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Liquid Chromatography-Multiple Reaction Monitoring-Mass Spectrometry Assay for Quantitative Measurement of Therapeutic Antibody Cocktail REGEN-COV Concentrations in COVID-19 Patient Serum

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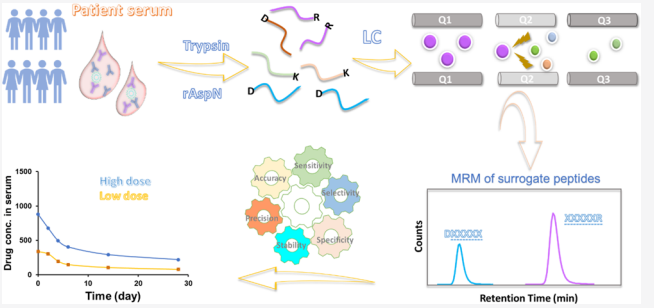
ABSTRACT: REGEN-COV is a cocktail of two human IgG1 monoclonal antibodies (REGN10933 + REGN10987) that targets severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike protein and has shown great promise to reduce the SARS-CoV-2 viral load in COVID-19 patients enrolled in clinical studies. A liquid chromatography-multiple reaction monitoring-mass spectrometry (LC-MRM-MS)-based method, combined with trypsin and rAspN dual enzymatic digestion, was developed for the determination of total REGN10933 and total REGN10987 concentrations in several hundreds of pharmacokinetic (PK) serum samples from COVID-19 patients participating in phase I, II, and III clinical studies. The performance characteristics of this bioanalytical assay were evaluated with respect to linearity, accuracy, precision, selectivity, specificity, and analyte stability before and after enzymatic digestion. The developed LC-MRM-MS assay has a dynamic range from 10 to 2000 μg/mL antibody drug in the human serum matrix, which was able to cover the serum drug concentration from day 0 to day 28 after drug administration in two-dose groups for the clinical PK study of REGEN-COV. The concentrations of REGEN-COV in the two-dose groups measured by the LC-MRM-MS assay were comparable to the concentrations measured by a fully validated electrochemiluminescence (ECL) immunoassay.



INTRODUCTION

REGEN-COV (REGN10933 + REGN10987, also referred to as casirivimab and imdevimab, respectively) is an investigational antibody cocktail therapy developed by Regeneron Pharmaceuticals, Inc. for the treatment of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).[1−3] The antibody cocktail includes two humanized IgG1 monoclonal antibodies (REGN10933 and REGN10987), which are designed to target nonoverlapping epitopes on the SARS-CoV-2 spike protein, thereby blocking the interaction of SARS-CoV-2 virus with human ACE2[1] and preventing viral escape due to rapid genetic mutation of the virus.[4] A recent clinical study has shown that REGEN-COV therapy can reduce viral load and improve symptoms for nonhospitalized COVID-19 patients, especially those who were seronegative or had high viral loads at baseline.[3] Based on the promising results from the clinical investigation, REGEN-COV was granted Emergency Use Authorization (EUA) by the U.S. Food and Drug Administration (FDA) in November 2020 for the treatment of recently diagnosed, mild-to-moderate COVID-19 in adults and pediatric patients at least 12 years of age and weighing at least 40 kg and are at high risk for progressing to severe COVID-19 and/or hospitalization.

Due to the urgent need for an effective therapy to treat COVID-19, the timelines for drug discovery and preclinical validation processes of REGEN-COV were highly compressed after the outbreak of the virus was designated as a global pandemic. Within 2 months of lead candidate selection for potent neutralizing antibodies against SARS-CoV-2, several clinical trials of REGEN-COV were initiated in hospitalized and ambulatory patients. As part of the clinical study, the

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determination of circulating drug concentrations in patients is critical for pharmacokinetic (PK) characterization of protein therapeutic and drug dose optimization. To meet this need and manage the accelerated development for a COVID-19 therapy, we developed and qualified a fit-for-purpose liquid chromatography-multiple reaction monitoring-mass spectrometry (LC-MRM-MS) assay for the REGEN-COV PK study in 1 month—a much shorter timeframe than that required for the development of a conventional ligand-binding assay. Unlike the ligand-binding assay, the LC-MRM-MS assay does not require highly specific affinity capture and detection reagents for antibody therapeutics, which typically take several months to develop and produce. In addition, the LC-MRM-MS assay also provides a wide dynamic range, good accuracy and precision, excellent selectivity and specificity for the quantification of protein-based biopharmaceuticals in serum matrix.[5] Recently, LC-MRM-MS has become a more frequently adopted bioanalytical strategy for both preclinical[6−8] and clinical[9−11] sample analysis due to continuous improvement in the performance of LC-MS instrumentation.

The quantification of total antibody drug concentration, including free and bound antibodies, in human serum samples by LC-MRM-MS assay is based on the measurement of ion intensities of the surrogate peptides derived from the variable complementarity-determining regions (CDRs) of the antibody drugs.[12] To process patient serum samples, typically, a few microliters of serum sample was reduced, alkylated, and then underwent protease digestion. Stable heavy isotope-labeled proteins or surrogate peptides are usually used as internal standards (ISs) to normalize the signal variation from sample processing and instrument performance fluctuation. The sensitivity, selectivity, and specificity of the assay are reliant on the unique CDR peptides that have been selected for quantification. For the REGEN-COV antibody cocktail, the LC-MRM-MS can be readily multiplexed to measure multiple drug analytes simultaneously. Despite limited throughput due to the chromatographic separation, the developed LC-MRM-MS method met the required dynamic range, sensitivity, selectivity, stability, and specificity for the early measurement of drug concentrations of REGEN-COV in a limited number of serum samples in the clinical trials. The method development and performance characterization for this LC-MRM-MS assay are presented below. The concentrations of REGEN-COV in two-dose groups of ambulatory patients measured by LC-MRM-MS assay were compared with the results obtained from a fully validated ligand-binding immunoassay, which demonstrated that the two assays were in good agreement. This work sets an example as a fit-for-purpose application of LC-MRM-MS for clinical sample analysis when there are challenges to deliver a validated immunoassay to meet the urgent timeline, or high-quality anti-idiotypic antibody reagents for ligand-binding assay are not available.

MATERIALS AND METHODS

Chemicals and Reagents. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl), trifluoroacetic acid (TFA, LC-MS grade), 0.1% formic acid (v/v) in water (LC-MS grade), and 0.1% formic acid (v/v) in acetonitrile (LC-MS grade) were purchased from Thermo Fisher Scientific (Rockford, IL). Ultrapure 1 M Tris−HCl pH 8.0 was obtained from Invitrogen (Carlsbad, CA). Urea and iodoacetamide (IAM) were purchased from Sigma-Aldrich (St. Louis, MO). Trypsin (Mass Spectrometry grade) and rAspN were purchased from Promega (Madison, WI). Pooled human serum and single human serum from 10 individuals were purchased from Innovative Research (Novi, MI). AUQA grade custom synthetic heavy peptides for internal standards, LLIYAASNLETGVPSR*(10 Da), DTAV*(6 Da) YYCASGS, were ordered from Thermo Fisher Scientific (Rockford, IL). REGN10933 and REGN10987 drug substance (DS) were developed and obtained from Regeneron Pharmaceuticals (Tarrytown, NY). The COVID-19 patient serum samples were from a clinical trial of REGEN-COV sponsored by Regeneron Pharmaceuticals (ClinicalTrials.gov Identifier: NCT04425629).

Preparation of Standard Solutions. Stock solutions of REGEN-COV in human serum were made by co-spiking REGN10933 and REGN10987 DS into pooled human serum. Calibration standards (20, 25, 30, 50, 100, 250, 500, 1000, 2000 μg/mL for REGN10933; 10, 20, 25, 30, 50, 100, 250, 500, 1000, 2000 μg/mL for REGN10987) were made through a serial dilution of the stock solution using the pooled human serum. Five qualification QC standards including the upper limit of quantitation (ULOQ, 2000 μg/mL REGN10933, 2000 μg/mL REGN10987), high QC (HQC, 1500 μg/mL REGN10933, 1500 μg/mL REGN10987), mid QC (MQC, 750 μg/mL REGN10933, 750 μg/mL REGN10987), low QC (LQC, 60 μg/mL REGN10933, 30 μg/mL REGN10987), and the lower limit of quantitation (LLOQ, 20 μg/mL REGN10933, 10 μg/mL REGN10987) were also prepared by co-spiking REGN10933 and REGN10987 DS into the pooled human serum and serial dilutions.

LLOQ (20 μg/mL REGN10933, 10 μg/mL REGN10987) spiked individual human serum samples were prepared by co-spiking REGN10933 and REGN10987 DS into 10 individual human serum blanks. For the drug specificity assay, the QC standards containing one drug were made by serial dilution of stock solution of the antibody drug using the pooled human serum as the diluent. The QC standards containing one drug with the presence of coadministered drug as a matrix background were made from serial dilution of the stock solution using 2 mg/mL of the coadministered drug in the pooled human serum as diluent.

Digestion of Serum Samples. Prior to sample processing, serum samples (calibration standards, QC standards, and patient samples) were thawed on ice. The serum sample digestion was conducted in a 96-well plate (0.5 mL, polypropylene, Agilent Technologies, Santa Clara, CA). Five microliters of the serum sample was added to each sample well prefilled with 80 μL of denaturation solution (10 mM TCEP, 8 M urea). The 96-well plate was sealed with an adhesive plate seal (Waters, Milford, MA) and heated at 80 °C for 10 min on Thermomixer C (Eppendorf, Hamburg, Germany) at 650 rpm. After cooling to room temperature, 15 μL of 0.25 M IAM was added to each sample well and the plate was incubated by shaking at 650 rpm in dark for 30 min under room temperature. Prior to use, digestion solution containing two enzymes and two IS peptides was made by reconstitution of 200 μg of trypsin, 100 μg of rAspN, 150 μL of REGN10933 IS stock solution (5 pmol/μL), and 100 μL of REGN10987 IS stock solution (5 pmol/μL) in 9 mL of 0.1 M Tris buffer. Following alkylation, 10 μL of each sample was transferred to a second 96-well plate and mixed with 90 μL of digestion solution containing two enzymes and IS peptides. The sample plate was sealed and incubated at 37 °C for 3 h with 650 rpm shaking. When the digestion finished, 10 μL of 10% TFA was

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added to each sample well to quench the reaction. The sample plate was spun at 700 rpm for 1 min prior to LC-MRM-MS analysis.

Reduced Peptide Mapping of Antibody Drug Standards. REGN10933 and REGN10987 DS stock solutions were diluted to 80 mg/mL by 0.1 M Tris buffer, and then digested under similar conditions as described for the serum samples. Five microliters of the antibody drug standard was added to 80 μL of denaturation solution (10 mM TCEP, 8 M urea) and heated for 10 min at 80 °C. After alkylation with IAM, the drugs were digested by two enzymes with 2:1:40 trypsin/rAspN/substrate ratio, in 0.1 M Tris buffer (pH 8) at 37 °C for 3 h. The digestion reaction was quenched by adding 10% TFA solution prior to reversed-phase (RP) LC-MS/MS analysis. The trypsin/rAspN digests of antibody drug substances were analyzed by a Waters I-Class UPLC system (Waters, Milford, MA) coupled with Thermo Scientific Q Exactive plus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Approximate 6 μg of the digested sample was loaded onto a C18 column (ACQUITY UPLC peptide BEH 1.7 μm, 2.1 mm × 150 mm, Waters) and separated by a 90 min gradient with 0.05% TFA in water as mobile phase A, and 0.045% TFA in acetonitrile as mobile phase B (0−5 min, 0.1%B; 5−80 min, 0.1−35%B; 80−85 min, 35−90%B; 85−90 min, 90%B). The mobile phase flow rate was 0.25 mL/min. The column temperature was set at 40 °C. The instrument parameters of the HESI source of MS were set as follows: spray voltage 3.8 kV, auxiliary gas 10, auxiliary gas temperature 250 °C, capillary temperature 350 °C, and S-lens RF level 50. A top five data-dependent acquisition method (DDA) was applied for MS/MS data acquisition during online LC separation. The following settings were applied for MS1 scans: resolution 70k, AGC target 1×10^6, maximum ion injection time 50 ms, and scan range 300−2000 m/z. For HCD MS/MS scans, the following settings were applied: isolation window 4 m/z, NCE 27, scan range 200−2000 m/z, 17.5k resolution, AGC target 1×10^5, and maximum ion injection time 100 ms. The raw data files were searched against the antibody fasta sequences by Byonic (version 3.9.4, Protein Metrics, San Carlos, CA) for protein sequence coverage analysis.

LC-MRM-MS Method. The LC-MRM-MS experiments were performed using an Agilent Infinity II UPLC system coupled with a 6495 Triple Quadrupole Mass Spectrometer (Agilent Technologies, Santa Clara, CA). Ten microliters of a digested serum sample, proportional to approximately 45 nL of the original serum, was loaded onto a C18 column (ACQUITY UPLC BEH300 1.7 μm, 2.1 mm × 100 mm, Waters) and separated by reversed-phase gradient elution using mobile phase A as 0.1% formic acid in water and mobile phase B as 0.1% formic acid in acetonitrile at a flow rate of 0.3 mL/min. Prior to each injection, the sample injection path was sequentially flushed with IPA/ACN/H_2O v/v/v (3:1:1), ACN/H_2O/FA v/v/v (25:75:0.1), and ACN/H_2O/FA v/v/v (5:95:0.1). The LC gradient for MRM experiments was set as follows: 0−0.5 min, 5%B; 0.5−16 min, 5−25%B; 16−18 min, 25−90%B; 18−20 min, 90%B; 20−20.5 min, 90−5%B, and 20.5−25 min, 5%B. The column temperature was set at 60 °C, and the autosampler was maintained at 7 °C during sample analysis.

The triple quadrupole MS ion source parameters were set as follows: gas temperature 200 °C, gas flow rate 12 L/min, nebulizer gas 20 psi, sheath gas temperature 300 °C, sheath gas flow 11 L/min, capillary voltage 3500 V, and nozzle voltage 500 V. Time-scheduled MRM transitions for two surrogate peptides and two IS peptides with parameters of each transition channel are listed in Table S1 and were applied for all of the quantitative analysis experiments.

Data Analysis. The raw data from LC-MRM-MS experiments were analyzed using Agilent MassHunter Quantitative Analysis software. The extracted ion chorogram (XIC) peak areas of the monitored transitions were integrated with the Agile2 algorithm. To construct the calibration curve for each drug, the peak areas of a surrogate peptide from calibration standards normalized by the peak areas from the corresponding coeluting IS peptide were plotted against their respective nominal concentrations using a $1/x^2$ weighted three-parameter quadratic model (with variable weight for each point of the standard curve), from which all other readings were subsequently calculated. The equation for quadratic fit is

$$y = ax^2 + bx + c$$

where y is the ratio of the XIC peak area of the surrogate peptide and that of the corresponding IS peptide, x is the concentration of drug (μg/mL), and a, b, c are quadratic coefficient, linear coefficient, and constant term, respectively.

The weight for each point of the standard curve is inversely proportional to the analyte concentration. The calibration curve parameters were automatically computed by Agilent MassHunter Quantitative Analysis software.

Electrochemiluminescent Immunoassay. The assay procedures employed streptavidin microplates coated with either biotinylated mouse anti-REGN10933 monoclonal antibody or biotinylated mouse anti-REGN10987 monoclonal antibody. REGN10933 and REGN10987 captured on plates specific for each molecule were detected using two ruthenylated, noncompeting mouse monoclonal antibodies that are specific to either REGN10933 or REGN10987. The electrochemiluminescent signal generated from the ruthenium label when a voltage applied to the plate was measured by the MSD reader. The measured electrochemiluminescence is proportional to the concentration of total REGN10933 or total REGN10987 in the serum samples.

RESULTS AND DISCUSSION

Method Development for LC-MRM-MS Assay. Both REGN10933 and REGN10987 are dimer molecules that are composed of a pair of light chains and a pair of heavy chains. REGN10933 light chain contains 221 amino acid residues, and its heavy chain contains 450 amino acid residues. REGN10987 light chain contains 216 amino acids residues, and its heavy chain contains 450 amino acid residues. Because IgG proteins exist with high abundance in human serum, and greater than 93% of amino acid sequence of these two humanized IgG1 drugs are identical to endogenous serum IgG, the selection for suitable surrogate peptides for MRM-based IgG antibody drug quantification are restricted to the peptides derived from CDR regions (typically three from heavy chain, and three from light chain) of the variable domains. The peptides from the constant regions of antibody drugs cannot be differentiated from those derived from endogenous antibodies in human serum.

To select suitable surrogate peptides for MRM quantification, the following considerations were applied to screen the candidate peptides generated by protease cleavage in the CDR region of the human IgG drugs: (1) no identical BLAST match

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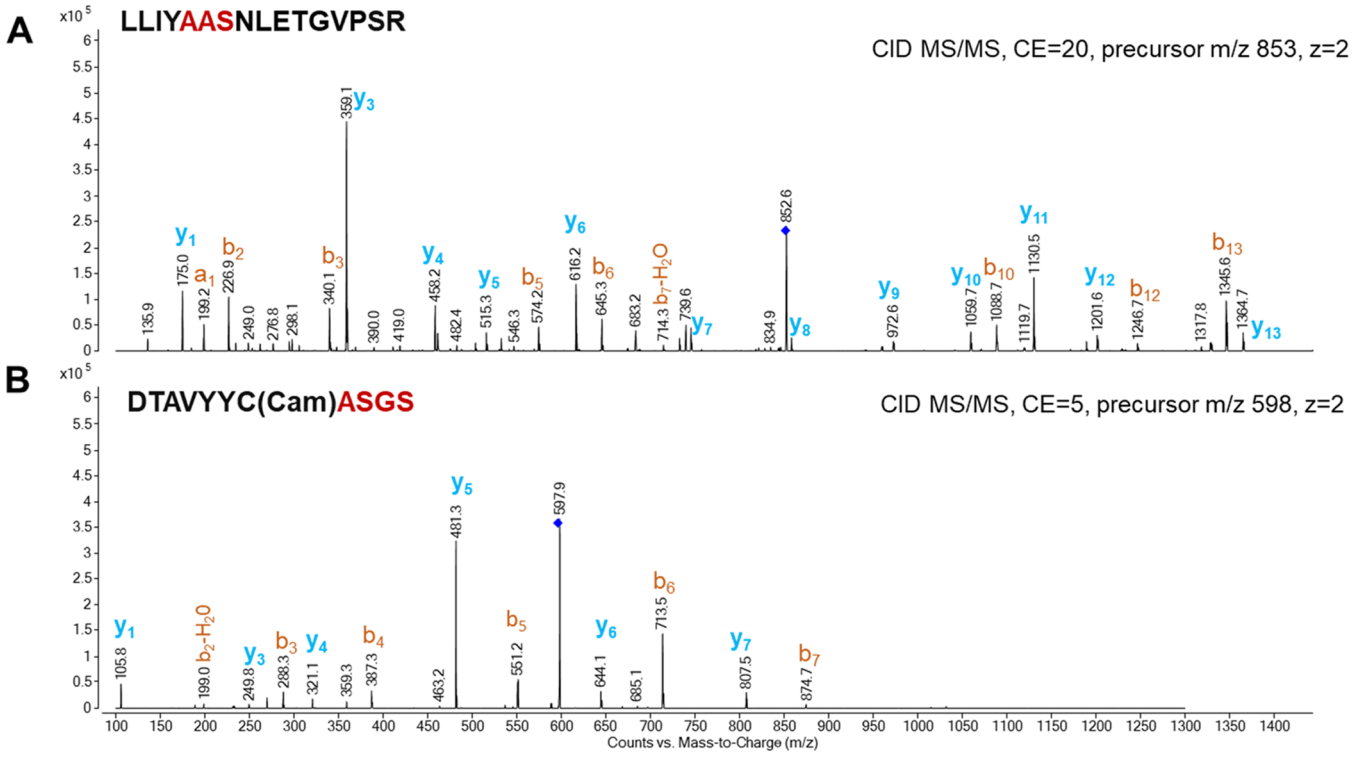

Figure 1. CID MS/MS spectra of surrogate peptides for REGN10933 (A) and REGN10987 (B) generated from trypsin and rAspN digestion. The red letters in the peptide sequences indicate CDRs of the IgG antibody. The spectra were acquired under the time-scheduled product ion scan mode of QQQ instrument during online LC separation of digests of antibody drug standard.

hit in Uniprot human proteome database (https://www.uniprot.org/blast/); (2) peptide length shorter than 20 amino acid residues; (3) sequence does not contain sites prone to missed cleavages during enzymatic digestion (e.g., KR, RDR) for trypsin cleavage; and (4) sequence does not contain sites susceptible to *in vivo* biotransformation or residues prone to partial modification during sample processing, such as methionine. By applying these criteria to examine the *in silico* trypsin digestion-generated CDR peptides of REGN10933 and REGN10987, we found that only one peptide, LLIYAASNLETGVPSR, which is from the light chain CDR2 of REGN10933, can serve as the surrogate peptide for REGN10933 quantification; none of the tryptic peptides from REGN10987 CDR regions could satisfy all of the criteria list above (Table S2). In this case, we had switched to another protease, rAspN, to generate a unique surrogate peptide with appropriate length from heavy chain CDR3, DTAVYYCASGS, for REGN10987 quantification (Table S2).

Prior to serum sample digestion, reduced peptide mapping of antibody drug substances was performed by RPLC coupled with DDA MS/MS to evaluate the trypsin/rAspN digestion efficiency. To mimic the patient serum digestion condition, antibody drug standards were first heated in 8 M urea and 10 mM TECP solution at 80 °C for 10 min, for the purpose of viral deactivation, protein denaturation, and disulfide reduction. Following this step, the reduced antibodies were alkylated and digested by two enzymes in Tris buffer, with 2:1:40 trypsin/rAspN/substrate ratio, at 37 °C for 3 h. The HCD MS/MS data acquired with Orbitrap MS were searched against the FASTA sequences of the two IgG drugs, and the results showed close to 100% sequence coverage for both antibody drugs, with only one missing short peptide not retained by reversed-phase LC. Similar digestion conditions were later applied to serum samples spiked with antibody drug substances. To ensure a good digestion efficiency, the actual enzyme: substrate ratio applied for serum sample analysis was slightly greater compared to that applied for antibody drug substances digestion, considering that the normal range for total protein amount in human serum is 60−80 mg/mL. Although heating the proteins in 8 M urea under high temperature causes some carbamylation at protein N-termini and lysine side chains, this modification does not impact the LC-MRM-MS quantification of REGN10933 and REGN10987 as the surrogate peptide candidates do not contain lysine residues.

The trypsin digests of REGN10933 DS and rAspN digests of REGN10987 DS were used to optimize MRM transition parameters on an Agilent QQQ system. Time-scheduled product ion scan experiments for surrogate peptide candidates during reversed-phase LC separation were performed to select the best transition and collisional energy. Based on the CID MS/MS spectra acquired (Figure 1), the transition from the +2 precursor ion to y_3 product ion was selected to monitor the abundance of the REGN10933 surrogate peptide LLIYAASNLETGVPSR (Figure 1A); and transition from the +2 precursor ion to y_5 product ion was selected to monitor the abundance of the alkylated REGN10987 surrogate peptide DTAVYYC(Cam)ASGS (Figure 1B). Notably, the optimal collisional energy for this doubly charged REGN10987 surrogate peptide is around 5 V, which is much smaller compared to the typical collisional energy required for doubly charged tryptic peptides.

The selected transition channels of surrogate peptides and their corresponding transitions for internal standard peptides were examined for the human serum matrix background interference. Pooled human serum blank as well 10 individual serum blank samples (five female, five male) digested with a

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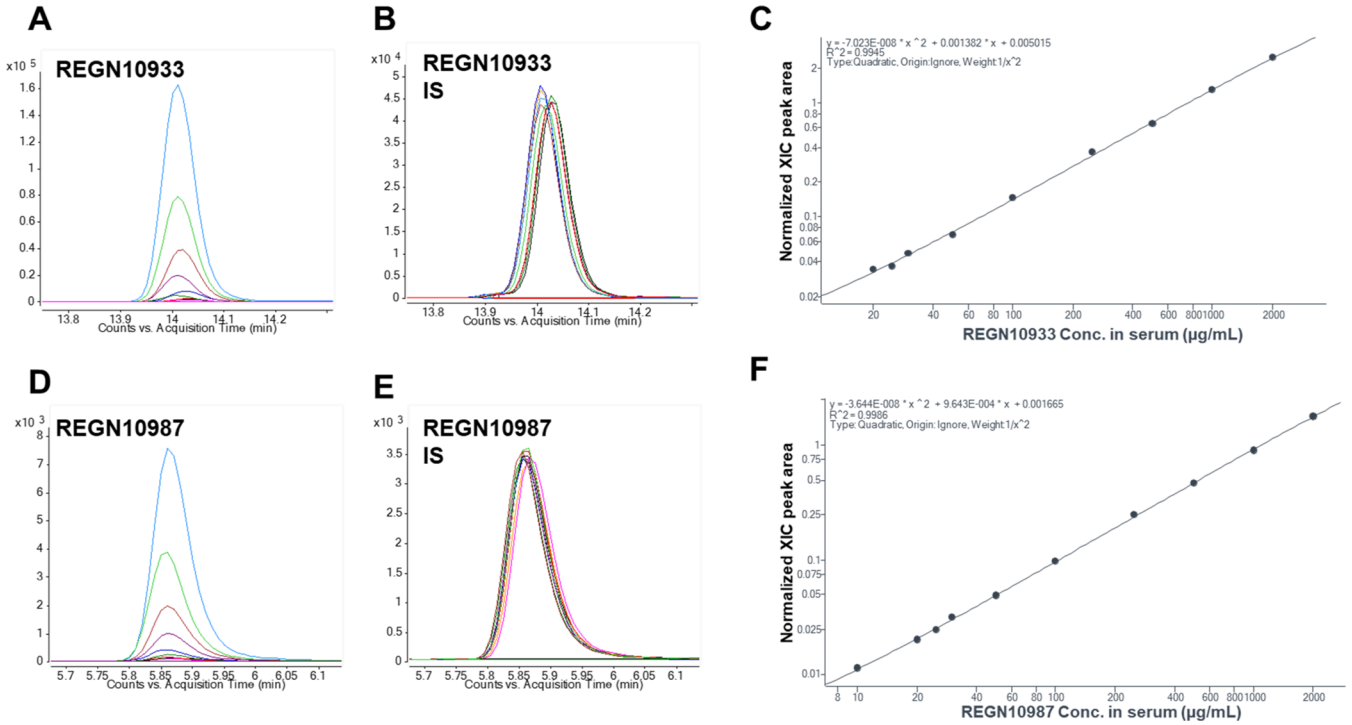

Figure 2. Extracted ion chromatograms of surrogate peptides (A, D), internal standards (B, E), and calibration curve plots (C−F) of REGN10933 (A−C) and REGN10987 (D−F) generated from LC-MRM-MS of calibration standards with individual drug concentration ranging from 10 to 2000 μg/mL in pooled human serum.

combination of trypsin and rAspN were analyzed with a 16 min reversed-phase LC gradient and time-scheduled MRM acquisition of the four transition channels. Signal interference was not observed from neither the light nor the heavy transition channels of the two surrogate peptides. After evaluation of the matrix interference of the selected transitions for the two surrogate peptides, the instrument parameters were further optimized under MRM mode, and the parameters list in Table S1 were applied for the assay qualification and patient sample analysis.

LC-MRM-MS Assay Qualification. Prior to application on clinical sample analysis, we evaluated the performance of the developed LC-MRM-MS assay using the following most critical parameters to fit the purpose of this study: (1) linearity, (2) accuracy and precision, (3) selectivity, (4) specificity, and (5) analyte stability before and after sample digestion.

Linearity. Linearity is defined as the proportionality of the instrument's response to standard concentrations with the appropriate statistical model of linear or nonlinear regression. In this LC-MRM-MS assay, linearity was determined by the normalized extracted ion chromatogram (XIC) peak areas of 9 nonzero standards for REGN10933 and 10 nonzero standards for REGN10987 over 3 days. Representative XICs of surrogate peptides and IS peptides from the calibration standards, as well as the calibration curves for the two antibody drugs, are shown in Figure 2. The back-calculated drug concentrations of the calibration standards using the normalized responses and the respective standard curve equation were used to estimate the accuracy of the standards using the following equation.

$$\text{accuracy \% (\%ACC)} = 100\% \times (\text{measured concentration}/\text{nominal concentration})$$

The statistical profiles of the measured concentrations of all of the nonzero standards for both drugs from three independent experiments are summarized in Table S3. The average %ACC values of all of the standards ranged from 89 to 105% for REGN10933 and 92 to 106% for REGN10987; the CV% of measured concentration values for all nonzero standards varied from 2.6 to 11% for REGN10933 and 0.7 to 12% for REGN10987. These results met the criteria for bioanalysis that %ACC should be within ±20% of the nominal value for nonzero standards, except for standards at the LLOQ or ULOQ level, which must be within ±25%, and CV% must be ≤20% for all nonzero standards, except for standards at the LLOQ or ULOQ level, which must be ≤25%.[13]

Accuracy and Precision. Accuracy is the closeness of agreement between a measured result and its theoretical true value and is expressed as %ACC. Precision is the quantitative measure of the random variation between repeated measurements of the same sample, which is expressed as the percentage of coefficient of variation (CV% Conc). The intraday accuracy and precision were determined by five replicates of qualification QCs per run, in three independent measurements over 3 days. The interday accuracy and precision were determined by three independent measurements from sample preparation to LC-MRM-MS analysis, each with five replicates of qualification QCs, over 3 days. Data for the intraday and interday accuracy and precision parameters are presented in Table S4. The statistical profiles of the interday accuracy and precision assessment show that %ACC values for REGN10933 of all five QCs ranged from 95 to 103% and %ACC for

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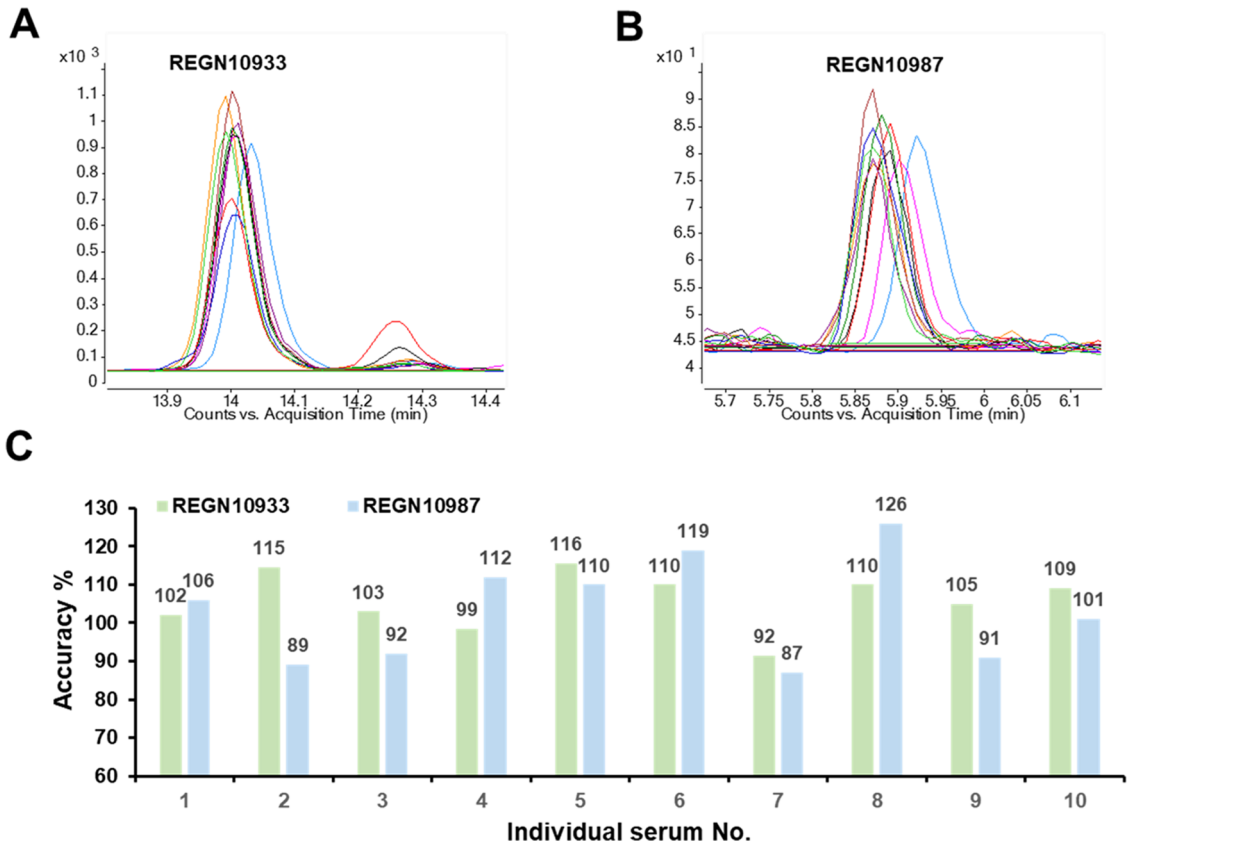

Figure 3. LC-MRM-MS assay selectivity for REGN10933 and REGN10987 at LLOQ. (A, B) Overlaid XICs of surrogate peptides of REGN10933 (A) and REGN10987 (B) from 10 individual naïve human serum samples cospiked with 10 μg/mL REGN10987 and 20 μg/mL REGN10933. (C) Measured accuracy percentage of drug concentrations in 10 individual human serum samples with drugs spiked at the LLOQ level.

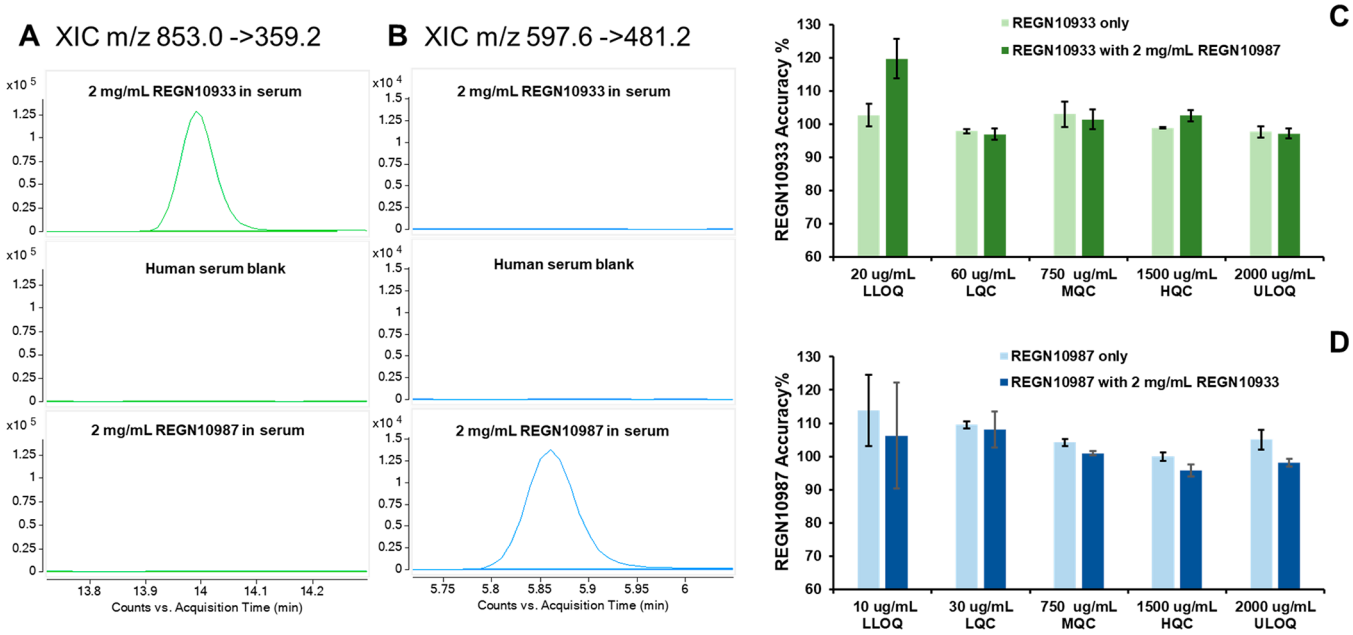

Figure 4. LC-MRM-MS assay specificity for REGN10933 and REGN10987 five QC levels. (A, B) XICs of MRM transitions for surrogate peptides of (A) REGN10933 and (B) REGN10987. Top panel, 2 mg/mL REGN10933 in human serum; middle panel, pooled human serum blank; and bottom panel, 2 mg/mL REGN10987 in human serum. (C) Comparison of accuracy percentage of drug concentrations of REGN10933 at five QC levels measured without the presence of REGN10987 in serum matrix and with 2 mg/mL REGN10987 in serum matrix. The error bar represents the standard deviation of the accuracy percentage measured from two QC replicates. (D) Comparison of accuracy percentage of drug concentrations of REGN10987 at five QC levels measured without the presence of REGN10933 in serum matrix and with 2 mg/mL REGN10933 in serum matrix. The error bar represents the standard deviation of the accuracy percentage measured from two QC replicates.

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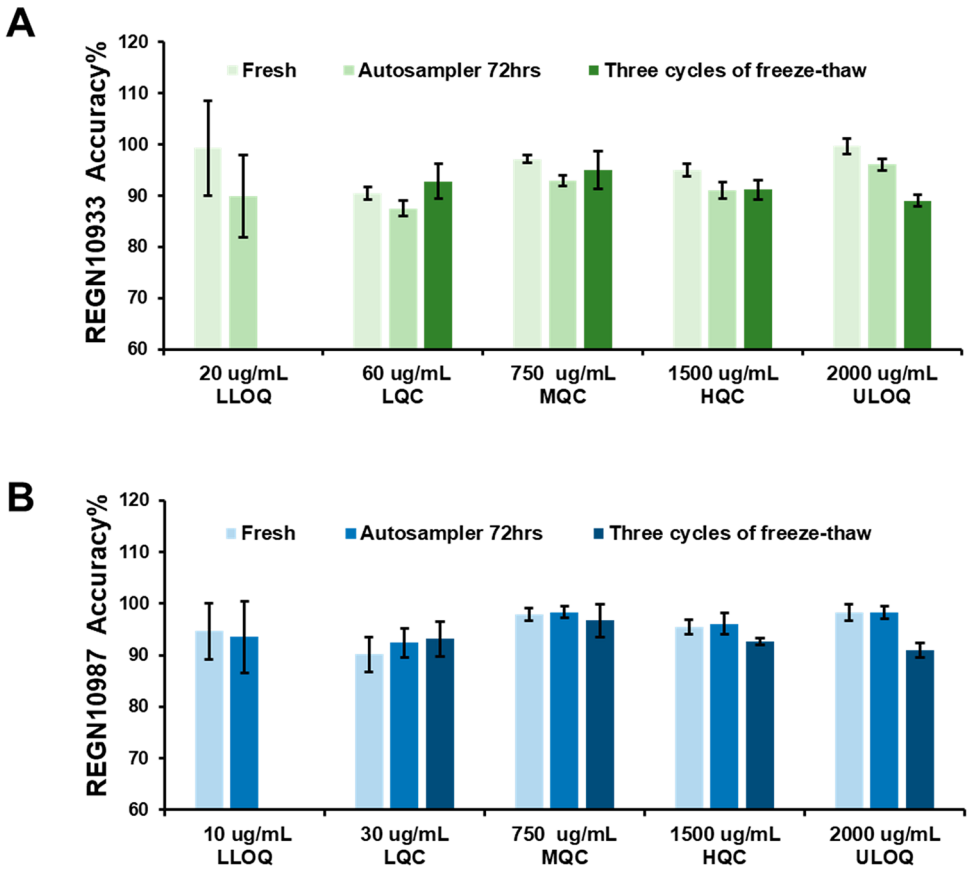

Figure 5. Assessment of REGEN-COV drug stability after exposure to three freeze−thaw cycles before sample processing and digested analyte stability in an instrument autosampler at different QC levels. The error bar represents the standard deviation of the accuracy percentage measured from five QC replicates.

REGN10987 of all five QCs ranged from 96 to 106%; the interday CV% Conc for all QCs varied between 3 and 13% for REGN10933 and 3 and 9% for REGN10987. For the intraday assessment, the %ACC for REGN10933 of the five QCs were between 89 and 114%, with CV% Conc between 1 and 6%, and the %ACC for REGN10987 of the five QCs were between 92 and 111%, with CV% Conc between 2 and 9%. These results demonstrated that, for both REGN10933 and REGN10987, both intraday and interday accuracy of this LC-MRM-MS assay are between 80 and 120% for HQC, MQC, and LQC, and between 75 and 125% for LLOQ and ULOQ. Both intraday and interday CV% of measured concentration are within 20% for HCQ, MQC, and LQC, and within 25% for LLOQ and ULOQ.

Selectivity. Selectivity is the selective and specific quantitation of the analyte in the presence of varying endogenous and non-study-specific matrix constituents. A set of 10 individual naïve human serum samples were analyzed to examine if the assay was subject to nonspecific matrix interference. For both REGN10933 and REGN10987, all samples were shown to be below the limit of quantitation (BLQ), which is 20 μg/mL for REGN10933 and 10 μg/mL for REGN10987. Further evidence of selectivity was evaluated by accuracy assessment of LLOQ-spiked individual naïve human serum samples. As shown in Figure 3, for all 10 individual serum samples cospiked with 20 μg/mL REGN10933 and 10 μg/mL REGN10987, the measured concentrations of REGN10933 were within ±25% of the nominal value, with %ACC for REGN10933 ranging from 92 to 116%, and the %ACC for REGN10987 from 87 to 126%, with a measured concentration of REGN10987 in one sample (sample no. 8) out of the ±25% of the nominal value. These results all met the acceptance criteria stated in the Bioanalytical Method Validation Guidance for Industry:[13] at least 80% of the LLOQ-spiked naïve samples must meet the acceptance criteria of %ACC within ±25% of the nominal value. From these evaluations, it was demonstrated that the LC-MRM-MS assay developed is selective for human serum samples containing REGN10933 and REGN10987. In addition, the results obtained with LLOQ-spiked samples confirmed that the assay in neat serum can quantitate the levels of total REGN10933 and REGN10987 as low as 20 and 10 μg/mL, respectively, further establishing the LLOQ for the LC-MRM-MS assay.

Specificity. Specificity is the ability of a method to assess the analyte in the presence of other components that are expected to be present. Because REGN10933 and REGN10987 are coadministered as an antibody cocktail therapy, we evaluated the interference from concomitant medication for the method specificity of each drug. As shown in the XICs of Figure 4, the signal from the transition channel of REGN10933 surrogate peptide (m/z 853.0 → m/z 359.2) at a retention time of 14 min was not detectable in 2 mg/mL REGN10987 in human serum (Figure 4A), and a similar response was observed for the signal from the transition channel of REGN10987 surrogate peptide (m/z 597.6 → m/z 481.2) in the sample of 2 mg/mL REGN10933 in human serum (Figure 4B). To systematically evaluate the drug specificity for each drug, the accuracies of

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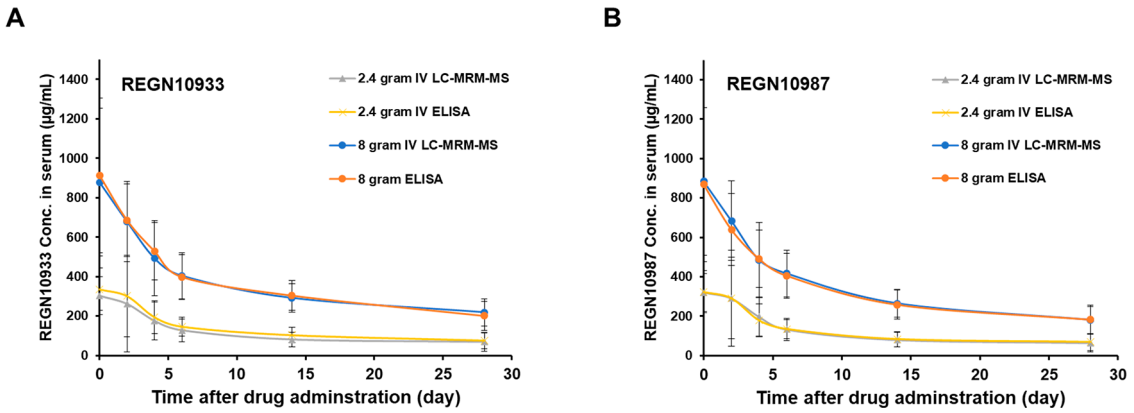

Figure 6. Concentrations of REGN10933 (A) and REGN10987 (B) measured from serum samples from two-dose groups of ambulatory COVID patients involved in phase I clinical trial of REGEN-COV by the LC-MRM-MS assay and a fully validated electrochemiluminescence immunoassays assay. Number of patients: 15−20 in each group. The error bar represents the standard deviation of drug concentrations from multiple patient samples at each time point.

QC standards made with one drug alone were compared with the accuracies of QC standards containing 2 mg/mL coadministered drug, at five different QC concentration levels. The ACC% values of REGN10933 QCs ranged from 98 to 103% without the presence of REGN10987, which was comparable to the ACC% range (97−120%) measured for REGN10933 QCs with 2 mg/mL REGN10987 in serum (Figure 4C). The ACC% values of REGN10987 QCs ranged from 100 to 114% without the presence of REGN10933, which was also comparable to the ACC% range (96−108%) measured for REGN10987 QCs with 2 mg/mL REGN10933 spiked in serum (Figure 4D). The ACC% values of QCs for each drug at all five QCs level, with or without the presence of the coadministered drug, met the acceptance criteria of %ACC within ±20% of the nominal value, except %ACC of ULOQ and LLOQ within ±25% of nominal value.

Analyte Stability. Analyte stability is the ability to accurately measure the analyte within the acceptance criteria of the assay after the sample has been subjected to stress or different storage conditions. To fit for the purpose of our study, we specifically evaluated the stability of intact antibody drug in human serum after exposure to three freeze−thaw (FT) cycles and the stability of digested analyte during storage in the UPLC autosampler.

To assess analyte stability under the conditions of freeze−thaw cycles, five replicates of ULOQ, HQC, MQC, and LQC were frozen/thawed for three cycles before digestion. To assess the analyte stability in the instrument autosampler, five replicates of ULOQ, HQC, MQC, LQC, and LLOQ were analyzed after 72 h of storage at 7 °C in the autosampler. The measured accuracy of fresh QC samples was compared with that of QC samples subjected to freeze−thaw cycles before digestion, as well as QC samples stored for long hours in an autosampler after digestion. As shown in Figure 5, no significant changes in the measured accuracy of drug concentrations were observed for the conditions we evaluated. All QCs met the acceptance criteria that %ACC of all QC should be within ±20% of the nominal value, except %ACC of ULOQ within ±25% of the nominal value, indicating that both REGN10933 and REGN10987 are stable in human serum under the conditions tested.

Measurement of REGEN-COV Concentrations in COVID-19 Patient Serum Samples. Due to the urgent need for a reliable quantitative PK assay for the characterization of REGEN-COV in the early stages of the clinical studies, the developed LC-MRM-MS assay served as an interim method to determine the total concentrations of REGN10933 and REGN10987 in serum samples collected from patients in the clinical trial of REGEN-COV in outpatients with COVID-19.[3] The patients participating in the double-blind clinical study were randomly assigned (1:1:1) to receive placebo, 2.4 g of REGEN-COV (1.2 g of each antibody), or 8.0 g of REGEN-COV (4.0 g of each antibody) by intravenous injection. The serum samples were collected predose, 1 h after drug infusion, and on days 2, 4, 6, 14, and 28 after drug administration from patients in the 2.4 and 8.0 g dose groups and analyzed by the qualified LC-MRM-MS assay. Calibration standards, as well as QC standards at four concentration levels in triplicates (LLOQ, LQC, MQC, HQC), were digested and analyzed together with each batch of patient samples. The run acceptance criteria for the LC-MRM-MS assay were defined by the %ACC of nonzero calibration standards, as well as the %ACC and coefficients of variation of QCs. The accuracy of the measured concentration of the calibration standard must be within 25% of the nominal concentration at LLOQ, and within 20% of the nominal concentration at all other concentrations. At least two-thirds of the measured QC concentrations must be within 20 or 25% (LLOQ) of their respective nominal values. At least two replicates of the QCs at each level should be within 20 or 25% (LLOQ) of their nominal concentrations. Acceptance criteria for precision are determined at each concentration level and should be within 20%. All of the predose serum samples measured had responses below LLOQ for both REGN10933 and REGN10987, which further validated the selectivity of this LC-MRM-MS assay.

The time profile of serum drug concentration after a single dose injection (Figure 6) was used to determine the PK parameters of REGN10933 and REGN10987. The results showed that the antibody drug concentration reaches a maximum within 1 h of intravenous injection and decays over time. The PK of each antibody was linear and dose-proportional, and the drug concentration in serum at day 29 remained above the predicted neutralization target concentration based on *in vitro* and preclinical data.[1−3] The linearity range of our developed assay covers all of the clinical samples from both dose groups and six PK sampling time points over 1 month after drug infusion.

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Immunoassay has been applied traditionally for the determination of antibody drug concentration in serum matrix for clinical sample analysis. It measures protein target as an intact molecule, instead of surrogate fragments derived from protease digestion being measured by the LC-MRM-MS assay. The most critical reagents for clinical PK immunoassay development are the anti-idiotypic antibodies that specifically bind to the idiotypes of antibody drugs, and it normally takes a few months to screen and produce these reagents. Immunoassay typically provides good sensitivity in the low ng/mL range, which could be challenging for LC-MRM-MS direct assay to reach without additional immunoaffinity enrichment steps. Another advantage of the immunoassay is that the large batch of patient sample analysis can be carried out in a high-throughput format once the method is established. After an electrochemiluminescence immunoassay for REGEN-COV was fully validated at a later time, we reanalyzed these clinical samples, and the comparison indicates that there is good agreement between the results from both assays (Figure 6). The differences of average drug concentrations from individual patients (n = 15−20 per dose group) measured by the two assays were within 23% for REGN10933 and 11% for REGN10987.

CONCLUSIONS

An LC-MRM-MS-based assay was developed and qualified to determine the individual total concentration of REGN10933 and REGN10987 in human serum samples for the first-in-human clinical study of an antibody cocktail therapy for COVID-19. The method was thoroughly evaluated and met the acceptance criteria set forth in the Bioanalytical Method Validation Guidance for Industry.[13] Acceptable inter- and intra-assay accuracy and precision were demonstrated. The dynamic range of the assay covers the drug concentrations in COVID-19 patient samples collected from day 1 to day 29 in both high and low dose groups. The assay was selective for the quantitation of total REGN10933 and total REGN10987, with no interference from the human serum matrix in the dynamic range. The specificity of this assay for each drug when administered in combination was evaluated, and no interference was observed from the coadministered therapeutic within the dynamic range. The freeze−thaw stability of REGN10933 and REGN10987 in human serum, and autosampler stability of digested REGN10933 and REGN10987 in human serum, was not affected by the treatment conditions. In the clinical study across different dose groups, the concentrations of REGEN-COV measured by the LC-MRM-MS assay are in good agreement with the results obtained from a fully validated ligand-binding immunoassay.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.1c01613.

Table S1, Instrument parameters for time-scheduled LC-MRM-MS experiments; Table S2, Prediction of peptide sequences containing CDRs by trypsin digestion or combined trypsin and rAspN digestion of REGEN-COV; Table S3, Accuracy and precision for all of the nonzero standards of REGN10933 and REGN10987 from three independent experiments; and Table S4, Intraday and interday accuracy and precision of REGN10933 and REGN10987 at five QC levels (PDF)

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Notes

The authors declare the following competing financial interest(s): X.Z., S.N., L.G., S.R., Y.Z., R.W., M.A., C.E., G.S., S.C.I., M.A.P., H.Y., S.Y.E., H.Q., Y.M., A.T., and N.L. are current or former employees and shareholders of Regeneron Pharmaceuticals Inc.

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