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A sample preparation process for LC–MS/MS analysis of total protein drug concentrations in monkey plasma samples with antibody

Qin C. Ji *, Ramona Rodila **, Tawakol A. El-Shourbagy

Department of Drug Analysis, Abbott Laboratories, Abbott Park, IL 66048, United States

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Abstract

The determination of protein concentrations in plasma samples often provides essential information in biomedical research, clinical diagnostics, and pharmaceutical discovery and development. Binding assays such as ELISA determine meaningful free analyte concentrations by using specific antigen or antibody reagents. Concurrently, mass spectrometric technology is becoming a promising complementary method to traditional binding assays. Mass spectrometric assays generally provide measurements of the total protein analyte concentration. However, it was found that antibodies may bind strongly with the protein analyte such that total concentrations cannot be determined. Thus, a sample preparation process was developed which included a novel "denaturing" step to dissociate binding between antibodies and the protein analyte prior to solid phase extraction of plasma samples and LC–MS/MS analysis. In so doing, the total protein analyte concentrations can be obtained. This sample preparation process was further studied by LC–MS analysis with a full mass range scan. It was found that the protein of interest and other plasma peptides were pre-concentrated, while plasma albumin was depleted in the extracts. This capability of the sample preparation process could provide additional advantages in proteomic research for biomarker discovery and validation. The performance of the assay with the novel denaturing step was further evaluated. The linear dynamic range was between 100.9 ng/mL and 53920.0 ng/mL with a coefficient of determination (r^2) ranging from 0.9979 and 0.9997. For LLOQ and ULOQ samples, the inter-assay CV was 12.6% and 2.7% and inter-assay mean accuracies were 103.7% and 99.5% of theoretical concentrations, respectively. For QC samples, the inter-assay CV was between 2.1% and 4.9%, and inter-assay mean accuracies were between 104.1% and 110.0% of theoretical concentrations.

Keywords: Protein quantitation; LC–MS/MS; Antibody binding; Kringle 5; Bioanalytical assay validation; Solid phase extraction

1. Introduction

The determination of protein concentrations in plasma samples often provides essential information in biomedical research, clinical diagnostics, and pharmaceutical discovery and development. Typically used for this purpose are binding assays, such as ELISA, that require an antigen or antibody that specifically binds to the protein analyte. The binding between the reagent antigen or antibody and the protein gives an analytical response that is in direct correlation to the concentration of the analyte. Although such assays are sensitive, rapid, and low cost, it is often very challenging to discover, prepare, and purify the specific antigens or antibodies required. In addition, binding assays often have a nonlinear calibration curve. Due to their mechanism of detection, only "free" analyte proteins, whose active site is not blocked by binding to plasma proteins and antibodies, can give a response. The reported "free" concentration is usually only a fraction of the total concentration of the analyte in the plasma matrix. This obtained concentration is highly dependent on the binding strength of the analyte to the reagent, and binding to other antibodies or background proteins in the matrix. In the case where strong binding complexes with high antibody concentrations are formed, the available free analyte concentration may be well below the quantitation limit of the binding assay. Although meaningful free analyte concentration results can be obtained by careful selection of the antigen or antibody reagent, the ability to measure the total analyte concentration may provide additional information that could potentially open new doors in biomedical research.

* Corresponding author. Present address: Covance Laboratories Inc., 3301 Kinsman Boulevard, Madison, WI 53704, United States. Tel.: +1 608 2422652.
** Corresponding author. Tel.: +1 847 938 5715; fax: +1 847 938 7789.
E-mail addresses: qin.ji@covance.com (Q.C. Ji), ramona.rodila@abbott.com (R. Rodila).

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Mass spectrometric technology is becoming a promising complimentary tool to the traditional binding assays [1–8]. The strategies of mass spectrometric assay for protein quantitation could be (1) through quantitative analysis of a representative peptide fragment of analyte of interest or (2) through direct monitoring of the intact protein by extension of traditional sample preparation techniques from small molecules to the larger protein molecules. In plasma samples where no strong-binding antibody is generated, mass spectrometry normally detects the total protein concentration with only a small percentage bias between the measured and the theoretical concentration, demonstrated during the assay validation. However, as discussed further in this report, an approach using mass spectrometry may not be able to detect the total concentration of a protein analyte when an antibody exists in the plasma sample.

Solid phase extraction has traditionally been used for the analysis of small organic molecules in biological matrices. In our previous publication, we proposed and demonstrated that this sample preparation technique could be extended for the analysis of some protein analytes in plasma samples [6–8]. rK5 is a small protein with molecular weight 10464 amu. It is a specific and potent angiogenesis inhibitor that may be effective in the treatment of human brain glioma and other tumors [9,10]. In recent pre-clinical studies, it was found that dosing of animal subjects resulted in an immune response that induced the generation of rK5 antibodies. The discovery of this antibody generated during pre-clinical studies underscored the need for further method development with a goal of breaking the binding between antibodies and rK5 in order to allow LC–MS/MS measurement of the total rK5 concentration. In the method presented here, we include a novel "denaturing" sample preparation step added to the solid phase extraction and LC–MS/MS analysis of total protein concentration in plasma samples. To our knowledge, there is no report where such a step was intentionally added. This additional denaturing step in the solid phase extraction sample preparation process provides the potential for complete dissociation of the binding between the analyte of interest and any background proteins, even antibodies with strong binding affinity. In this case denaturing allows us to obtain the total rK5 concentration in plasma even in the presence of strongly binding anti-rK5 antibodies. A method to monitor free rK5 concentrations using equilibrium dialysis and LC–MS/MS detection was further developed with this sample preparation procedure [11]. Assay evaluation also demonstrated that accurate and precise measurement could be achieved with the addition of the denaturing step.

Sample preparation with denaturing and solid phase extraction was further evaluated by LC–MS analysis, where it was observed that an additional advantage of using this technique is a depletion of plasma albumin. Furthermore, the denaturing step potentially releases other proteins and peptides that can consequently be extracted. The benefits of dissociation of complexes by employing an additional denaturing step in solid phase extraction, and albumin depletion by solid phase extraction may also provide added benefits in proteomic research for biomarker discovery and development.

2. Experimental

2.1. Chemicals and reagents

The stock solutions of rK5 and internal standard used were produced at Abbott Laboratories (Abbott Park, IL, USA). Guanidine hydrochloride (Guanidine HCl) was purchased from Sigma (St. Louis, MO, USA). The rK5 hyper-immunized monkey serum as a source of polyclonal anti-rK5 antibodies was provided by Abbott Laboratories. Monoclonal anti-rK5 antibody was purchased from Green Mountain Antibodies (Burlington, VT, USA). All Omnisolv® grade methanol, acetonitrile, and water, along with HPLC grade hexanes were purchased from EMD, formerly EM Science (Gibbstown, NJ, USA). Trifluoroacetic acid was also purchased from EM Science. Glacial acetic acid was purchased from Aldrich (St. Louis, MO, USA) and the normal Monkey (Cyno) plasma with potassium EDTA as anticoagulant (NCP-KEDTA) was purchased from Lampire Biological Laboratories (Pipersville, PA, USA).

2.2. Instrumentation

For plasma solutions, a Gilson (Middleton, WI, USA) single channel positive displacement hand-held pipette and BioHit (Helsinki, Finland) multi-channel hand-held electronic pipettes were used. A Hamilton (Reno, NV, USA) MicroLab AT 2 Plus automated liquid handler was used for adding and mixing the internal standard. Solid phase extraction (Oasis HLB 60 mg) plates were purchased from Waters Corporation (Milford, MA, USA). The solid phase extraction process was performed using a Beckman–Coulter (Fullerton, CA, USA) square well plate collar and vacuum manifold base. The mass spectrometer used was an API-3000 from PE Sciex (Toronto, ON, Canada), along with the computer control system. The three-piece HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-10 AD HPLC pump, a Shimadzu SIL-10A XL autosampler, and a Shimadzu SCL-10A system controller. An inline filter with an A-110X 2 μm titanium frit was from Upchurch Scientific Inc. (Oak Harbor, WA, USA). The LC flow between the mass spectrometer inlet and waste line were controlled by valves from Valco Instruments (Houston, TX, USA). A Hot Pocket column heater from Keystone Scientific (Bellefonte, PA, USA) was also used. Agilent Technologies (Palo Alto, CA, USA) provided an 1100 series HPLC pump and degasser system for delivering back wash solvent during pre-column regeneration. The analytical column used was a 2.1 mm × 150 mm Symmetry300 C18 5μ with a pre-column consisting of a 3.9 mm × 20 mm Symmetry300 C18 5μ cartridge, both from Waters Corporation. Lastly, MassChrom™ version 1.1.1 (or Analyst version1.3.2) software was used for data acquisition.

2.3. Preparation of standards and QC samples for assay evaluation

All of the standard and QC samples were prepared using the stock solutions from the same source and the same concentration. Separate working solutions for standard and

QC preparation were made by diluting the stock solution of the analyte with water. Standards one through ten were prepared by adding the correct volume of the working solutions to class A volumetric flasks and diluting to volume with pooled NCP-KEDTA. The following concentrations were prepared: 100.9 ng/mL, 201.8 ng/mL, 672.8 ng/mL, 1681.9 ng/mL, 3363.8 ng/mL, 10091.3 ng/mL, 20182.5 ng/mL, 33637.5 ng/mL, 43728.8 ng/mL, and 53820.0 ng/mL. Accordingly, QC samples were also prepared with the same method at concentrations of 239.2 ng/mL, 1196.0 ng/mL, 5980.0 ng/mL, 17940.0 ng/mL, and 41860.0 ng/mL. The standards and QCs were aliquoted into polypropylene tubes and stored in freezers maintained at approximately −70 °C.

2.4. Sample preparation

Samples were thawed at room temperature and then vortexed to ensure sample solution homogeneity. First, 50 μL of each plasma sample were loaded into the designated wells of a 96 well plate using a handheld single channel pipette. Two hundred microliters of a 5 μg/mL ^{15}N rK5 solution was added as internal standard, except the well designated the double blank, then the samples were aspirated and dispensed six times using the Hamilton automated liquid handler. Two hundred and fifty microliters of an 8 M guanidine hydrochloride solution in water was then added to each well and mixed with the plasma sample. The plate was then covered with sealing film and let sit at room temperature for approximately one hour, after which 500 μL of water were added to each sample well, with the exception of the double blank where 700 μL of water were added to account for the volume of internal standard. A Waters Oasis HLB 60 mg solid phase extraction plate was conditioned by adding 1 mL methanol to each well and drawing through with vacuum, then equilibrated by adding 1 mL water to each well and drawing through with vacuum. Then, using a multi-channel pipette, the contents of the 96-well plate were transferred to the corresponding wells of the solid phase extraction plate, followed by drawing through with vacuum. Each well of the solid phase extraction plate was then washed by adding 1 mL of water with 0.2% trifluoroacetic acid and drawing through with vacuum, then 1 mL of hexane was added to each well followed by application of vacuum. The wells were then eluted into a clean 96-well plate by adding 0.8 mL of acetonitrile with 0.2% trifluoroacetic acid and drawing through with vacuum. Finally, the extract was dried down under room temperature nitrogen and reconstituted with 100 μL water. Samples were then injected for LC–MS/MS analysis.

2.5. LC–MS/MS analysis

The same LC–MS/MS method described in previous publication was used [6]. Briefly, a gradient HPLC method was utilized for separation with mobile phases A and B. Mobile phase A consisted of 0.1% acetic acid and 0.02% trifluoroacetic acid in water; and mobile phase B consisted of 0.1% acetic acid and 0.02% trifluoroacetic acid in 80/20 (v/v) acetonitrile/water. Mobile phase A was also used as the injector wash solution with the injector being rinsed with 1 mL following each injection. The analytical column was maintained at a temperature of 40 °C and the injection volume was 40 μL. The first 7.30 min of LC effluent were diverted to the solvent waste line. The LC system was also configured to provide backwash of the pre-column after the analyte and internal standard were eluted in each LC–MS/MS run to improve the ruggedness of the assay. The mass spectrum acquisition was started 7.30 min after sample injection so the actual chromatographic peak retention time is 7.30 min more than that shown in Fig. 6. A PE Sciex API 3000 triple quadrupole mass spectrometer with a Turbo Ionspray® ionization source operated in the positive ion mode was used for LC detection. The mass spectrometry conditions were similar to the ones used for the quantitative analysis of rK5 in plasma samples without antibodies [6]. Briefly, the SRM (selective reaction monitoring) channels for the precursor and product ions were 1495 → 1463 for rK5, and 1513 → 1481 for the ^{15}N labeled internal standard. Mass spectrometer parameters were optimized by infusion of the analyte with a mixture of 50:50 mobile phase A:B at a flow rate of 200 μL/min. The following are examples of typical tuning parameters used by Analyst™ software in analysis: the nebulizer gas setting was 9, the curtain gas was 10, the ion spray voltage was 5000 V, the source temperature was 350 °C, and the collision gas (CAD) setting was 5. Other compound specific parameters used were: DP (declustering potential) at a setting of 85 V, EP (entrance potential) was 10 V, FP (focusing potential) was 380 V, CXP (collision cell exit potential) was 48 Volts, and the CE (collision energy) setting was 51 eV. A unit resolution (full width half maximum) setting was used for both the Q1 and Q3 mass filters.

The peak areas of rK5 and internal standard were determined using the SCIEX MacQuan™ software (version 1.6) (or Analyst software version 1.3.2). For assay evaluation of each analytical batch, a calibration curve was derived from the peak area ratios (analyte versus internal standard) using weighted linear least squares regression of the area ratio versus the concentration of the standards. For curve fit, a weighting of 1/concentration^2 was used. The measured concentration at each standard level was back-calculated using the regression equation from the generated calibration curve. The results were then compared to the theoretical concentrations to obtain accuracy, expressed as percentage of the theoretical value, for each standard level measured. Likewise, the concentrations of the QC samples were also calculated from the regression equation using the observed area ratio for each QC sample. The accuracies for the QC samples were determined using the same method as the standards.

2.6. LC–MS analysis

LC–MS detection was used for the further evaluation of the extracted components from Solid Phase Extraction. The same HLPC conditions as described in the previous section were used, except the mass spectrum acquisition was started 2 min after sample injection so the actual retention time is 2 min more than that shown in Figs. 3 and 4. The mass range was from *m*/*z* 1200 to 2400.

3. Result and discussion

3.1. Sample preparation process

After the study subjects were dosed with rk5, it was found that antibody was generated for some subjects. The hyper-immunized monkey serum was collected as a source of polyclonal anti-rK5 antibody. Following sample loading into the solid phase extraction plate, the previously published sample preparation process [6] involved washing each well with 1 mL of water with 0.2% trifluoroacetic acid, 1 mL of hexane and then eluting by adding 0.8 mL of acetonitrile with 0.2% trifluoroacetic acid. Our initial thought was that any non-covalent complex including rK5 with antibodies should be disassociated because the wash steps involved the use of strong acid and high organic solvent. However, it was observed that when the rK5 plasma sample was spiked with the hyper-immunized monkey serum, there was a significantly lower analytical recovery compared to the same rK5 plasma sample not spiked with the hyper-immunized monkey serum. As shown in Fig. 1A, using the original extraction method [6], when rK5 monkey plasma has an increased amount of antibody added (by spiking an increasing volume of the hyper-immunized monkey serum), the resulting LC–MS/MS peak area of rK5 decreased significantly. The peak area of the internal standard was decreased as well, but not as significantly as rK5. As a result, the area ratio of rK5 versus internal standard still decreases as shown in Fig. 1B. This led us to believe that a complex of rK5 (or internal standard) with antibody was formed and that this complex was not completely broken apart during the sample preparation process. The amount of time of mixing samples with the internal standard was increased in hope that the same degree of binding could be achieved for both labeled and unlabeled rK5, such that the effect of rK5 binding to the different amounts of antibodies in the plasma could be compensated by the same degree of binding of the internal standard. Therefore, the total amount of the rK5 concentration could still be obtained. However, this approach was not successful as the binding strength of ^{15}N rK5 could be less than the non-labeled rK5. Alternatively, we endeavored to find a method that denatures and dissociates the rK5/anti-rK5 antibody complex before subjecting the sample to solid phase extraction. Due to the wash steps during solid phase extraction we could test a broad range of substances without significantly changing the solvent composition in the eluting mixture. Reagents such as 0.5% trifluoroacetic acid, 8 M urea in water, saturated sodium chloride solution, and sodium dodecyl sulfate (SDS) were tested. The addition of the 8 M guanidine HCl gave the best results as shown Fig. 1. Both intensities of rK5 and internal standard for the samples with addition of various amounts of antibody remained unchanged from the samples without addition of antibody. This fully demonstrated that a total rK5 concentration is obtained when 8 M guanidine HCl was introduced into the sample preparation process. The addition of 8 M guanidine HCl most likely denatured the antibody and therefore deactivated the binding activity, thus releasing the rK5 for solid phase extraction and LC–MS/MS detection. The dilution of the sample-guanidine mixture with water prior to solid phase extraction also allows better retention of the analyte before the elution step.

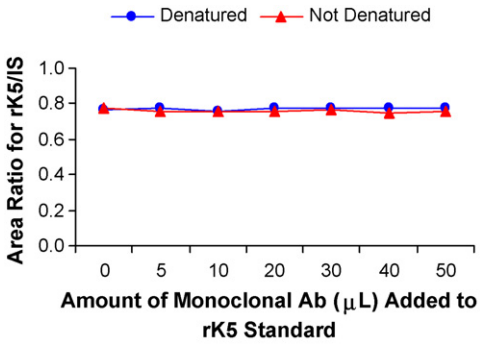

Fig. 2. LC–MS/MS analysis of rK5 plasma samples with monoclonal antibody extracted with and without "denaturing" step in sample preparation.

A similar experiment was repeated for rK5 in monkey plasma spiked with various amounts of monoclonal antibody (results shown in Fig. 2). It can be seen that results from the samples processed with or without the 8 M guanidine HCl give total rK5 concentrations in plasma samples. This is suspected to be due to the weak binding activity of the monoclonal antibody.

As proposed and demonstrated in previous papers [6–8], the strategy of bioanalysis of small molecules could be extended to the analysis of protein analytes in plasma matrices. The sample preparation process using solid phase extraction was further evaluated using the same LC–MS conditions, but with full mass

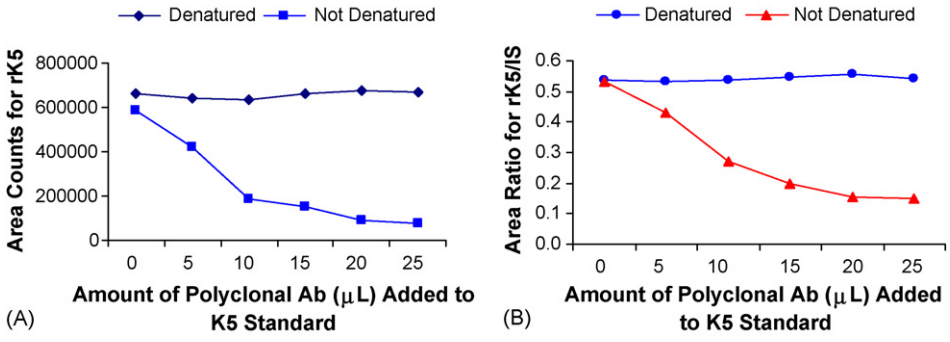

Fig. 1. LC–MS/MS analysis of rK5 plasma samples with polyclonal antibody extracted with and without "denaturing" step in sample preparation. (A) rK5 peak area vs. increasing amount of anti-rK5 antibody in the plasma; (B) rk5/internal standard peak area ratio vs. increasing amount of anti-rK5 antibody in the plasma.

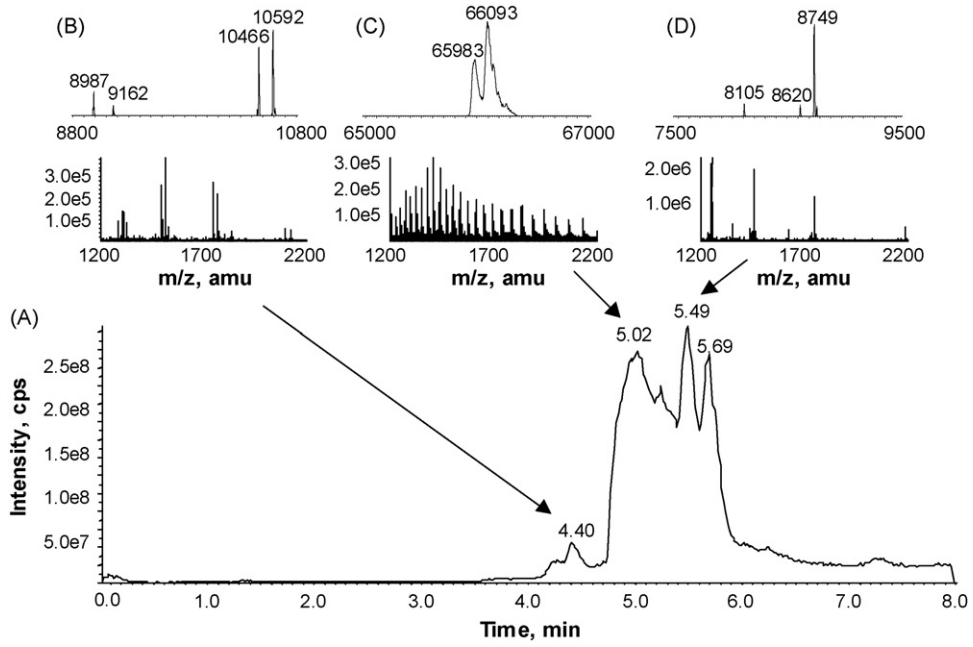

Fig. 3. (A) LC–MS total ion chromatograph; and the related mass spectra with de-convoluted molecular weight spectra of an extracted sample of 1 μg/mL rK5 in plasma. (B) Mass spectra and de-convoluted molecular peak of rK5, internal standard and plasma peptides. (C) Mass spectra and de-convoluted molecular peak of albumin proteins. (D) Mass spectra and deconvoluted molecular peak of some additional plasma peptides.

scan. The results of LC–MS analysis of the extracts of rK5 plasma sample at a concentration of 1 μg/mL are shown in Fig. 3. In addition to the rK5 and internal standard peak, the main chromatographic peak is from albumin. The analyte peak is well separated from the albumin peak, although there are some co-eluting peptides extracted from the blank plasma. An example of the additional peptides extracted from plasma matrices is also shown in Fig. 3. The same plasma sample was subjected to LC–MS analysis after a 100-fold dilution with water and is shown in Fig. 4. Because there is significant column carryover of the albumin peak, multiple blanks were injected before the evaluation sample was injected. LC–MS analysis of the diluted sample shows the albumin peak intensity is similar to the extract from undiluted sample. Comparison between Figs. 4 and 3 demon-

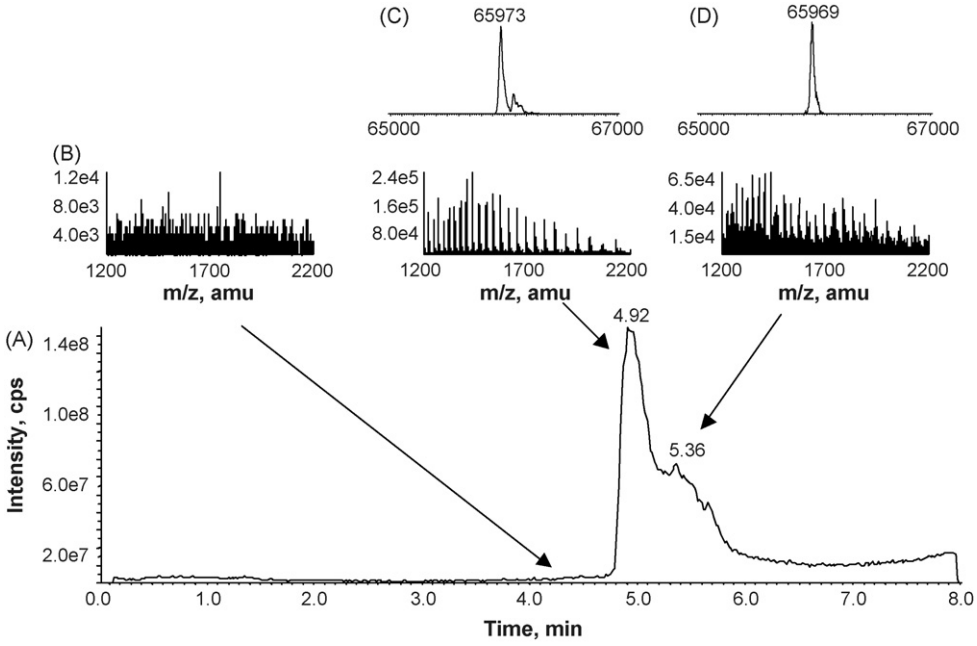

Fig. 4. (A) LC–MS total ion chromatograph, and the related mass spectra with de-convoluted molecular weight spectra of a diluted plasma sample. Plasma sample of rK5 at 1 μg/mL was diluted one hundred times with water. (B) Mass spectra obtained at rK5 elution time. (C) Mass spectra and de-convoluted molecular peak of albumin proteins. (D) Mass spectra and deconvoluted molecular peak at the elution time of some additional plasma peptides.

Table 1
Statistical calculation of calibration standards for assay linearity evaluation

	Standard level									
	1	2	3	4	5	6	7	8	9	10
Theoretical concentration (ng/mL)	100.91	201.83	672.75	1681.88	3363.75	10091.25	20182.50	33637.50	43728.75	53820.00
	100.43	204.43	663.81	1673.28	3422.85	10148.69	20352.63	33966.60	43792.42	52060.14
Calculated concentration (ng/mL)	104.94	184.12	680.03	1718.15	3453.24	10349.27	20542.46	34200.73	41399.67	52847.84
	103.01	195.77	640.88	1650.83	3649.04	9925.35	19945.97	35687.56	42577.39	52946.73
Mean	102.79	194.77	661.57	1680.75	3508.38	10141.10	20280.35	34618.30	42589.83	52618.24
% CV	2.2	5.2	3.0	2.0	3.5	2.1	1.5	2.7	2.8	0.9
% Theoretical	101.9	96.5	98.3	99.9	104.3	100.5	100.5	102.9	97.4	97.8

strate that solid phase extraction has pre-concentrated the rK5 analyte while significantly reducing the amount of albumin in the extracts. The removal of a substantial amount of albumin also greatly improves assay ruggedness. In addition, in the field of proteomic research for biomarker discovery and development, it is often very difficult to identify potential protein and peptide biomarker as they may be masked by highly abundant plasma albumin background. The benefits of disassociation of complexes by adding a denaturing reagent, and albumin depletion by solid phase extraction may prove to be another useful tool in this research area.

3.2. Assay performance evaluation

The assay performance with the denaturing and disassociation steps integrated into the sample extraction and subsequent LC–MS/MS analysis were evaluated using standards and QC samples prepared by spiking rK5 into monkey plasma. The experimental design and results of the most important aspects of method evaluation are presented in the following sections

3.2.1. Linearity, LLOQ, ULOQ and dilution

The linearity of the calibration curve was determined and evaluated from three consecutively prepared batches. The dynamic range of linearity was evaluated to be from 100.9 ng/mL to 53820.0 ng/mL. Within this assay range, the coefficient of determination (r^2) was between 0.9979 and 0.9997 (not shown in table). The accuracy of the standards was between 96.5% and 104.3% of the theoretical concentrations (Table 1). An example of a derived calibration curve is shown in Fig. 5.

The accuracy and precision at the low end of the assay was evaluated using eighteen replicates of lower limit of quantitation (LLOQ) samples from three separate runs. The accuracy was 103.7% of theoretical and the calculated CV was 12.6%. For the upper limit of quantitation (ULOQ), the same method was utilized, where the mean accuracy was 99.5% of theoretical and the CV was equal to 2.7% (Table 2). LC–MS/MS chromatograms of ULOQ and LLOQ samples are represented in Fig. 6A and B.

3.2.2. Accuracy and precision

Eighteen replicates of QC samples from three consecutive runs were used to evaluate the precision and accuracy at each concentration level. The intra-assay CV (not shown in the table) was between 0.3% and 3.6% and the inter-assay CV (Table 2) was between 2.1% and 4.9%. The inter-assay mean analytical recoveries were between 104.1% and 110.0% of the theoretical concentrations (Table 2).

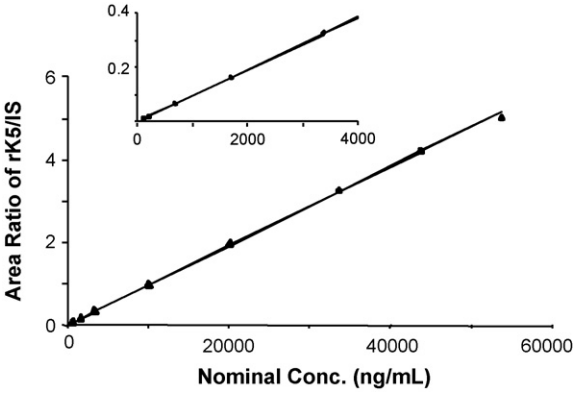

Fig. 5. An example calibration curve.

3.2.3. Selectivity

Selectivity was evaluated by extracting blank plasma samples from six different lots of matrix and comparing the response at the retention time of rK5 to the response at the LLOQ. No significant peaks were observed in any of the blank plasma samples. The addition of the denaturing step into the solid phase extraction did not introduce any additional interference peaks. As shown in Fig. 6A, the intensity of LC–MS/MS response of the LLOQ sample was approximately 1000 counts per second (cps) while intensities of LC–MS/MS response for the blank plasma samples extracted with internal standard was approximately 150 cps as shown in Fig. 6C and approximately 65 cps for the blank plasma sample extracted without internal standard Fig. 6D. In addition, the carryover from a ULOQ plasma extract injection followed directly by a blank plasma sample extract is approximately 0.1% as shown in Fig. 7A and B.

3.2.4. Extraction recovery

In order to determine extraction recovery, recovery control solutions were prepared in the reconstitution solvent at known

Table 2
Statistical calculation of LLOQ, ULOQ, and QC samples for assay accuracy and precision evaluation

	LLOQ	QC 1	QC 2	QC 3	QC 4	QC 5	ULOQ
	Theoretical concentration (ng/mL)						
	100.91	239.20	1196.00	5980.00	17940.00	41860.00	53820.00
	98.78	256.32	1277.99	6677.20	19680.25	43759.02	53651.24
	128.33	246.56	1286.61	6532.86	19507.08	43774.75	53927.90
	143.79	259.22	1269.76	6484.94	19565.80	44204.19	55345.65
	104.60	252.02	1303.05	6228.86	19342.76	44039.10	52000.12
	116.30	250.87	1252.61	6291.72	19140.86	42334.26	56147.92
	102.77	238.55	1256.92	6192.90	19085.57	43761.97	55366.78
	98.62	262.85	1294.06	6528.26	20415.75	44822.14	52075.63
	103.98	253.92	1307.16	6454.93	20347.24	44565.80	51843.05
	106.90	262.22	1316.70	6314.88	20222.99	44820.48	54782.61
	105.44	269.29	1348.86	6569.38	20643.50	44928.34	54541.96
	101.55	254.61	1350.64	6317.66	20168.18	44783.15	54092.11
	102.25	258.02	1319.45	6450.42	19918.70	44796.61	55036.78
	91.96	236.10	1234.54	6194.22	19587.99	44834.64	52408.03
	90.82	220.48	1174.52	6184.40	19761.40	44745.22	51633.09
	93.30	239.85	1177.58	6136.15	19817.29	44505.96	52544.47
	101.72	235.77	1205.21	6555.99	19255.11	43030.58	53065.70
	94.78	246.20	1184.87	6563.07	19354.53	42104.25	52153.90
	96.82	237.92	1208.57	6591.33	19256.58	42925.32	52959.65
Mean	104.60	248.93	1264.95	6403.84	19726.20	44040.88	53532.03
% CV	12.6	4.9	4.5	2.7	2.4	2.1	2.7
% Theoretical	103.7	104.1	105.8	107.1	110.0	105.2	99.5
n	18	18	18	18	18	18	18

concentrations. Fifty microliters of recovery control solution were added to extracted NCP-KEDTA with internal standard prior to the drying step. After drying, samples were reconstituted as normal. The area ratio (analyte/internal standard) for the recovery controls was then determined at each level, and compared to the area ratio obtained from extracted QC samples of the corresponding level. Extraction recovery was calculated by dividing the area ratios of individual QCs by the mean area ratio of the recovery control solutions. Overall mean extraction recovery evaluated at rK5 concentration levels of 5980.0 ng/mL and 41860.0 ng/mL were calculated to be 66.6% and 71.1% (Table 3). The introduction of the denaturing step with the solid phase extraction provided comparable extraction efficiency as sample preparation without the denaturing step [6]. Although the absolute extraction recovery could vary from well to well, the selection of the ^{15}N labeled rK5 internal standard provides effective compensation for this variation so that consistent results can be obtained.

Table 3
Statistical calculation of rK5 extraction recovery

	QC A	Control A	QC B	Control B
	Theoretical concentration (ng/mL)			
	5980.00		41860.00	
	0.5476	0.9088	3.8110	5.4180
	0.5533	1.0895	3.8590	5.2889
	0.5518	0.7782	3.8314	5.4188
	0.5531	0.7161	3.8438	5.4201
	0.5609	0.7455	3.9188	5.5027
	0.5513	0.7440	3.8441	5.4518
Mean	0.5530	0.8304	3.8514	5.4167
Mean % recovery	66.6		71.1	

3.2.5. Stability

The stability of rK5 in cynomolgus monkey plasma was evaluated and reported in our previous publication [6]. In addition, the stability of rK5 in the plasma with antibodies was also evaluated at this time. The freeze–thaw stability results, presented in Table 4, show the measured rK5 concentration from samples that went through three additional freeze–thaw cycles compared to samples from the same preparation that did not go through additional freeze thaw cycles. The % difference observed at the low and high QC concentration levels were 2.8% and 3.5%, respec-

Table 4
Statistical calculation for freeze/thaw stability of rK5 in plasma with anti-rK5 antibodies

	QC Low		QC High	
	0 F/T	3 F/T	0 F/T	3 F/T
Measured concentration (ng/mL)	211.55	212.40	35911.44	37441.12
	196.71	216.55	35843.70	36792.22
	213.25	209.74	34850.89	36084.89
Mean	207.17	212.90	35535.34	36772.74
% Difference	2.8		3.5	

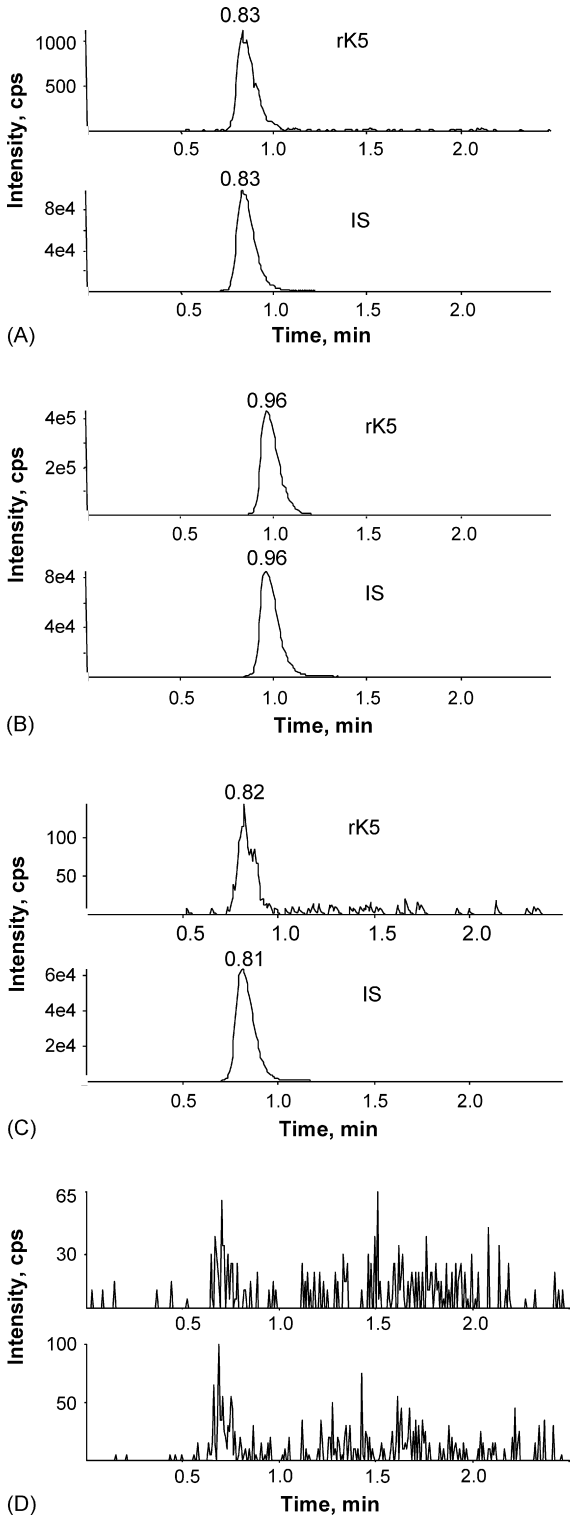

Fig. 6. Ion chromatographs of: (A) low standard (100.9 ng/mL rK5); (B) high standard (53820.0 ng/mL rK5); (C) blank plasma with internal standard; and (D) blank plasma extract without internal standard.

tively. Other stabilities of rK5 in monkey plasma with antibodies such as long-term stability, and stability of the binding between rK5 and antibodies or plasma proteins will be presented in future publications [12].

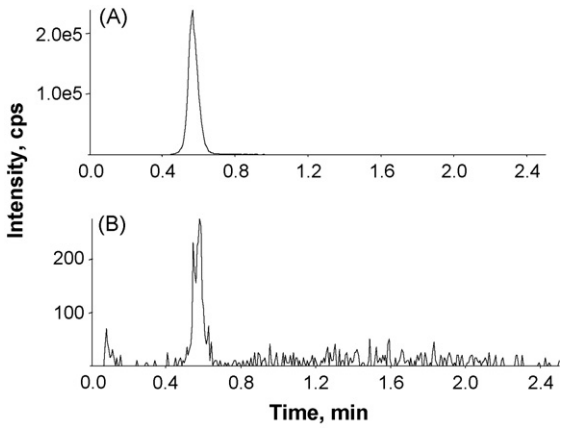

Fig. 7. Ion chromatographs of rK5 carryover from a (A) ULOQ to (B) blank plasma extract.

4. Conclusions

Here, we presented a novel sample preparation process for LC–MS/MS analysis of total rK5 concentrations in monkey plasma samples that contain polyclonal antibodies with strong binding activity to rK5. A denaturing step was incorporated to provide complete disassociation of the bindings between the analyte of interest and any background proteins including antibodies, and thus release rK5 for solid phase extraction. Assay evaluation also demonstrated that accurate and precise measurements could be achieved with the addition of the denaturing step. Furthermore, LC–MS evaluation of the extracts revealed that solid phase extraction not only pre-concentrated the analyte but also significantly depleted plasma albumin in the extracts. This capability could be used as an added advantage in proteomic research for biomarker discovery and development.

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