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Research paper

Trace level analysis of leached Protein A in bioprocess samples without interference from the large excess of rhMAb IgG

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ABSTRACT

Resins containing immobilized Staphylococcal Protein A (PA) are widely used in the commercial purification of recombinant human monoclonal antibody (rhuMAb IgG) biotherapeutics. Therefore, a sensitive assay for leached PA is needed to ensure that PA is not present at unacceptable levels as an impurity in the final product. PA impurities are measured by an ELISA using chicken anti-PA antibodies. However, PA in the presence of IgG product forms a PA/IgG complex that interferes in the assay. In this report a multi-product PA ELISA is described, wherein the PA/IgG complex is dissociated by heating in the presence of detergents and chelators prior to the ELISA. The dissociation facilitates the accessibility of the anti-PA antibodies to bind to PA in the immunoassay. Heat is provided by a novel microwave technology which allows brief heating time and high sample throughput using a microtiter plate for sample heating. Thus, broadly applicable dissociation conditions, suitable for all 21 rhMab IgGs tested to date were identified. This approach streamlines the measurement of leached PA, allows higher sample testing throughput, facilitates application across multiple products, and facilitates assay automation. Data comparing in-process samples tested with both the former product-specific ELISA and this new multi-product assay are shown.

1. Introduction

Recombinant monoclonal antibodies are an important class of biopharmaceuticals, widely used to treat diseases such as cancer and autoimmune diseases. Commercially, recombinant monoclonal antibodies often are purified by affinity chromatography using resins immobilized with Protein A (PA) ligand (Jungbauer and Boschetti, 1994; McCue et al., 2003; Jungbauer and Hahn, 2004). PA affinity chromatography offers the benefits of high selectivity and high capacity, achieving high product purity in a single chromatography step. However, the PA ligand may leach from the affinity resin and co-elute with the monoclonal antibody product. PA may have immunogenic (Gomez et al., 2004) and mitogenic effects (Bensinger et al., 1984; Bertram et al., 1985; Kraft and Reid, 1985); therefore, immunoglobulin clinical products must be shown to be free of detectable trace PA impurities.

This paper compares two immunoassays used for trace PA quantification using the chicken anti-PA antibodies. Leached PA is quantified by an enzyme-linked immunosorbent assay (ELISA) which uses chicken IgY raised specifically against PA. Unlike IgG, the Fc portion of IgY does not bind to PA. The first assay is the original product-specific PA (PS-PA) assay, and the second is the newly developed multi-product PA (MP-PA) assay.

The samples being tested for trace levels of leached PA contain a vast excess of the IgG biotherapeutic. In the PS-PA assay, the samples are tested without pretreatment. Little, if any, PA is free in solution, but rather present in PA/IgG complexes. While PA binds to the product IgG primarily via Fc

Abbreviations: AD, Assay diluent; BSA, Bovine serum albumin; DMAD, Dissociation maintenance assay diluent; DTPA, Diethylenetriaminepentaacetic acid; ELISA, Enzyme-linked immunosorbent assay; IgG, Immunoglobulin G; IgY, Immunoglobulin Y; MP-PA, Multi-product Protein A assay; PA, Protein A; PCDB, Protein (or Protein A/IgG) complex dissociation buffer; PS-PA, Product-specific Protein A assay; rhMAb, recombinant humanized monoclonal antibody; SDS, Sodium dodecyl sulfate.

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region there are also binding sites in the Fab region (Sandor and Langone, 1982; Moks et al., 1986). As will be demonstrated, these IgG interactions with PA interfere with the binding of IgY anti-PA, thus interfering in the ELISA. Furthermore, highly homologous, but different, IgG therapeutics inhibit the ELISA to differing degrees, requiring that the assay standards and controls are diluted in the specific IgG product to a level equivalent to that in the samples. The requirement that the current PS-PA assay must control for unique product-specific inhibition effects limits assay throughput, and requires that the assay be optimized for each new rhuMab introduced into the product pipeline.

As such, a MP-PA method was developed, wherein the PA/IgG complex is dissociated prior to the ELISA (Fig. 1). This dissociation does not denature PA to the extent that recognition by the anti-PA IgY antibodies is compromised. This MP-PA ELISA is independent of the specific IgG in the sample and, therefore, may be applied across multiple rhuMab therapeutic products without the need for product-specific standards and controls. Also, the product independency of MP-PA ELISA requires minimum assay optimization for each new rhuMAb.

2. Materials and methods

2.1. Reagents and buffers

The following reagents were used in this study: ProSep vA (Millipore, Billerica, MA); MabSelect and MabSelect SuRe (GE Healthcare, Piscataway, NJ); Chicken anti-Staphylococcal PA (Cat. No. C5-B01, OEM Concepts, Saco, ME); Protein A/IgG Complex Dissociation Buffer (PCDB) — 8.5 mM Diethylenetriaminepentaacetic acid (DTPA)/1.5% BSA/0.1 M Sodium Phosphate Buffer/1% SDS pH7.2; Assay Diluent (AD) — 0.15 M Sodium Chloride/0.1 M Sodium Phosphate/0.1% fish gelatin/0.05% Polysorbate 20/0.05% Proclin 300 pH 7.2; Dissociation Maintenance Assay Diluent (DMAD) — 0.15 M Sodium Chloride/0.1 M Sodium Phosphate/0.1% fish gelatin/0.05% Polysorbate 20/0.05% Proclin 300/1% polyvinylpyrrolidone (PVP)/0.15% SDS pH 7.2. 96-well plate polymerase chain reaction (PCR) plate (Cat. No. 72.1978.202.96, Sarstedt, Germany).

2.2. Instruments

DISCOVERY, a temperature and pressure controlled microwave and MARS, a temperature controlled microwave (both from CEM Corporation, Matthews, NC); micro-plate reader SpectraMax M5^e (Molecular Devices, Sunnyvale, CA); Agilent 2100 Bioanalyzer and Protein 230 kit (Agilent Technologies, Inc. Santa Clara, CA).

2.3. Software

SoftMax Pro (Molecular Devices, Sunnyvale, CA), JMP statistics software, (SAS institute, Cary NC); Agilent 2100 Expert Software (Agilent Technologies, Inc. Santa Clara, CA).

2.4. Assay procedure

Samples were diluted in PCDB with a ratio of 1:5 in a 96-well PCR plate prior to heating in microwave under defined conditions, e.g. at 80 °C for 10 min. The plate was then centrifuged briefly (650 g, for 2 min) and 5 µL from the supernatant of each well was transferred into 95 µL DMAD in the wells of an ELISA plate that was precoated with anti-PA antibody at 4 µg/mL and blocked with AD. The plate was then incubated with shaking for 2 h at ambient temperature and then washed three times with PBS-T (PBS/0.05% Tween-20) before incubating with 100 µL HRP-anti-PA conjugate (70 ng/mL) for 1 h at ambient temperature. The plate was washed as before, and100 µL TMB substrate was added. Color development was

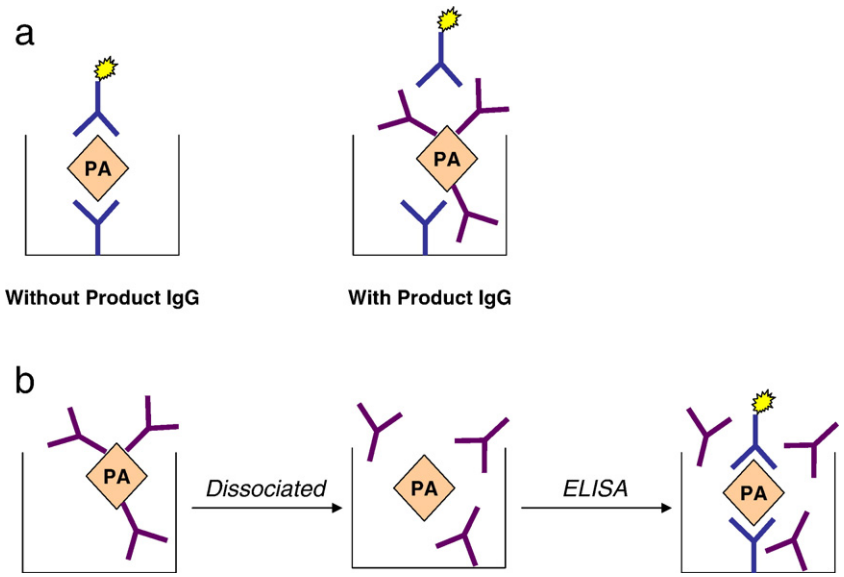


Fig. 1. Schematic drawing for the PA/IgG complex and its dissociation. a, PA forms a sandwich with its capture antibody (immobilized to the well of a microtiter plate) and detection antibody (blue color) in the leached PA ELISA; and in the presence of product rhMAbs (purple color), they form a PA/IgG complex. b, The PA/IgG complex is dissociated to allow full accessibility of PA for detection by the assay.

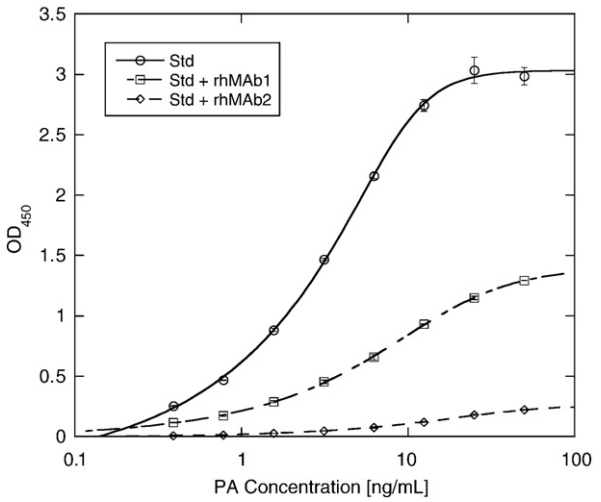


Fig. 2. Effect of rhMAbs on PA standard curves. Serial dilutions of PA with concentration ranging from 0.39 through 50 ng/mL were tested in duplicate, in the presence of rhMAb1 (□) or rhMAb2 (◊) at constant concentration of 0.2 mg/mL or in the absence of any rhMAb (○). The curves were plotted using the 5-parameter curve fitting function of software SoftMaxPro (Molecular Devices, Sunnyvale, CA).

read on a plate reader at 450 nm and the data reduced using SoftMax Pro software.

2.5. Bioanalyzer analysis

PA at 1.5 mg/mL was mixed with rhMAb1 or rhMAb2 at 5 mg/mL, or diluted directly 1:5 in PCDB. Samples were heated in Microwave MARS with predetermined assay condition (e.g. 90 °C for 5 min for a second microwave unit). Following heating, both heated and un-heated samples were prepared using Denaturing Solution according to manufacturing instruction prior to analysis on Bioanalyzer.

3. Results

3.1. The product-specific Protein A (PS-PA) ELISA

Fig. 1 illustrates the enzyme linked immunoabsorbent assay (ELISA) that was used to quantify the level of leached PA impurity. The capture antibodies and detection antibodies are both polyclonal chicken IgY anti-PA. They form a sandwich with the PA. Standard curves are generated using serial dilutions of pure PA in the presence or absence of various IgG biotherapeutics or neat. Even though most of the Genentech MAb IgGs are of the same humanized IgG1 framework, they show a different degree of inhibitory effects on the ELISA (Fig. 2). As shown in Fig. 2, rhMAb1 is less inhibiting than rhMab2 relative to the PA standard. These differences presumably reflect differences in the binding site or affinity to PA.

The practical consequence of these observations is that each rhMab therapeutic must have a standard curve and controls, diluted in the presence of the specific MAb product. For routine testing all samples were diluted to 0.2 mg/mL IgG biotherapeutic.

3.2. Development of the multiproduct-Protein A (MP-PA) ELISA

Following a method previously reported in which the PA/IgG complexes were dissociated using a combination of detergents, chelators, and heat (Steindl et al., 2000), PA was

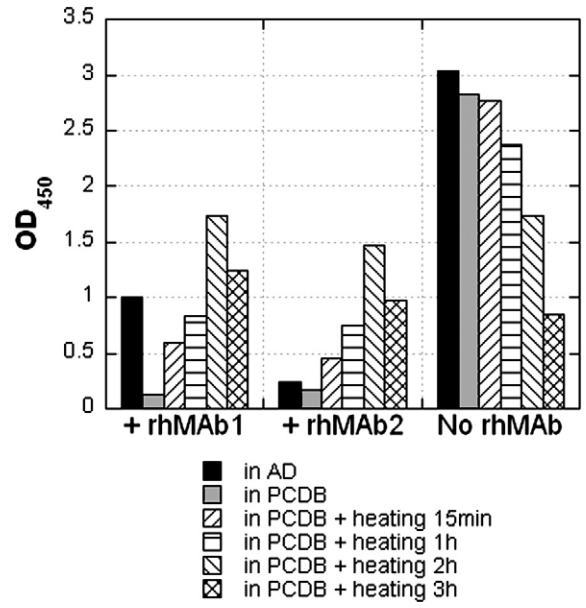


Fig. 3. Heat dissociation effect on PA detection in the presence or absence of rhMAb, rhMAb1, or rhMAb2. Assay diluent containing PA at 12.5 ng/mL was spiked with rhMAb1, rhMAb2 at 5 mg/mL or no rhMAb. Samples were diluted in assay diluent with no further treatment, or in PCDB, followed by heating at 96 °C for various length of time as indicated in the figure, prior to analysis by PA ELISA.

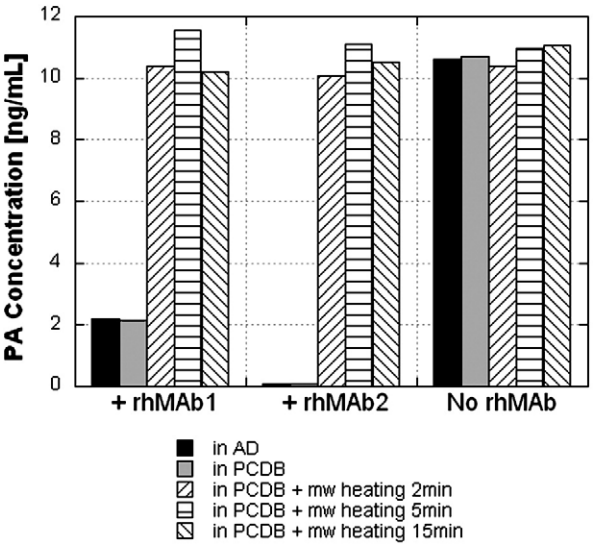


Fig. 4. Measurement of PA in the presence or absence of rhMAb1 or rhMAb2 after microwave-assisted heating. Assay diluent containing PA at 12.5 ng/mL was spiked with rhMAb1, rhMAb2 at 5 mg/mL or no rhMAb. Samples were diluted in assay diluent with no further treatment, or in PCDB, followed by heating in the microwave oven at 96 °C for 2, 5, or 15 min, prior to analysis by PA ELISA.

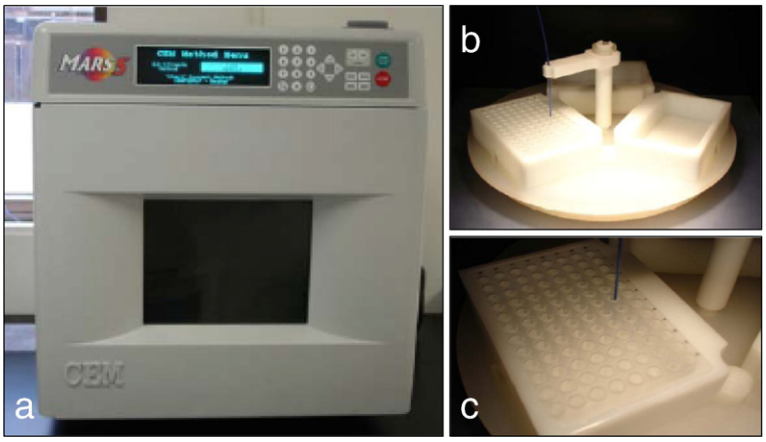


Fig. 5. CEM MARS microwave instrument. a, exterior image, b and c, interior images; the turntable designed to hold three 96-well plates (b), and the fiber optic temperature probe inserted in a 96-well PCR plate (c).

measured in the presence of rhMAb1 or rhMAb2. In-process PA pool samples containing either rhMAb1 or rhMAb2 were spiked with varying levels of PA. The samples were diluted 1:20 in PCDB and incubated at 96 °C (in a PCR thermocycler) for 1/4, 1, 2 or 3 h. Following heating, samples were diluted 1:5 in DMAD prior to the analysis in the ELISA. As shown in Fig. 3, at short heating times (1 h or less) the PA detected in samples containing MAbs was significantly lower compared to the PA level in the absence of product, suggesting insufficient dissociation of the PA/IgG complex. When heated for 2 h or longer the PA assay results were independent of the MAbs, suggesting adequate dissociation of the PA/IgG complex. Heating for 3 h in the absence of MAbs showed the PA level was significantly lower than in unheated or briefly heated samples, suggesting possible protein degradation caused by the prolonged heating.

To avoid potential protein degradation caused by extended conventional heating, and to improve sample throughput, microwave-assisted heating was explored. Samples were prepared by spiking 12.5 ng/mL PA into the same in-process rhMab1 and rhMab2 pools used above. These were then heated at 95 °C using the microwave oven Discover for 2, 5 or 15 min. As shown in Fig. 4, comparable levels of PA could be detected in the samples independent of the presence of rhMAb, and independent of heating time. The lack of a significant decrease in signal even with 15 min heating suggests that microwave heating was sufficient to dissociate the PA/IgG complex without degradation. Furthermore, similar heating conditions dissociated both the rhMAb1 and rhMAb2 PA/IgG complexes.

While the microwave oven Discover allowed dissociation of the PA/IgG complex, its throughput was limited to ten samples at a time. The MARS Model microwave was configured to permit three 96-well plates to be heated simultaneously on a turntable (Fig. 5). Temperature resistant 96-well thin wall polypropylene plates designed for polymerase chain reactions (PCR), together with the sealers to prevent evaporation, were used.

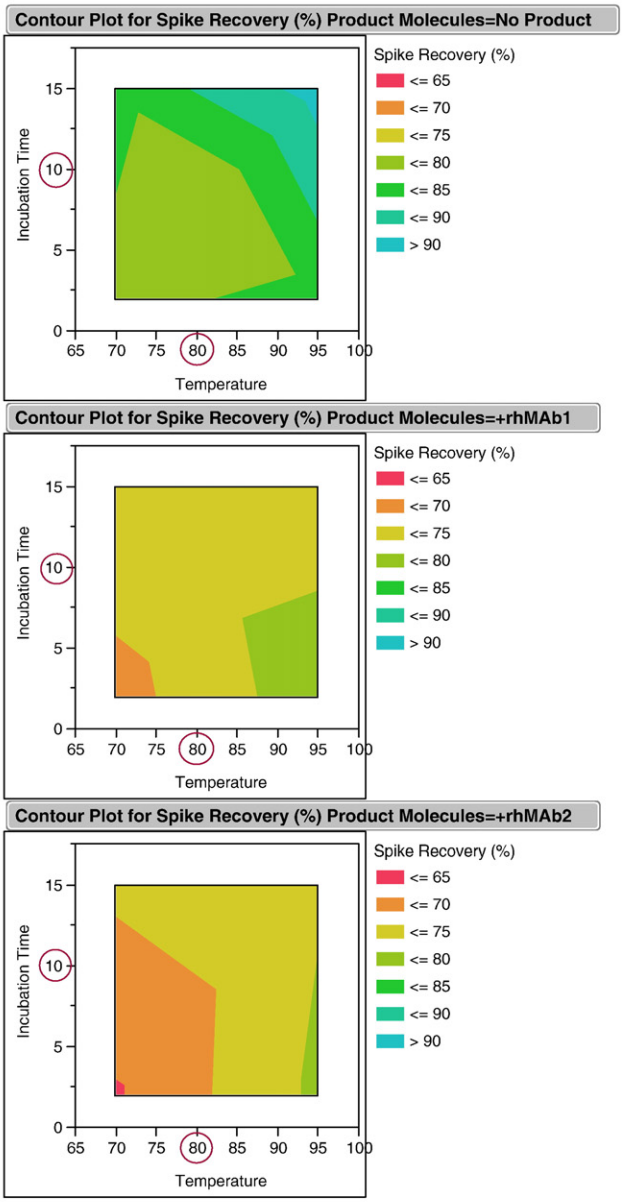
First, the uniformity of the heating using 96-well plates was assessed. PA at a final concentration of 12.5 ng/mL was mixed with 5 mg/mL rhMAb2 and pipetted into the wells. The plate was heated in a microwave oven, and 5 μL of the contents of each well was transferred to the PA ELISA. The results showed that the coefficient of variation across the plate was no greater than 3%, which is within the expected variability for the ELSA.

Next, universal heating conditions were developed that could be applied to multiple MAb molecules. rhMAb1 used as a representative of a group of MAb molecules that had minimal impact in the PS-PA ELISA, while rhMAb2 represented a group of MAb molecules with maximum interference. Applying a full factorial Design of Experiments (DOE), the ranges of two continuous factors were explored: temperature (70 to 95 °C) and heating time (2 to 15 min) with a center point of each for these two MAbs. 80 °C for 10 min was identified as the optimum condition (using the particular microwave unit), allowing both rhMAb1 and rhMAb2 to be dissociated from PA. This set of conditions was confirmed with a second DOE with narrower ranges for the factors (Fig. 6).

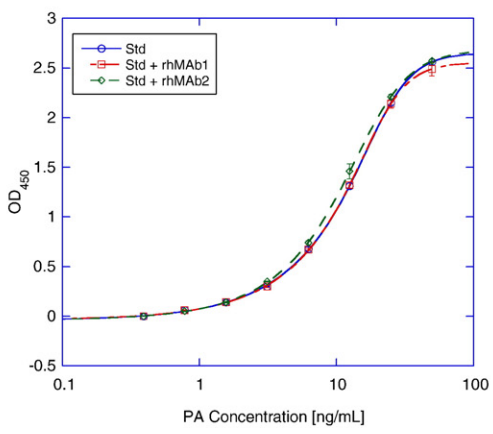
Then to verify that the optimal assay conditions identified by DOE applied to other product IgG molecules, the recovery of PA spiked at final concentrations of 10, 2.5 and 0.5 ng/mL in the presence of 21 different rhMAb product molecules was determined. Spike recovery was calculated relative to samples with no rhMAb. 90% of the 66 spiked samples were within the acceptable range of spike recovery, 70–130% (Fig. 7). It is worth to note that, none of the rhMAbs caused under recovery (less than 70%) for all three spikes. This confirmed that the product molecules were sufficiently dissociated from PA under the universal dissociation conditions to allow ELISA measurement of PA in the presence of different IgG product molecules.

Fig. 6. Determination of a universal dissociation condition by DOE. a, Contour plot of ProSep vA PA spike recovery values based on the incubation time and temperature in the presence of rhMAb1, rhMAb2, or no rhMAb (No Product); b, Standard curves with microwave heating. The optimum values of 80 °C and 10 min (for this particular instrument) are indicated on each graph.

a


Contour Plot for Spike Recovery (%) Product Molecules=No Product
Spike Recovery (%)
<= 65
<= 70
<= 75
<= 80
<= 85
<= 90
> 90
Incubation Time
Temperature
Contour Plot for Spike Recovery (%) Product Molecules=+rhMAb1
Contour Plot for Spike Recovery (%) Product Molecules=+rhMAb2

b


Std
Std + rhMAb1
Std + rhMAb2
OD450
PA Concentration [ng/mL]

In order to determine the concentration range of the product MAbs that could be effectively dissociated from the PA molecules under the selected condition, serial dilutions of PA in the presence of product rhMAb3 at concentrations ranging from 1 mg/mL to 90 mg/mL were tested. After heating, precipitation was observed in the samples containing rhMAb3 with concentrations of 25 mg/mL and above. However, no precipitation was observed for samples containing rhMAb3 at concentrations from 1 to 20 mg/mL. These samples were analyzed by ELISA (Fig. 8). All the curves are superimposable. In unheated controls the results showed an rhMAb3 dose-dependent inhibition in the ELISA. These results demonstrate that up to 20 mg/mL product IgG in the samples can be effectively dissociated from PA, allowing accurate PA detection in the ELISA.

Finally, to demonstrate that the heating with microwave had minimum impact to the integrity of PA, a set of samples containing PA was heated, using the assay condition for microwave heating, in the presence or absence of rhMAb1 or rhMAb2 compared to another set of samples without pretreatment of microwave heating. Both sets of samples were analyzed using BioAnalyzer under non-reducing condition (Fig. 9). PA migrated close to the 63 kDa band of the ladder, and comparable intensity of band was observed with or without heating by microwave (Fig. 9, Lanes 1, 4, 5 *cf.* lanes 6, 9, 10, respectively), in the presence or absence of rhMAbs (Fig. 9, Lane 1 *cf.* lanes 4 and 5). Moreover, no additional bands with faster migration were observed. This lack of significant changes of PA with microwave heating compared to that not heated suggests that microwave heating did not cause substantial fragmentation of PA.

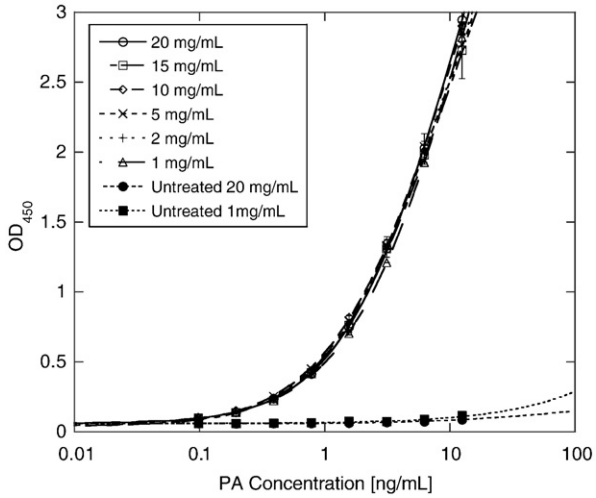


Fig. 8. Concentration range of rhMAb that can be dissociated from PA. PA standard curves spiked with rhMAb3 at concentrations of 1 (△), 2 (+), 5 (×), 10 (◊), 15 (□) and 20 (○) mg/mL with heat treatment and 1 (■) and 20 (●) mg/mL without heat treatment prior to the ELISA analysis.

3.3. Application of the MP-PA ELISA

3.3.1. Comparison of the universal dissociation condition with different forms of the PA ligand: MabSelect and MabSelect SuRe

Our Process Development organization has evaluated different recombinant PA (rPA) resins for affinity purification of rhuMab IgGs. The resins MabSelect and MabSelect SuRe differ in the number of IgG binding domains of the PA, and

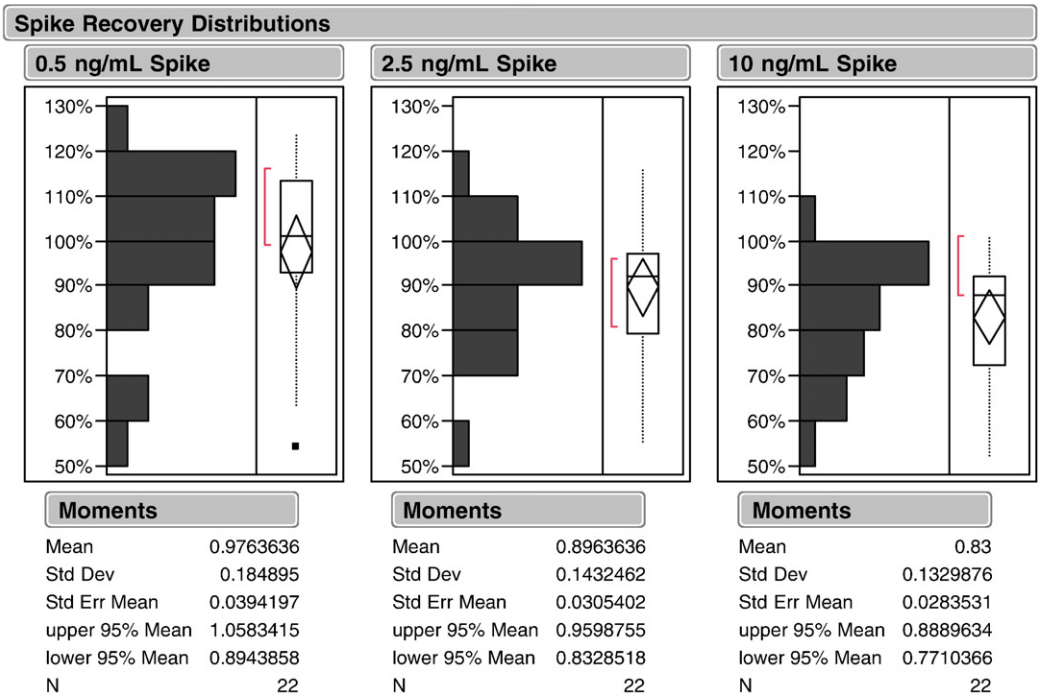


Fig. 7. Spike recovery of PA in the presence of different rhMAbs after microwave-assisted heating. Spike samples of PA at three different concentration levels; 0.5, 2.5 and 10 ng/mL in the presence of 21 different rhMAbs as well as no rhMAb were heat treated before analysis by Protein ELISA. The distributions of the spike recovery of these samples were plotted.

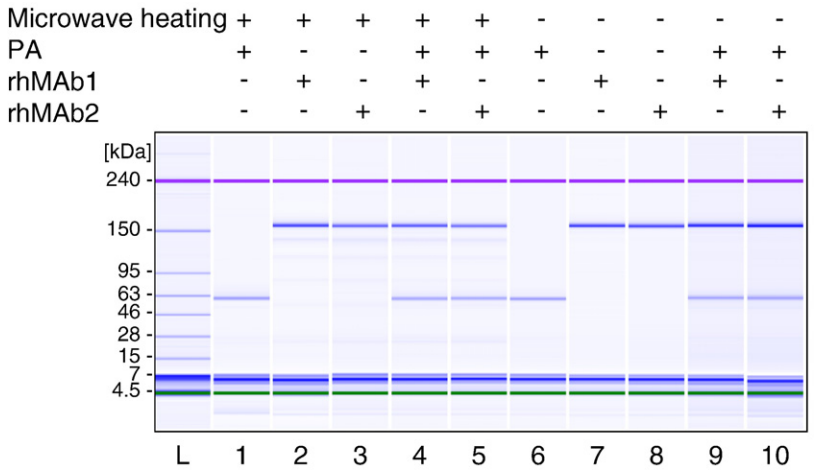


Fig. 9. PA remains intact after microwave heating. PA was heated with microwave in the presence or absence of rhMAb1 or rhMAb2. The samples were analyzed by Bioanalyzer. System markers at 240 kDa and 4.5 kDa are indicated as purple and green bands. rhMAbs migrated at around 150 kDa, PA migrated just below 63 kDa.

include engineered amino acid mutations for improved coupling efficiency and increased tolerance for alkaline conditions often used in column-cleaning procedures. These rPA ligands react similarly with the chicken anti-PA polyclonal antibody used in the ELISA, except that for MabSelect, the signals generated in the ELISA were lower. The MabSelect ligand is smaller in size and, therefore, may offer fewer binding sites for the chicken anti-PA antibodies. The product-specific inhibitory effects by different rhMAbs were also observed in the PS-PA ELISA for these rPAs (data not shown).

Using the optimized microwave-assisted dissociation, both MabSelect and MabSelect SuRe could be dissociated from product MAbs, thus allowing reliable quantification of leachate from these resins (data not shown).

3.3.2. MP-PA ELISA has comparable performance to the PS-PA method

The PS-PA method uses a standard curve that ranges from 0.39 to 50 ng/mL PA, using the standardized assay conditions of 0.2 mg/mL rhuMab. Re-expressing the standard curve PA concentrations relative to the rhuMab concentrations assay range is between 2 and 250 ng/mg or ppm (parts per million). Using the MP-PA method, samples are first diluted 1:5 in PCDB, heated, and further diluted 1:20 in DMAD, for a total 100-fold dilution. While samples up to 20 mg/ml IgG could be used, more typical in-process pool samples contain 5 mg/mL IgG. Therefore, in order to match the assay range with the PS method, the standard curve was shifted to lower concentrations, 0.098–12.5 ng/mL. Expressing these values relative to a 5 mg/mL samples, the assay range is 2–250 ppm. Thus, the new MP-PA ELISA can be used under conditions of comparable sensitivity and range as the previously used PS-PA ELISA. Further more, both methods had comparable precision (both intra and inter assay, Table 1), and improved spike recovery was observed with MP method.

3.3.3. Testing process development samples

Five sets of in-process pool samples from bioprocess development were tested for leached PA ligand (either ProSep vA or MabSelect SuRe). These in-process pools were from the PA chromatography (pH adjusted PA Pool), cation-exchange chromatography (Cation Pool), anion-exchange chromatography (Anion Pool), and the final ultrafiltration and diafiltration step (UF/DF Pool). PA levels at each step were analyzed using both the PS-PA and MP-PA methods, and the results are summarized in Table 2. Leached PA was detected in most PA pool samples by both methods, except for rhMAb2 and rhMAb4 with ProSep vA using the PS-PA method and for rhMAb4 with MabSelect SuRe ligand using the MP-PA method. With MP-PA method, some PA Pool samples showed two to three fold higher PA level as compared to that obtained with PS-PA method. However, with both methods, leached PA levels were below detectable throughout all downstream pools, indicating clearance of leached PA in later steps in the process.

Table 1
Assay performance comparison

		MP	PS
Recovery	Standard	96–110%	94–104%
	Control spike	88–106%	97–102%
	Real sample spike	80–121%	62–70%
Precision	Intra-assay	≤9%	≤4%
	Inter-assay	≤15%	≤9%
Sensitivity (ng/mL)	LOD	0.043	0.081
Working range (ng/mL)	ULOQ	12.5	50
	LLOQ	0.098	0.391
ng/mg=ppm=Protein A in sample (ng/mL)÷Mab in sample (mg/mL)			
MAb in samples (mg/mL)		5	0.2
Sensitivity (ng/mg)	LOD	0.86	0.41
Working range (ng/mg)	ULOQ	250	250
	LLOQ	2	2

Assay performance was determined for the Multi-Product (MP) and Product-Specific (PS) leached PA assay. Minimum of three assay runs were performed on different days, and key parameters of assays were obtained. Sensitivity is expressed as the limit of detection (LOD) in ng/mL. The assay working range is flanked by the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ). Concentrations are also expressed as parts per million (ppm), which is equivalent to ng PA per mg IgG.

Table 2
In-process sample testing result comparison

Process steps		rhMAb1		rhMAb2		rhMAb3		rhMAb4		rhMAb4	
		MP	*PS*	*MP*	*PS*	*MP*	*PS*	*MP*	*PS*	*MP*	*PS*
	Ligand	Prosep vA		Prosep vA		Prosep vA		Prosep vA		MabSelect SuRe	
PA Pool	(ng/mL)	50.8	16.9	34.6	LTR	103.7	46.8	37.9	LTR	LTR	0.7
	(ng/mg)	7.3	2.7	3.6	LTR	15.9	7.1	6.4	LTR	LTR	3.7
Cation Pool	(ng/mL)	LTR	LTR	LTR	LTR	LTR	LTR	LTR	LTR	LTR	LTR
	(ng/mg)	LTR	LTR	LTR	LTR	LTR	LTR	LTR	LTR	LTR	LTR
Anion Pool	(ng/mL)	LTR	LTR	LTR	LTR	LTR	LTR	LTR	LTR	LTR	LTR
	(ng/mg)	LTR	LTR	LTR	LTR	LTR	LTR	LTR	LTR	LTR	LTR
UF/DF Pool (or Bulk)	(ng/mL)	LTR	LTR	LTR	LTR	LTR	LTR	LTR	LTR	LTR	LTR
	(ng/mg)	LTR	LTR	LTR	LTR	LTR	LTR	LTR	LTR	LTR	LTR

Process samples from the recovery of different rhMAbs were tested with either the multi-product PA (MP) immunoassay or its product specific (PS) version. Either ProSep vA or MabSelect SuRe was used as the PA ligand for PA Chromatography for these samples. Concentration of PA is in ng/mL, or converted to ng/mg (highlighted in grey). LTR, stands for "lower than range".

To evaluate assay reliability, spike recovery was assessed. Five in process samples were spiked with PA at final concentration of 2.5 ng/mL and 10 ng/mL, and quantified using either PS- or MP-PA ELSA. The measurement was repeated and the spike recovery is listed in Table 3. Except for one spike with recovery of 67%, all the spikes recovered within 70–130% when quantified by MP-PA ELISA. However, using PS-PA ELISA, greater number of spikes had recovery outside the 70–130% range. This indicates that the measurement of MP-PA ELISA is more reliable.

4. Discussion

PA is a bacterial cell wall protein. Its high affinity to immunoglobulin is widely exploited for affinity purification of rhuMAbs used as therapeutic drugs. PA potentially may leach from affinity resins and co-elute with the protein. To monitor the level of the leached PA during process development, as well as in the final product, PA is analyzed using a sandwich ELISA based on chicken anti-PA antibodies. Most bio-process samples have a high level of IgG that forms complexes with the leached PA. The presence of the product has been observed to interfere with the analysis of PA in the ELISA.

Table 3
Spike recovery comparison

	Spike conc. [ng/mL]	*PS (%)*		*MP (%)*	
		Assay1	Assay2	Assay1	Assay2
Sample 1	2.5	***138***	***106***	***81***	***95***
	10	***113***	***120***	***88***	***85***
Sample 2	2.5	***159***	***129***	***71***	***94***
	10	***127***	***137***	***67***	***114***
Sample 3	2.5	***174***	***120***	***76***	***106***
	10	***137***	***134***	***95***	***93***
Sample 4	2.5	***189***	***122***	***81***	***105***
	10	***141***	***144***	***83***	***99***
Sample 5	2.5	***165***	***108***	***85***	***113***
	10	***134***	***135***	***76***	***92***

PA was spiked into the in-process samples (1 through 5) at final concentration of 2.5 and 10 ng/mL. These samples were tested either by the PS-PA or the MP-PA. The spike recovery was calculated as the percentage of the concentration obtained by the assays compared to the expected concentration. Samples that fall within the desired spike recovery values of 70–130% are shaded.

Genentech has been using a PS-PA ELISA in which samples are diluted to 0.2 mg/mL rhMab and standards and controls are spiked with the same rhMAb at this level. The standards and controls must be spiked with the same rhMAb as in the test samples because different antibodies inhibit the ELISA to different extents. This PS-PA method is labor intensive, has low sample throughput, and limited potential for automation. With an increasing number of rhMAbs in our product pipeline, we have the need for a method independent of the specific rhuMAb matrix.

In this paper the optimization of sample pretreatment methods that dissociate the leached PA from the IgG matrix is described. This work builds on the previous observation that the PA/IgG complex can be dissociated with a combination of detergents, chelators, and heat (Steindl et al., 2000). The conventional heating was replaced with a microwave heating system, optimized to work with samples in 96-well microtiter plates.

Microwave-assisted heating under controlled conditions (such as temperature and or pressure) has become an invaluable technology for medicinal chemistry and drug discovery, as it allows a dramatic decrease of reaction time and the achievement of extreme reaction conditions (Kappe and Dallinger, 2006). This technology has been widely used in organic synthesis and proteolytic cleavage reactions.

The goal was to develop a multi-product PA assay that can be readily applied to different rhMAbs. Our optimized MP-PA dissociation method uses a 1:5 dilution of the sample into dissociation buffer, microwave heating for 80 °C for 10 min (qualified for the individual microwave unit), followed by a 1:20 dilution of the sample into DMAD. No loss of signal was cobserved with longer heating, suggesting that protein stability was maintained. The MP-PA method demonstrated spike recoveries generally within the acceptable range of 70–130% for 21 different rhMabs spiked at three different levels. In addition, this universal dissociation treatment could be used with different rPA ligands, such as Prosep vA, MabSelect, and MabSelect SuRe. Finally, up to 20 mg/mL rhMab could be dissociated from PA using this method.

In these studies, samples spiked with PA ligand often demonstrated recoveries that were outside the 70–130% range of the expected value of PA when quantified using the product-specific ELISA, e.g., over-recovery was seen with PA spiked into five samples tested by PS-PA method (see Table 3).

However, when applying the dissociation treatment with microwave-assisted heating to the same sample set, the number of assay values showing a spike recovery outside of the desired range of 70–130% of the expected value was only 1 out of 10. Using an orthogonal method, i.e. by Bioanalyzer, PA was shown mostly intact after microwave heating compared to not heated sample (Fig. 9) indicating that little fragmentation of PA was caused by microwave heating. Thus, a plausible explanation for improved spike recovery is that, the dissociation facilitated by microwave heating allowed improved recovery due to minimized interference by the pA/IgG complex. As a consequence, the quantification of PA is more reliable using the MP-PA assay based on dissociation.

In summary, a multi-product leached PA assay based on the dissociation of PA/IgG complexes was developed. We have utilized a novel technology, microwave-assisted heating, to facilitate the dissociation. This product independent assay will be useful to test leached PA in a variety of the rhMAbs intended for therapeutic purposes, and it is automation-ready for higher throughput and efficiency.

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