presence of PYX-201 after being extracted. Aur0101 was able to be reinjected with or without the presence of PYX-201. Aur0101 was stable in human whole blood for up to two hours on ice or at RT prior to processing to plasma in either a 2 to 8 °C or RT centrifuge with no presence of PYX-201. Aur0101 was stable in human whole blood for up to one hour at RT and for up to two hours on ice prior to processing to plasma in either a 2 to 8 °C or RT centrifuge with the presence of PYX-201. There was no hemolysis or lipemic effects in this assay. The overall recovery is 89.7% for Aur0101 and 88.7% for the IS Aur0101–d_8. Matrix effect is consistent in this assay and this assay is robust for at least a total of 95 injections.

This sensitive, rapid, and robust assay has been successfully implemented for analysis of human plasma samples and is supporting an ongoing, phase I clinical trial. To our knowledge, this is the first publication of a fully validated bioanalytical assay for unconjugated Aur0101 in any matrix, from any species.

Author statement

The authors state that no data presented in this manuscript has been published in any other journals.

CRediT authorship contribution statement

Feng Yin: Methodology, Investigation, Validation, Writing – original draft. **Diana Adhikari:** Methodology, Investigation, Validation, Writing – review & editing. **Yan Li:** Investigation. **Devan Turner:** Investigation. **M. Shane Woolf:** Visualization, Writing – original draft. **Diane Lebarbenchon:** 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

Data will be made available on request.

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