RESEARCH PAPER

Quantification of the anti-murine PD-1 monoclonal antibody RMP1-14 in BALB/c mouse plasma by liquid chromatography-tandem mass spectrometry and application to a pharmacokinetic study

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Abstract

RMP1-14 is a monoclonal antibody that targets the murine PD-1 protein, and has been used extensively to probe the effects of PD-1 inhibition in preclinical murine models. However, to date, no quantitative analytical methods have been published for RMP1-14. To evaluate its anti-tumor activity in BALB/c mice inoculated with CT26.WT murine colon cancer cells, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to quantify RMP1-14 in BALB/c mouse K_3EDTA plasma was developed and validated. The methodology used a signature peptide (GFYPPDIYTEWK) as a surrogate for RMP1-14 quantitation and an isotopically labeled analog of the signature peptide as the internal standard. Initial method development focused on a hybrid LC-MS/MS assay involving Protein G immunoprecipitation, but this strategy was abandoned due to lack of selectivity. The final validated method consisted of dilution with Tris-buffered saline, trypsin digestion, and desalting using micro solid-phase extraction. Analytical run time was 3.50 min, and the method demonstrated linearity between 0.500 and 50.0 μg/mL of intact RMP1-14. Accuracy, precision, and robustness were all acceptable, and the method was demonstrated to be comparable to a commercially available fit-for-purpose enzyme-linked immunosorbent assay (ELISA) capable of measuring RMP1-14. The validated method was used to generate pharmacokinetic parameters from tumor-bearing BALB/c mice dosed with RMP1-14 at either 2.50 or 7.50 mg/kg. Overall, the validated method represents a novel tool that can be used to evaluate RMP1-14 activity in future immuno-oncology studies.

Keywords RMP1-14 · LC-MS/MS · Monoclonal antibody · Pharmacokinetics · Bioanalysis · Immune checkpoint inhibitor

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Introduction

Immune checkpoint inhibition as an anti-cancer therapy has enjoyed recent clinical success, leading to the development of several new treatment modalities, particularly monoclonal antibodies (mAbs) [1]. Targets of immune checkpoint inhibition include cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), and programmed death-ligand 1 (PD-L1) [1]. At present, regulatory approval has been granted to the mAbs ipilimumab (anti-CTLA-4), nivolumab and pembrolizumab (anti-PD-1), and atezolizumab, durvalumab and avelumab (anti-PD-L1) for the treatment of various cancers [1]. Continued development of novel immune checkpoint inhibitors requires robust preclinical models, and more specifically, utilization of murine syngeneic tumor models to evaluate anti-tumor activity and mechanism of action.

RMP1-14 is an anti-murine PD-1 mAb that was first developed in 2005 by immunizing Sprague–Dawley rats with murine PD-1 protein [2]. It has been used in a variety of studies as a probe drug to evaluate the effect of PD-1 inhibition on various cancers and other disease states [2–8]. To evaluate the effect of intravenous administration of RMP1-14 on tumor growth, a murine colon tumor model was generated by subcutaneous inoculation of immunocompetent BALB/c mice with CT26.WT murine colon cancer cells. To correlate observed experimental outcomes to plasma RMP1-14 concentrations, a validated analytical method was required.

Traditionally, analysis of mAbs has been performed using enzyme-linked immunosorbent assays (ELISAs) due to their high specificity, sensitivity, and simplicity for sample analysis [9, 10]. Recently, liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based approaches have been utilized due to shorter development time, larger dynamic range, and selectivity against nontarget matrix interferences [11–13]. At the present, LC-MS/MS analysis requires enzymatic digestion of the mAb to form smaller peptide fragments termed "signature peptides" prior to analysis, as the mass of an intact mAb (> 100 kDa) exceeds the mass range of MS/MS systems, as MS/MS systems have limited resolution for masses > 2000 Da [12]. Furthermore, standard LC-MS/MS sample preparation procedures such as protein precipitation or dilution tend to have higher limits of quantitation (LOQs) compared to ELISA due to ion suppression by nontarget matrix components that are present in the final extracted sample [14]. By comparison, the specificity of the ELISA immunocapture minimizes the presence of these components in the final extracted sample. Hybrid methodologies utilizing antibodies to isolate ("pull down") intact protein from complex biological matrices coupled to LC-MS/MS analysis may also be used for the possibility of lower detection limits while maintaining the increased selectivity and dynamic range of the LC-MS/MS analysis [15].

The primary objective of this study was to develop and validate the first known LC-MS/MS method for the quantification of RMP1-14 in BALB/c mouse plasma to support pharmacokinetic (PK) analysis of RMP1-14 in the previously described murine colon tumor model. A comparison of two different RMP1-14 extraction techniques (immunoprecipitation and digestion of a diluted sample), comparison of the final validated LC-MS/MS method to ELISA, and results of the PK analysis are discussed in detail.

Materials and methods

Chemicals and reagents

RMP1-14 reference material was purchased as a pre-dissolved solution (6–8 mg/mL in 1× phosphate buffered saline, pH 7.0) from Bio X Cell (West Lebanon, NH, USA). A lyophilized isotopically labeled analog of the signature peptide (GFYPPDIYTEWK-[$^{13}C_6$,$^{15}N_2$]) for use as internal standard (IS) was custom synthesized by New England Peptide, Inc. (Gardner, MA, USA) at purity > 98%. Control BALB/c mouse K_3EDTA plasma was acquired from BioIVT (Hicksville, NY, USA). Rapid Digestion-Trypsin kits were obtained from Promega, Inc. (Madison, WI, USA). Acetic acid, acetonitrile (ACN), ammonium bicarbonate, formic acid, and methanol (MeOH) were all purchased from either Sigma Aldrich (St Louis, MO, USA) or ThermoFisher Scientific (Waltham, MA, USA) and were of HPLC grade or higher purity. Glycine, hydrochloric acid, Tris base, and 20× Tris-buffered saline (TBS) were all purchased from ThermoFisher Scientific; ammonium hydroxide was purchased from MilliporeSigma (Burlington, MA, USA); and 10× TBS with 0.5% Tween 20, pH 7.5, was purchased from Teknova, Inc. (Hollister, CA, USA). Type 1 water used in this study was deionized and filtered through a Barnstead Nanopure Diamond system (Dubuque, IA, USA) prior to use.

Stock and working solutions

Pre-dissolved RMP1-14 reference material was directly used as a stock solution at the concentration specified on the manufacturer-supplied certificate of analysis. Two independent working solutions (one for calibrators and one for quality control (QC) samples) were prepared daily in Eppendorf Protein LoBind microcentrifuge tubes (Hauppage, NY, USA) at 1000 μg/mL in BALB/c mouse plasma and disposed after use.

IS stock solution was prepared at 1.00 mg/mL in water and diluted with 1× TBS to prepare an intermediate solution at 4.20 μg/mL. The intermediate solution was diluted to 16.8 ng/mL in 1× TBS for use as an IS working solution. All IS solutions were subaliquoted into Eppendorf Protein LoBind microcentrifuge tubes and stored at − 70 °C. All aliquots were thawed on wet ice prior to use and never refrozen.

Determination of signature peptide

In order to identify signature peptides for RMP1-14, a 100 μL aliquot of the manufacturer-supplied reference material was subjected to an 18 h trypsin digest at 37 °C using MS-grade Trypsin Gold (Promega, Inc.). The trypsin working solution was prepared by reconstituting 100 μg of lyophilized trypsin with 1 mL of 5 mM acetic acid and diluting it to a final volume of 13 mL using a 50 mM ammonium bicarbonate solution prepared in 10/90 (v/v) MeOH/water adjusted to pH 8 with ammonium hydroxide. A 300 μL aliquot of the trypsin working solution was used to digest the RMP1-14 reference material, and after the incubation was completed, trypsin activity was quenched by adding 400 μL of 1% (v/v) formic acid. A 10 μL aliquot of this mixture was separated on an Acclaim™ 120 C18 2.1 × 250 mm, 3 μm column (ThermoFisher

Scientific) using gradient elution at a flow rate of 0.250 mL/ min and analyzed using a Vanquish UHPLC system (ThermoFisher Scientific) coupled to a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer (ThermoFisher Scientific) running the Xcalibur™ software suite and Q Exactive™ BioPharma platform. The mobile phases consisted of 0.1% (v/v) formic acid in water (mobile phase A) and 0.1% (v/v) formic acid in ACN (mobile phase B), and the gradient (% mobile phase B) used was as follows: initial–5%, 5.0 min–5%, and 95.0 min–95%. The sample manager and column oven were maintained at 7 and 70 °C, respectively. Mass spectrometric acquisition settings used the "Full MS/dd MS^2" experiment with electrospray ionization in positive polarity mode, full MS resolution of 140,000 and dd-MS^2 resolution of 17,500, scan range of 300 to 2500 m/z, and collision energy of 27. The following source parameters were also used: sheath gas, auxiliary gas, and sweep gas flow rates of 35, 10, and 0, respectively; ion spray voltage of 3.50 kV; capillary temperature of 250 °C; S-lens RF level of 50; and auxiliary gas heater temperature of 250 °C.

Obtained masses from the digested RMP1-14 reference material were compared against tryptic peptides obtained from in silico digestion of rat IgG2a, since the intact protein sequence of RMP1-14 is unknown, but the mAb is an isolate of rat IgG2a following immunization of Sprague–Dawley rats with murine PD-1 protein [2]. The rat IgG2a protein sequence was obtained from UniProt (accession ID: Q5M842) [16], and in silico tryptic digest was performed using Skyline version 3.5 [17]. Obtained in silico tryptic peptides were filtered to exclude peptides with missed cleavage sites, peptides containing either cysteine or methionine resides, and peptide greater than 20 amino acids in length or less than 7 amino acids in length. Using this approach, eight candidate signature peptides were identified. When queried against the masses obtained from the digested RMP1-14 reference material using the BioPharma Finder software package (ThermoFisher Scientific), four matches were obtained: SVSELPIVHR, VNSGAFPAPIEK, GFYPPDIYTEWK, and FSWFIDDVEVHTAQTHAPEK. Each of these peptides was queried using the US National Institutes of Health's Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi) against the mouse proteome (taxid: 10088) using the *blastp* algorithm [18] and the UniProtKB/SwissProt sequences database [16]. No matches with 100% identity were obtained for proteins having E value ≤ 10 and query coverage ≥ 80%, which suggests that the candidate signature peptides are specific to RMP1-14 in murine biological matrices.

The four candidate signature peptides were optimized on an AB Sciex Triple Quad™ 5500 mass spectrometer (Framingham, MA, USA) by post-column infusion, and the sensitivity and robustness of each peptide was evaluated during optimization of the tryptic digest and desalting conditions. Based on the overall sensitivity and robustness, GFYPPDIYTEWK was chosen as the signature peptide for RMP1-14.

Preparation of calibration standards and QC samples

The RMP1-14 working solution was diluted in control BALB/c mouse plasma to obtain the upper limit of quantitation (ULOQ) calibration standard (50.0 μg/mL). Seven additional calibration standards at concentrations of 45.0, 25.0, 10.0, 5.00, 2.50, 1.00, and 0.500 μg/mL were obtained by serial dilution of the highest calibration standard. For immunoprecipitation experiments, the two lowest calibration standards were at concentrations of 0.500 and 0.200 μg/mL.

The dilution QC (DQC, 250 μg/mL), high QC (HQC, 40.0 μg/mL), and medium QC (MQC, 20.0 μg/mL) samples were prepared by dilution of the RMP1-14 working solution in control BALB/c mouse plasma such that the final nonplasma component of each QC level was ≤ 5%. The low QC (LQC, 1.50 μg/mL for TBS dilution or 0.600 μg/mL for immunoprecipitation experiments) and lower limit of quantitation QC (LLOQ QC, 0.500 μg/mL for TBS dilution or 0.200 μg/mL for immunoprecipitation experiments) were prepared by dilutions of the MQC samples in control BALB/c mouse plasma. All QC samples were stored at − 80 °C and thawed on wet ice prior to use.

All calibration standards and QC samples were prepared in Eppendorf Protein LoBind microcentrifuge tubes.

Sample preparation

Protein G immunoprecipitation

Immunoprecipitation experiments were performed in a 96-well format using Pierce™ Protein G magnetic beads and a KingFisher™ Flex Purification System (both from ThermoFisher Scientific). Samples were prepared by mixing 25 μL of the sample with 50 μL of 1× TBS, and adding 25 μL of the diluted sample to 475 μL of 1× TBS with 0.05% Tween 20. Prior to use, 50 μL of the beads were aliquoted into 150 μL of 1× TBS with 0.05% Tween 20, washed with 1 mL of 1× TBS with 0.05% Tween 20, and then mixed gently with the samples for 1 h at room temperature. Beads were then washed twice with 500 μL of 1× TBS with 0.05% Tween 20 and once with water before bound proteins were eluted into 100 μL of 100 mM glycine adjusted to pH 2 with hydrochloric acid. Samples were neutralized using 15 μL of 1 M Tris base adjusted to pH 8.5 with hydrochloric acid, after which 25 μL of IS working solution was added. A 100 μL aliquot of the IS-containing sample was then subjected to trypsin digest.

TBS dilution

TBS dilution experiments were performed in a 96-well format using a Hamilton Microlab Nimbus automated liquid handling system (Reno, NV, USA) for all liquid transfers. A 25 μL aliquot of the samples was mixed with 475 μL of 1× TBS,

and 75 µL was transferred to a clean 96-well plate. IS working solution (25 µL) was added to the samples, which were then subjected to trypsin digest.

Trypsin digest and desalting

Trypsin working solution was prepared by reconstituting 100 µg of lyophilized MS-grade Rapid Trypsin Gold with 500 µL of manufacturer-supplied resuspension buffer and adding 250 µL of the reconstituted trypsin to 29.75 mL of manufacturer-supplied digest buffer. A 300 µL aliquot of trypsin working solution was added to all samples following either dilution or immunoprecipitation, and samples were incubated at 70 °C for 2 h in a New Brunswick Innova42R shaker-incubator (Eppendorf) with shaking at 250 rpm. Following incubation, samples were allowed to cool at room temperature for 5 min and then acidified with 400 µL of 1% (v/v) formic acid to quench further trypsin activity.

Desalting was performed using a 2 mg Oasis HLB 96-well µElution Plate (Waters Corporation, Milford, MA, USA). Briefly, the plate was conditioned with 200 µL of ACN followed by 200 µL of water, after which samples were loaded in 400 µL aliquots. The plate was washed twice with 200 µL of water after which samples were eluted with two 25 µL aliquots of 80% (v/v) ACN in water. The eluate was diluted with 200 µL of water and stored at refrigerated conditions prior to LC-MS/MS analysis.

LC-MS/MS analysis

LC-MS/MS experiments were performed on a Waters Acquity UPLC system coupled to an AB Sciex Triple Quad™ 5500 mass spectrometer. Mobile phases consisted of 0.1% (v/v) formic acid in water (mobile phase A) and MeOH (mobile phase B), and tryptic peptides were separated using a Waters Acquity Peptide BEH C18 2.1 × 50 mm, 1.7 µm column fitted with a Waters Acquity 0.2 µm in-line filter using gradient elution at a flow rate of 0.700 mL/min for 3.50 min. The gradient (% mobile phase B) used was as follows: initial–15%, 0.20 min–15%, 0.40 min–25%, 1.20 min–40%, 2.00 min–74%, 2.10 min–90%, 2.70 min–90%, and 2.80 min–15%. The sample manager and column oven were maintained at 5 and 60 °C, respectively.

MS/MS was performed using electrospray ionization operated in positive ion mode. Analytes were acquired by scheduled multiple reaction monitoring experiments (target scan time 0.5 s, scan window 45 s) using the mass transitions of 758.5 > 574.9 (GFYPPDIYTEWK) and 762.5 > 578.9 (GFYPPDIYTEWK-[$^{13}C_6$,$^{15}N_2$]). Mass spectrometric parameters included an ion spray voltage of 5500 V; source temperature of 600 °C; collision, curtain, nebulizer, and auxiliary gases set at 7, 30, 60, and 80 arbitrary units, respectively; entrance potential of 10 V; declustering potential of 80 V;

collision cell exit potential of 15 V; and collision energy of 22 V. Data were processed using Analyst version 1.6.2 (Sciex).

Method validation

A full method validation was conducted for both sample preparation techniques based on the US Food and Drug Administration guidelines for bioanalytical method validation of chromatographic assays [19]. In all cases, calibration standards were freshly prepared for analytical runs. Analytical batches consisted (at a minimum) of duplicate calibration curves (one at the start and one at the end of each run); two replicates of the LQC, MQC, and HQC; a matrix double blank sample; a matrix blank sample spiked with IS working solution; a reagent (water) blank sample; and two matrix double blank samples placed after each of the highest calibration standards to evaluate carryover.

Calibration model

Eight nonzero calibration standards were freshly prepared in BALB/c mouse plasma in the range of 0.200–50.0 µg/mL (Protein G immunoprecipitation) or 0.500–50.0 µg/mL (TBS dilution) for each analytical run and analyzed in duplicate (once each at the start and end of each analytical run). A linear regression model with $1/x^2$ weighting was applied to the concentration–response plot, where x was the ratio of analyte to internal standard concentration. For the calibration model to meet acceptance in an analytical run, at least 75% of the calibration standards had to have calculated concentrations of ± 15.0% of the nominal concentration (except at the LLOQ where the threshold was ± 20.0%). For the LLOQ to meet acceptance, the peak area at the LLOQ in the calibration standard sample had to be greater than five times the blank sample analyte response. Additionally, in the case of accuracy and precision runs, at least one replicate of the LLOQ and ULOQ calibration standards had to meet the acceptance criterion.

Selectivity and specificity

Selectivity was established by analyzing double blank matrix samples from six pooled lots of BALB/c mouse plasma (unique animals in each lot), and also evaluating the same lots when spiked at the LLOQ with RMP1-14. For these lots to meet acceptance, at least five lots had to demonstrate no significant peaks in the chromatographic region of GFYPPDIYTEWK and its IS when analyzed as double blank samples, and had to demonstrate bias within ± 20.0% of the nominal concentration when spiked at the LLOQ concentration (CV ≤ 20.0% if analyzed in multiplicate). Additionally, control BALB/c mouse plasma was spiked with RMP1-14 only at the ULOQ and cross-analyte/IS interference was

evaluated. All analytical runs also contained matrix double blank samples and blank matrix samples spiked with IS working solution only to evaluate intra-run interferences. Any interfering peaks detected were deemed significant if the peak area was > 20.0% of the average peak area for the LLOQ calibration standard or > 5.00% of the average IS peak area.

Carryover

Carryover was evaluated in all analytical runs by placing two double blank matrix samples immediately after each ULOQ calibration standard. Carryover was deemed significant if the first blank sample after the ULOQ calibration standard had an analyte peak area > 20.0% of the average peak area for the LLOQ calibration standard or > 5.00% of the average IS peak area, and this response did not decrease to < 20.0 or < 5.00% in the next blank sample.

Accuracy and precision

At least three accuracy and precision runs for each extraction type were performed on separate days by two analysts to ensure assay robustness. All accuracy and precision runs consisted of duplicate calibration curves (one each at the start and end of the analytical run), six replicates of the LLOQ QC, LQC, MQC, and HQC, as well as all blank samples specified above. For an individual run to meet acceptance, a minimum of five data values had to be generated at each QC sample concentration, and each QC level had to have an average bias of ± 15.0% of the nominal concentration (± 20.0% for the LLOQ QC) and a coefficient of variance (CV) of ≤ 15.0% (≤ 20.0% for the LLOQ QC). Inter-run accuracy and precision were also determined, and all QCs had to meet the same acceptance criteria described above.

Matrix effects and recovery

Matrix effects were evaluated as matrix factors using a surrogate analyte approach in six pooled lots of BALB/c mouse plasma (unique animals in each lot). Each lot of plasma was extracted as a double blank sample and spiked at the final step of sample preparation with an IS solution that represented the peptide-equivalent concentration of an extracted LQC or HQC sample demonstrating 100% recovery. Similarly, reagent (water) blank samples were extracted in the absence of IS and spiked post-extraction with the same IS solutions. The ratio of the average responses for the plasma and reagent samples was used to determine the matrix factors for the assay at each concentration.

Matrix effects were also evaluated at the HQC and LQC concentrations in hemolyzed plasma samples. Hemolyzed plasma was prepared by adding 2% (v/v) lysed BALB/c mouse whole blood to control BALB/c mouse plasma, and

QCs were prepared by serial dilution of the RMP1-14 working solution into the hemolyzed plasma. Hemolyzed QC samples (n = 6 per QC concentration) were extracted in an analytical run containing a calibration curve and plasma-based QCs, and acceptance criteria were set at an average bias of ± 15.0% of the nominal concentration and a CV of ≤ 15.0%.

For samples processed by Protein G immunoprecipitation, recovery was evaluated post-immunoprecipitation but pre-digestion. Double blank matrix samples were spiked after immunoprecipitation with solutions representing RMP1-14 at the LQC, MQC, and HQC concentrations assuming 100% recovery (n = 3 per concentration) and the IS working solution. Recovery was determined by comparing the average analyte responses for extracted LQC, MQC, and HQC samples in the analytical run (n = 6 per concentration) to their respective recovery samples.

For samples processed by TBS dilution, recovery was determined post-desalting. Aliquots of the LQC, MQC, and HQC samples (n = 3 per concentration) were extracted without IS, and post-desalting was spiked with an IS solution that represented the peptide equivalent concentration of an extracted sample demonstrating 100% recovery. Recovery was determined by comparing the average analyte to internal standard response for each QC level.

Dilution integrity

To evaluate dilution integrity, six replicates of the DQC were diluted 50× in control BALB/c mouse plasma prior to sample processing, and then analyzed in their diluted state. Accuracy and precision for the diluted samples was determined, with acceptance criteria set at an average bias of ± 15.0% of the nominal concentration and a CV of ≤ 15.0%. Additionally, for samples processed by Protein G immunoprecipitation, dilution integrity post-immunoprecipitation was determined by subjecting six undiluted DQC samples to immunoprecipitation and diluting the neutralized sample 50× with 1× TBS prior to IS addition and trypsin digest. Acceptance criteria for these samples were the same as above.

Stability

Replicates of the HQC and LQC samples (three tubes in each case) were exposed to either ambient conditions or wet ice for at least 24 h to evaluate benchtop stability, subjected to four freeze–thaw cycles (frozen at either − 20 or − 80 °C and thawed on wet ice), or stored at − 20 or − 80 °C to evaluate long-term stability. Two aliquots were taken from each tube subjected to the various stability tests (six aliquots in total) and analyzed against freshly prepared calibration curves and stored QC samples (except for the long-term stability assessment, where QCs were freshly prepared). Acceptance criteria were set at an average bias of ± 15.0% of the nominal concentration and a CV of ≤ 15.0%.

Extract stability was evaluated at the LQC and HQC concentrations by re-injecting previously processed samples that met acceptance criteria ($n = 6$ for each QC level) in an analytical run that contained freshly extracted calibration standards and QC samples. Re-injection reproducibility was evaluated at the LQC, MQC, and HQC concentrations by re-injecting the calibration curve, QC samples, and blank samples from an analytical run that had previously met acceptance criteria. Acceptance criteria were set at an average bias of $\pm 15.0\%$ of the nominal concentration and a CV of $\leq 15.0\%$.

Sample collection and harvesting stability was assessed using one fresh (never frozen) BALB/c mouse K_3EDTA whole blood sample spiked at the MQC concentration. The whole blood MQC sample was incubated for at least 10 min at 37 °C and then subaliquoted, with half the aliquots stored at room temperature and half on wet ice. After 5 min, one aliquot from each storage condition was harvested to plasma and stored at -80 °C ("t_0 sample"), and 2 h later, a second aliquot from each storage condition was harvested to plasma and stored at -80 °C. Plasma harvest was performed by centrifugation at either ambient or refrigerated conditions for 10 min at $2000 \times g$, depending on the storage conditions of the whole blood. Each plasma harvest was analyzed in triplicate, and stability was considered acceptable if the average peak area ratio of the stored sample was within $\pm 15.0\%$ of the t_0 sample for that storage temperature.

Stability of the RMP1-14 stock solution was evaluated under both benchtop and stored conditions. To evaluate benchtop stability, an aliquot of the stock solution was exposed on wet ice for 29 h and then HQC samples were prepared by dilutions of the stock in control BALB/c mouse plasma. Samples ($n = 6$ aliquots) were extracted by the TBS dilution method and the average peak area ratio was compared against extracted HQC samples ($n = 6$) prepared from an aliquot of the same stock solution that had been stored under refrigerated conditions for the same duration. Acceptance criteria were set at an absolute difference of $\pm 10.0\%$ of the control sample area ratio and CV $\leq 10.0\%$. Stored stock stability was evaluated by extracting HQC samples ($n = 6$) freshly prepared from a previously purchased lot of RMP1-14 reference material and quantifying against a calibration curve and QC samples prepared from a newly purchased lot of RMP1-14 reference material. Acceptance criteria were set at an average bias of $\pm 15.0\%$ of the nominal concentration and a CV of $\leq 15.0\%$, and the storage duration was determined to be the difference between dates of receipt of the two lots of RMP1-14 reference material.

Benchtop stability of the IS stock and working solutions was also determined after 25 h of exposure on wet ice. Solutions were diluted and the average peak area was compared against aliquots of the same solutions that had been stored at refrigerated conditions for the same duration ($n = 6$ aliquots in each case). Acceptance criteria were set at an absolute difference of $\pm 10.0\%$ of the control sample peak area and CV $\leq 10.0\%$. Stored stability for the IS stock and working solutions was not evaluated; instead, these solutions were assigned an arbitrary stability of 365 days.

Preclinical application of the validated method

This method was developed and validated to support a PK study examining the anti-tumor activity of RMP1-14 in female BALB/c mice (Envigo, Fredrick, MD, USA) subcutaneously inoculated with CT26.WT murine colon cancer cells (ATCC #CRL-2638, Manassas, VA, USA) to generate a subcutaneous solid tumor. Mice were inoculated with 1×10^6 cells per animal via subcutaneous injection into the right flank, and tumor-bearing animals were intravenously dosed at either 2.50 or 7.50 mg/kg of RMP1-14 ($n = 48$ per dose group). Dosing was performed on day 1 and day 8 of the study, with the first dose occurring 15 days post-inoculation (average tumor volume of 370 mm³). Blood samples were collected by cardiac puncture in K_3EDTA tubes at 1, 4, 8, 24, 72, 120, and 168 h post-dose administration ($n = 3$ per dose group, per timepoint). Blood samples were stored on wet ice prior to plasma harvest, which was conducted by centrifuging the blood at $3000 \times g$ for 5 min at 4 °C. Plasma samples were stored at -80 °C until analysis. Animals were euthanized by carbon dioxide asphyxiation followed by cervical dislocation after blood collection. In vivo studies were performed under protocols approved by the Covance Institutional Animal Care and Use Committee, and were in compliance with the U.S. Department of Agriculture's Animal Welfare Act (9 CFR Parts 1, 2, and 3) and the Guide for the Care and Use of Laboratory Animals [20]. Whenever possible, procedures in this study were designed to avoid or minimize discomfort, distress, and pain to animals.

Plasma analysis was conducted using the validated TBS dilution analysis method described above. Where necessary, samples were diluted up to 10× using control BALB/c mouse plasma, with DQCs included in the analytical batches to assure dilution integrity.

Comparison of ELISA and LC-MS/MS analysis of RMP1-14

As indicated previously, mAbs are usually analyzed either by ELISA or LC-MS/MS. While there are no published or commercially available ELISA methods for RMP1-14 specifically, communication with Bio X Cell suggested that a commercial ELISA kit that analyzed for rat IgG2a may be useful for the quantification of RMP1-14, since RMP1-14 is an isolate of rat IgG2a following immunization of Sprague–Dawley rats with murine PD-1 protein [2]. To compare against the ELISA method, an analytical batch consisting of duplicate plasma-based calibration standards, triplicate plasma-based

QCs (including the DQC), and 14 pooled replicates of in-curred samples (seven per dose group distributed throughout collected timepoints) was analyzed by the Invitrogen IgG2a Rat Uncoated ELISA Kit (ThermoFisher Scientific) using manufacturer-provided instructions, and by the validated TBS dilution analysis method. Concentrations for each sample by each method were compared by simple linear regression, Deming regression ($\lambda = 0.867$) [21], and Bland–Altman ratio analysis [22], with all analyses being performed using GraphPad Prism 8 (San Diego, CA, USA).

Results

Method validation

Accuracy and precision and calibration model

For samples extracted by both the Protein G immunoprecipitation and TBS dilution techniques, at least three independent accuracy and precision batches met acceptance criteria of bias ± 15.0% of the nominal concentration (± 20.0% at the LLOQ) and CV ≤ 15.0% (≤ 20.0% at the LLOQ). At least 75% of the calibration standards in each analytical run had bias within ± 15.0% of the nominal concentration (± 20.0% at the LLOQ), including at least one replicate each of the LLOQ and ULOQ calibration standards. The average signal to noise at the LLOQ was 5:1 for samples extracted by Protein G immunoprecipitation and 16:1 for samples extracted by TBS dilution. Intra- and inter-run precision and accuracy, as well as the correlation coefficients for the calibration curves for each batch, are summarized in Table 1 for Protein G immunoprecipitation and in Table 2 for TBS dilution. Representative chromatograms of the LLOQ, ULOQ, double blank, and blank matrix spiked with internal standard samples extracted by Protein G immunoprecipitation and TBS dilution are shown in Fig. 1.

Carryover

The highest carryover observed in any analytical run was 47.7% of the LLOQ and 0.766% of the IS in the first blank following a ULOQ sample for samples extracted by Protein G

immunoprecipitation, and this carryover decreased to 18.7% of the LLOQ in the second blank following the same ULOQ sample. Therefore, while initially significant, the carryover can be mitigated by the use of an additional blank sample following samples with high RMP1-14 concentration. For samples extracted by TBS dilution, the highest carryover observed in any analytical run was 9.21% of the LLOQ and 0.684% of the IS in the first blank following a ULOQ sample, suggesting a lack of significant carryover.

Dilution integrity

For both the Protein G immunoprecipitation and TBS dilution sample preparation techniques, a dilution factor of 50× using control BALB/c mouse plasma as a diluent was established pre-sample preparation. Average bias and CV were − 0.200 and 7.89% for Protein G immunoprecipitation and − 1.60 and 6.70% for TBS dilution, thus meeting acceptance criteria. For samples extracted by Protein G immunoprecipitation, 50× dilution with 1× TBS was also evaluated post-immunoprecipitation, and the average bias and CV were determined to be − 76.1 and 16.7%, which did not meet acceptance criteria.

Selectivity and specificity

Of the six pooled lots tested by Protein G immunoprecipitation, five lots demonstrated no significant interferences in the chromatographic region of GFYPPDIYTEWK or its IS, which meets acceptance criteria. When spiked at the LLOQ, none of the six lots demonstrated bias within ± 20.0% of the nominal concentration, with the biases ranging from 24.0 to 131% (Table 3). Of the six pooled lots tested by TBS dilution, none of the lots demonstrated significant interference in the chromatographic region of GFYPPDIYTEWK or its IS, and five out of the six lots demonstrated bias within ± 20.0% of the nominal concentration and CV ≤ 20.0% when spiked at the LLOQ concentration and tested in triplicate (Table 3), which meets acceptance criteria.

Blank plasma samples spiked at the ULOQ and analyzed without IS or spiked with IS working solution only demonstrated no significant interferences in other monitored MRM channels, suggesting a lack of cross-analyte/IS interferences.

Table 1 Precision and accuracy data for RMP1-14 when extracted by Protein G immunoprecipitation prior to trypsin digest and desalting. Data are represented as %bias (%CV) with $n = 6$ for each QC level in each analytical batch and $n = 18$ for each QC level for the inter-run data

Batch	R^2	LLOQ QC (0.200 μg/mL)	LQC (0.600 μg/mL)	MQC (20.0 μg/mL)	HQC (40.0 μg/mL)
1	0.993	− 17.8 (15.4)	0.861 (8.42)	7.08 (5.12)	7.58 (3.90)
2	0.995	2.92 (4.21)	0.917 (7.33)	1.00 (4.69)	4.96 (4.75)
3	0.996	9.50 (15.2)	3.53 (8.11)	− 3.00 (3.45)	1.21 (6.65)
Inter-run data		− 1.78 (16.9)	1.77 (7.59)	1.69 (5.97)	4.58 (5.50)

Table 2 Precision and accuracy data for RMP1-14 when extracted by TBS dilution prior to trypsin digest and desalting. Data are represented as %bias (%CV) with $n = 6$ for each QC level in each analytical batch and $n = 24$ for each QC level for the inter-run data, unless otherwise specified

Batch	R^2	LLOQ QC (0.500 μg/mL)	LQC (1.50 μg/mL)	MQC (20.0 μg/mL)	HQC (40.0 μg/mL)
1	0.991	− 0.200 (6.25)	− 0.667 (6.31)	− 1.00 (1.93)	1.75 (5.43)
2	0.995	16.4 (11.7)	− 1.33 (4.30)	2.50 (4.20)	2.50 (4.55)[a]
3	0.990	1.60 (10.0)	14.7 (3.33)	0.00 (5.54)	3.25 (5.37)
4	0.991	0.800 (16.6)	− 0.700 (9.80)	− 7.50 (4.90)	− 6.50 (5.60)
Inter-run data		4.65 (12.8)	2.92 (8.98)	− 1.58 (5.58)	0.141 (6.37)[b]

[a] $n = 5$ due to the presence of an outlier as determined by a two-tailed Grubb's test ($\alpha = 0.05$)

[b] $n = 23$ due to the presence of an outlier as determined by a two-tailed Grubb's test ($\alpha = 0.05$)

Matrix effects and recovery

Matrix factors for the Protein G immunoprecipitation method were determined to be 1.01 and 0.989 at the LQC and HQC concentrations, respectively. For the TBS dilution method, the matrix factors were determined to be 1.22 and 1.24 at the LQC and HQC concentrations, respectively. As stated previously, these matrix effects were determined using a surrogate analyte approach using GFYPPDIYTEWK-$[^{13}C_6,^{15}N_2]$. There appears to be slight ion enhancement in samples analyzed by the TBS dilution method; however, this enhancement appears to be consistent across the calibration range.

Analysis of RMP1-14 in 2% hemolyzed plasma by either Protein G immunoprecipitation or TBS dilution demonstrated no significant effects at either the LQC or HQC concentrations. Bias and CV for these samples are summarized in Table 4, and in all cases, the samples met acceptance criteria.

Recovery for samples analyzed by Protein G immunoprecipitation was evaluated post-immunoprecipitation and pre-digest, and was determined to be 35.5, 33.0, and 35.3% at the LQC, MQC, and HQC concentrations, respectively. Recovery for samples analyzed by TBS dilution was evaluated following trypsin digest and desalting, and was determined to be 27.5, 22.6, and 23.4% at the LQC, MQC, and HQC concentrations, respectively. Therefore, recoveries for both methods appear to be low, but consistent across the calibration range.

Stability

Stock solutions of RMP1-14 demonstrated stored stability for 226 days under refrigerated conditions (bias = − 0.917%; CV = 10.2%) and 29 h of stability on the benchtop when stored on wet ice (bias = 4.44%; CV = 8.21%). IS stock and working solutions demonstrated 25 h of stability on the benchtop when stored on wet ice (bias = 2.71% and 0.347%; CV = 1.04% and 0.472% for IS stock and working

solutions, respectively). Whole blood samples spiked at the MQC concentration harvested to plasma after 2 h of exposure at either room temperature or wet ice demonstrated no significant changes compared to aliquots of the same samples that were harvested to plasma immediately (% peak area ratio relative to $t_0 = 101\%$ for room temperature and 107% for wet ice). All other stability evaluations also met acceptance criteria, and results are summarized in Table 4.

Preclinical application of the validated method

The fully validated TBS dilution sample preparation method was applied to samples generated in a preclinical study to determine the PK profile of RMP1-14 in the CT.26WT tumor model in BALB/c mice. PK profiles of the 2.50 and 7.50 mg/kg i.v. dose groups are presented in Fig. 2, and basic PK parameters are presented in Table 5. In this study, 15% of the 2.50 mg/kg dose group and 100% of the 7.50 mg/kg dose group samples had to undergo 10× dilutions prior to sample analysis.

Comparison to ELISA

Precision and accuracy data for both the ELISA and LC-MS/MS methods met acceptance criteria (Table 6). Data generated for the same samples ($n = 45$) by the validated TBS dilution method and by ELISA demonstrated a linear relationship when evaluated by simple unweighted linear regression. The Pearson product-moment correlation coefficient (R) was determined to be 0.993 ($p < 0.00001$), and the equation of the best-fit line was $y = (1.09 \pm 0.0188) x + (0.253 \pm 1.40)$ where data are represented as average ± standard error. A graphical representation of the linear relationship between the two methods is presented in Fig. 3a.

Analysis of the same dataset by Deming regression ($\lambda = 0.867$) indicates a line of best-fit with the equation $y = (1.09 \pm 0.0535) x + (− 0.0510 \pm 1.40)$ where data are represented as average ± standard error. The 95% confidence interval of the
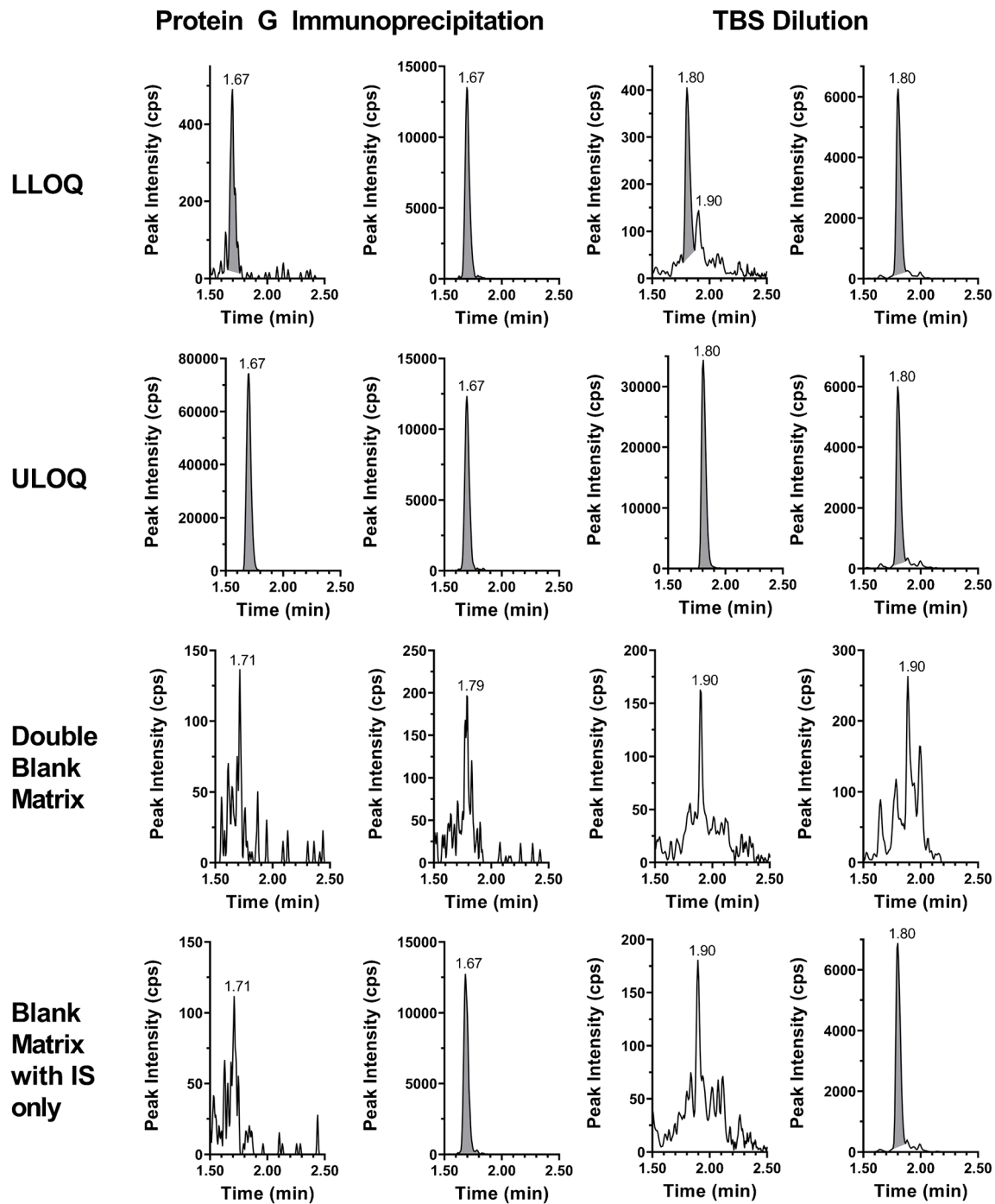
Protein G Immunoprecipitation   TBS Dilution

Fig. 1 Representative chromatograms of BALB/c mouse K_3EDTA plasma spiked with RMP1-14 and extracted by Protein G immunoprecipitation or TBS dilution, respectively, prior to trypsin digest and desalting. For each part of the figure, the signature peptide chromatogram is to the left, and the internal standard chromatogram is to the right. The figure was created using GraphPad Prism 8 based on the output from Sciex Analyst version 1.6.2

slope was determined to be 0.985 to 1.20, which includes the value of 1, therefore suggesting a lack of proportional difference between the two methods. The 95% confidence interval of the y-intercept was determined to be − 2.88 to 2.78, which includes the value of 0, therefore suggesting a lack of constant difference between the two methods.

Bland–Altman ratio analysis of the two methods suggested an average bias of 0.967 and a 95% confidence interval of 0.718 to 1.22. Of the 45 samples examined by the two methods, 44 (i.e., 97% of the samples) fell within the 95% confidence interval, suggesting that the two methods are comparable. A representation of the Bland–Altman ratio plot is presented in Fig. 3b.

Table 3 Selectivity of RMP1-14 when spiked into six unique pools of BALB/c mouse K$_3$EDTA plasma at the LLOQ concentration (0.200 μg/mL for Protein G immunoprecipitation and 0.500 μg/mL for TBS dilution). Lots analyzed are not the same for each extraction technique. Data are presented as %bias (%CV)

Matrix lot	Protein G immunoprecipitation ($n = 1$)	TBS dilution ($n = 3$)
1	91.5	12.8 (2.01)
2	73.5	15.3 (3.39)
3	28.5	15.7 (7.80)
4	131	4.73 (12.1)
5	24.0	26.2 (6.38)
6	27.0	18.7 (19.1)

Discussion

RMP1-14 is an anti-murine PD-1 mAb that has been used in research studies as a probe drug to evaluate the role of PD-1 in various disease states including cancer, autoimmune diseases, and inflammation [4–8]. Despite its extensive use, to date, no quantitative methods exist for RMP1-14 specifically. In this study, we utilized the CT.26WT murine tumor model in BALB/c mice administered RMP1-14 to evaluate the effect of intravenous administration of RMP1-14 on tumor growth, and to better correlate PD-1 inhibition to observed clinical outcomes, we aimed to generate a PK profile of RMP1-14 in BALB/c mouse plasma, which necessitated the development of a quantitative analytical method for RMP1-14. Quantitation of mAbs is usually achieved by either ELISA or LC-MS/MS assays, with LC-MS/MS-based approaches having shorter development time, larger dynamic range, and selectivity against nontarget matrix interferences [11–13]. Furthermore, when coupled to antibody-based approaches to isolate the mAb
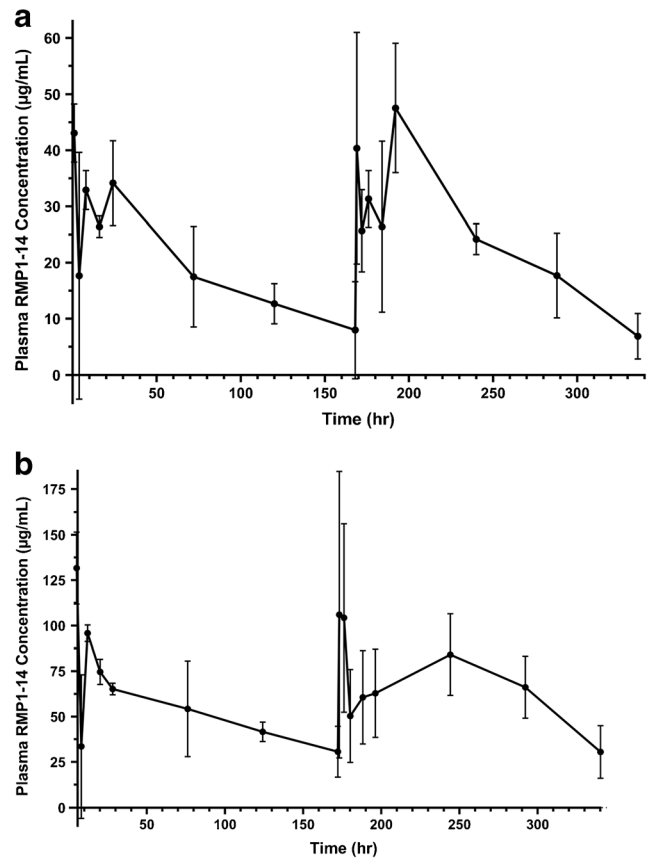
Fig. 2 Pharmacokinetic profiles of RMP1-14 administered intravenously on day 1 and day 8 of the study at 2.50 mg/kg (**a**) or 7.50 mg/kg (**b**) to BALB/c mice subcutaneously inoculated with CT26.WT murine colon cancer cells to generate a solid subcutaneous tumor ($n = 48$ per dose group). Data are presented as average ± standard deviation of RMP1-14 concentrations from three mice at each timepoint

from the biological matrix ("hybrid LC-MS/MS analysis"), LC-MS/MS methodologies offer comparable sensitivity to

Table 4 Stability data for RMP1-14 when extracted by either Protein G immunoprecipitation or TBS dilution prior to trypsin digest and desalting. Data are represented as %bias (%CV) with $n = 6$ in all cases, unless otherwise specified

Test	Protein G immunoprecipitation		TBS dilution	
	LQC (0.600 μg/mL)	HQC (40.0 μg/mL)	LQC (1.50 μg/mL)	HQC (40.0 μg/mL)
2% hemolyzed plasma	− 0.639 (9.24)	− 4.50 (6.23)[a]	− 5.33 (8.32)	10.6 (10.8)
Freeze–thaw stability at − 20 °C (4 cycles)	2.44 (11.0)	− 6.21 (7.29)	− 9.11 (9.73)	12.2 (3.44)
Freeze–thaw stability at − 80 °C (4 cycles)	− 0.694 (6.10)	− 0.875 (5.52)	1.78 (7.34)	− 3.13 (3.38)
Extract stability (146 h refrigerated)	1.03 (11.8)	2.00 (3.41)	− 0.111 (6.31)	1.38 (4.37)
Re-injection reproducibility (151 h refrigerated)	Not evaluated[b]		− 5.78 (4.69)	− 1.13 (4.43)
Matrix benchtop stability at room temperature (24 h)	2.25 (8.49)	− 4.71 (4.62)	1.78 (5.07)	4.08 (5.42)
Matrix benchtop stability on wet ice (24 h)	0.111 (14.9)	− 5.54 (5.40)	10.1 (10.7)	2.17 (3.57)
Matrix frozen stability at − 20 °C (169 days)	Not evaluated[b]		1.22 (7.16)	6.38 (9.53)
Matrix frozen stability at − 80 °C (169 days)	Not evaluated[b]		0.778 (3.11)	3.46 (2.23)

[a] $n = 5$ due to the presence of an outlier as determined by a two-tailed Grubb's test ($\alpha = 0.05$)

[b] Protein G immunoprecipitation was abandoned as a sample preparation strategy due to unacceptable selectivity at the LLOQ concentration prior to this test being performed

Table 5 Pharmacokinetic profiles of RMP1-14 administered intravenously on day 1 and day 8 of the study at 2.50 or 7.50 mg/kg to BALB/c mice subcutaneously inoculated with CT26.WT murine colon cancer cells to generate a solid subcutaneous tumor

Parameter	RMP1-14 dose	
	2.50 mg/kg	7.50 mg/kg
C_0 (μg/mL)		
Day 1	58.0	208
Day 8	46.9	107
C_{max} (μg/mL)		
Day 1	43.1	132
Day 8	47.5	106
AUC_{0-168} (h μg/mL)		
Day 1	3180	8820
Day 8	4100	11,100
$AUC_{0-\infty}$ (h μg/mL)		
Day 1	4150	13,900
$t_{1/2}$ (h)		
Day 1	84.6	116
Day 8	52.8	65.6
CL (mL/h/kg)		
Day 1	0.602	0.538
Day 8	0.610	0.674
V_{ss} (mL/kg)		
Day 1	69.0	89.9
Day 8	50.3	80.9

ELISA methods, while retaining their enhanced selectivity and dynamic range [15]. For these reasons, we decided to develop a hybrid LC-MS/MS-based method for RMP1-14.

Development of an LC-MS/MS assay for any mAb requires some knowledge of its peptide sequence in order to identify candidate signature peptides. The signature peptide needs to be specific to the target protein of interest for accurate quantitation. In the case of RMP1-14, the full intact protein sequence is unknown, but it is known that RMP1-14 is an isolate of rat IgG2a obtained following immunization of Sprague–Dawley rats with murine PD-1 protein [2], and the rat IgG2a sequence is publicly available. A query of tryptic peptides generated from the RMP1-14 reference material was performed and analyzed by high-resolution mass spectrometry against the rat IgG2a sequence to identify candidate signature peptides for further optimization. Four signature peptides were identified using this approach and were confirmed to be specific to the RMP1-14 target protein with a BLAST search of the murine proteome. Optimization of tryptic digest, desalting, and chromatographic and mass spectrometric parameters suggested that GFYPPDIYTEWK demonstrated the best sensitivity and reproducibility, which is why it was chosen as the signature peptide for RMP1-14. It should be noted that GFYPPDIYTEWK and all of the other candidate signature peptides are specific to RMP1-14 when analyzed in murine plasma, but all of these peptide sequences are present in the rat proteome and therefore are not specific to RMP1-14 if the mAb is analyzed in rat matrices.

Method development experiments involving hybrid LC-MS/MS analysis first used Protein G immunoprecipitation, since Protein G has high affinity for rat IgG2a [23]. Initial evaluation of bead binding capacity using 25.0 μL of a HQC sample suggested ~3× increase in analyte signal when the bead volume was changed from 25.0 to 50.0 μL, suggesting that bead binding capacity was being saturated at higher concentrations. To evaluate binding capacity further, a minimum required dilution (MRD) determination experiment was conducted by preparing a HQC and LQC sample in 1× TBS, BALB/c mouse plasma and BALB/c mouse plasma prediluted with 1× TBS to form 20, 33, 50, 67, and 80% plasma solutions. The plasma-based samples were compared to the TBS-based sample, and it was determined that samples prepared in 20% plasma and 33% plasma produced equivalent responses to samples prepared in 1× TBS. Further optimization in this range indicated that an MRD of 3× (i.e., 33% final plasma concentration) produced the best signal to noise at the chosen LLOQ concentration of 0.200 μg/mL. Validation of the Protein G immunoprecipitation approach with the 3× MRD demonstrated acceptable precision and accuracy, as well as stability of RMP1-14 in BALB/c mouse plasma; however, selectivity of a spiked LLOQ sample in individual pools of control plasma demonstrated unacceptable results. Examination of analyte recovery as well as QC samples diluted following Protein G immunoprecipitation suggested that bead binding capacity was still being saturated, since the observed recovery was low, and the diluted QC samples did not meet acceptance criteria.

Table 6 Precision and accuracy data for RMP1-14 in the same spiked samples when analyzed by either ELISA or LC-MS/MS. Data are represented as %bias (%CV) with $n = 3$ in all cases

Analytical technique	R^2	LLOQ QC (0.500 μg/mL)	LQC (1.50 μg/mL)	MQC (20.0 μg/mL)	HQC (40.0 μg/mL)	DQC (250 μg/mL)[a]
ELISA	0.995[b]	− 4.13 (10.4)	− 8.02 (5.87)	7.82 (2.84)	8.50 (3.23)	3.77 (3.03)
LC-MS/MS	0.996[c]	− 4.12 (4.56)	− 4.01 (14.8)	− 1.50 (5.02)	0.505 (3.96)	− 3.45 (7.65)

[a] Samples were diluted 10× using control BALB/c mouse K$_3$EDTA plasma prior to analysis

[b] Data fitted using a four-parameter logistic regression model with $1/y^2$ weighting.

[c] Data fitted using a linear regression model with $1/x^2$ weighting

Fig. 3 Comparison of data generated by ELISA and LC-MS/MS for RMP1-14 in the same samples by simple linear regression (**a**) and Bland–Altman ratio analysis (**b**). Key: filled triangle, calibration standards; filled square, QC samples; and filled circle, incurred samples
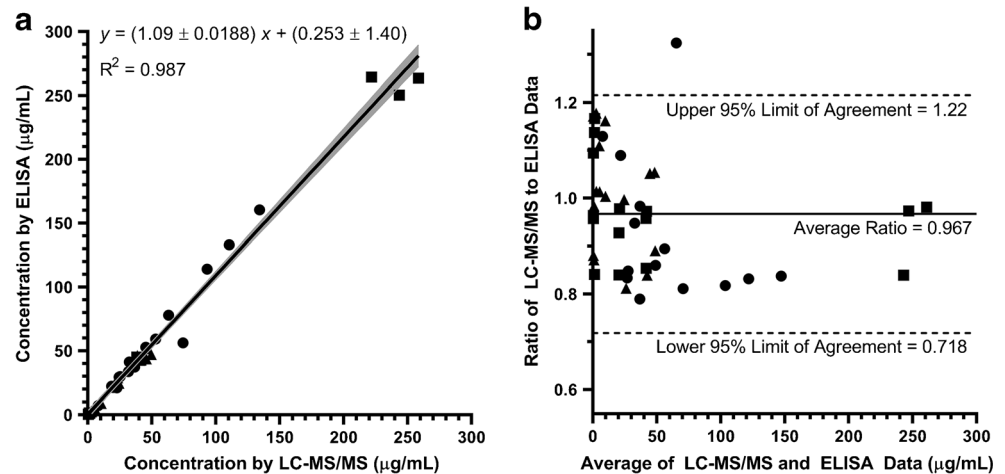
In addition to having high affinity for rat IgG2a, Protein G also has high affinity for immunoglobulins present in other animal species, particularly murine IgG1, IgG2a, and IgG2b [23]. For BALB/c mice, the endogenous serum immunoglobulin concentration is 200–350 μg/mL for IgG1, 100–250 μg/mL for IgG2a, and 150–730 μg/mL for IgG2b, depending on the age of the mice [24], all of which are considerably higher than the amount of RMP1-14 being spiked into the samples. Therefore, it is possible that endogenous immunoglobulins in the BALB/c mouse plasma were outcompeting RMP1-14 for binding sites on the Protein G beads, and therefore, a higher MRD would be sufficient to overcome this lack of selectivity at the LLOQ concentration. However, increasing the MRD to 20× and raising the LLOQ to 0.500 μg/mL was still not sufficient to overcome the observed lack of selectivity. Therefore, the use of Protein G immunoprecipitation was abandoned as a sample preparation strategy for RMP1-14 in mouse BALB/c plasma.

Alternative sample preparation strategies including the use of a stable isotope-labeled IgG (SILu™MAb, Sigma Aldrich) prior to Protein G immunoprecipitation, sample pre-treatment with Protein A followed by extraction using Protein G immunoprecipitation, ACN precipitation followed by analysis of the protein pellet, and direct digestion of the sample following MRD were all evaluated at MRDs of 3× and 20×. None of the strategies involving Protein G immunoprecipitation were able to overcome the lack of selectivity at the LLOQ concentration, and the ACN protein precipitation method clogged the μSPE plate used during desalting. However, direct digest of the matrix following a 20× MRD demonstrated acceptable selectivity at the LLOQ concentration in five out of the six unique pools of BALB/c mouse plasma, and therefore, this approach was evaluated during method validation. As seen from the "Results" section, this approach met acceptance criteria for all validation tests, and therefore, we were able to validate a quantitative LC-MS/MS method for RMP1-14 using a signature peptide approach. For this assay, we did not evaluate the

use of a customized immunoprecipitation approach to sample preparation (e.g., streptavidin magnetic beads coupled to biotinylated murine PD-1 protein, which would be highly selective for RMP1-14) due to the cost and labor that would be involved.

It is interesting to note that samples extracted by Protein G immunoprecipitation demonstrated acceptable results for all stability tests performed. These results appear to indicate that the Protein G binding site of RMP1-14 and the region of RMP1-14 that contains GFYPPDIYTEWK are both unaffected by up to four freeze–thaw cycles as well as exposure to room temperature or wet ice conditions for up to 24 h. Additionally, binding of RMP1-14 to Protein G appears to be unaffected by the presence of up to 2% hemoglobin in the plasma. These results are of little relevance to this analytical method, but may be useful in future method developments involving RMP1-14.

The validated sample analysis method was successfully applied to the analysis of plasma samples collected from BALB/c mice dosed intravenously with RMP1-14 at 2.50 or 7.50 mg/kg dose levels. None of the incurred samples demonstrated concentrations below the LLOQ, suggesting that the method was adequately sensitive, though 15% of the 2.50 mg/kg dose group and 100% of the 7.50 mg/kg dose group samples had to undergo 10× dilutions prior to sample analysis. However, as stated previously, the ULOQ for this assay was constrained by the manufacturer-supplied concentration of the reference material (6–8 mg/mL) and the desire to ensure that the final nonplasma component of each calibration standard and QC sample was ≤5%. Interpretation of the generated PK profiles and their relationship to observed clinical effects is beyond the scope of this manuscript.

Comparison of the validated LC-MS/MS method to a commercially available rat IgG2a ELISA kit indicated that the two analytical techniques were comparable. Simple linear regression suggested excellent correlation between the two techniques, and Deming regression suggested neither a constant

nor proportional bias existed between the methods. Bland–Altman ratio analysis also indicated comparability between the methods, but it would appear that a correction factor of 0.967 should be applied, should data acquired by one technique be compared against the other (i.e., divide LC-MS/MS data by 0.967 prior to comparison with ELISA data, or multiply ELISA data by 0.967 prior to comparison with LC-MS/MS data). While the ELISA method was not formally validated prior to use, no interferences were observed in the blank samples, and the calibration standards and QC samples met FDA-defined acceptance criteria for ligand binding assays [19].

In making a general comparison between ELISA- and LC-MS/MS-based approaches to mAb analysis, we should note that ELISA assays are generally easier to use, require less specialized equipment, have higher throughput, and are usually more sensitive than LC-MS/MS assays [14]. However, ELISA-based assays are also less selective and less amenable to multiplexing compared to LC-MS/MS assays [14]. Furthermore, ELISAs require significant initial investment in identifying or generating highly specific capture and detection antibodies and maintaining and bridging reagents across long-term studies [14]. All of this could lead to development and validation timelines spanning months, which impacts final data acquisition and delivery [14]. By comparison, LC-MS/MS method development and validation timelines are usually on the order of weeks, and the increased detector specificity for the target signature peptide ensures that more generic sample preparation approaches such as Protein G immunoprecipitation or even simple digests of the matrix are sufficient to obtain usable results [12, 13]. Additionally, the use of internal standards in LC-MS/MS analysis minimizes inter- and intra-assay variance, as well as mitigates lot-to-lot variance in any reagents used [12, 13].

For this study involving analysis of RMP1-14, all of the above are true, particularly the difference in sensitivity (LLOQ = 0.25 ng/mL for ELISA and 0.500 μg/mL for LC-MS/MS). The difference in sensitivity is somewhat unsurprising, since the ELISA method is able to analyze for RMP1-14 in its intact state whereas the LC-MS/MS method is dependent on the efficiency of the tryptic digest required to produce GFYPPDIYTEWK. However, we did not need the additional sensitivity offered by ELISA in the analysis of incurred samples, and we were also fortunate to find a commercially available fit-for-purpose ELISA kit which saved considerable time that would otherwise have been spent on method development for the ELISA analysis. By contrast, total development and validation time for the two approaches used for LC-MS/MS analysis of RMP1-14 was approximately 6 weeks each. While it appears that both ELISA and LC-MS/MS are suitable methodologies for the analysis of RMP1-14, the ultimate choice of analytical technique is dependent on the resources available to the individual laboratory, scope and challenges of the project, and timeline.

Conclusions

This manuscript presents the first known quantitative assay for the analysis of the anti-murine PD-1 mAb RMP1-14. The validated method in BALB/c mouse plasma demonstrated acceptable accuracy, precision, and robustness based on US FDA guidelines for validation of small molecules by LC-MS/MS and was comparable to a commercially available fit-for-purpose ELISA assay capable of measuring RMP1-14. Application to a preclinical PK study demonstrated that the LC-MS/MS method was adequately sensitive and suitable for the analysis of incurred samples. Overall, this method presents a novel tool that may be used in future immune-oncology studies to better evaluate RMP1-14 activity and its impact on observed clinical outcomes.

Compliance with ethical standards

In vivo studies were performed under protocols approved by the Covance Institutional Animal Care and Use Committee, and were in compliance with the U.S. Department of Agriculture's Animal Welfare Act (9 CFR Parts 1, 2, and 3) and the U.S. National Research Council's Guide for the Care and Use of Laboratory Animals.

Conflict of interest The authors declare that they have no conflict of interest.

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