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A novel method for quantitative measurement of a biomarker in the presence of a therapeutic monoclonal antibody directed against the biomarker

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ABSTRACT

Therapeutic monoclonal antibodies (MoAb) have become important tools in the treatment of numerous diseases. Many of these MoAb are present in the blood at very high levels due to high dosing and long half-lives. Quantification of biomarkers bound by these therapeutic MoAb can be an important factor in determining efficacy and dosing requirements. However, quantitation of these biomarkers with reasonable accuracy can be very difficult to accomplish due to concomitant binding of the therapeutic MoAb. We describe here a novel method for quantifying total (free plus bound) biomarker concentration in the presence of high levels of therapeutic MoAb using a single non-competing MoAb in a capture/elution format. This assay has the capability to accurately detect and quantitate circulating ng/ml biomarker levels in the presence of 200 μg/ml or more of therapeutic MoAb.

1. Introduction

Biomarkers are being used extensively to improve drug development processes including selection of lead compounds, animal model evaluations, early efficacy and safety studies and proof of concept studies [1–2]. These biomarkers come in many forms from physiological indicators such as blood pressure to molecular antigens, antibodies and genetic markers. Many molecular biomarkers are detected with immunoassays. The development and validation of an immunoassay method for a circulating biomarker in support of drug development can represent a challenging endeavor [3–4]. This challenge can be magnified substantially if the biomarker in question is the antigen recognized by a therapeutic monoclonal antibody directed against the biomarker of interest.

Immunoassay interference by antibodies other than those incorporated into the specific immunoassay can be a significant problem. These antibodies can include heterophile antibodies [5], autoantibodies [6], anti-drug antibodies [7] and therapeutic monoclonal antibodies. Typically, biomarkers are detected using sandwich immunoassays where the biomarker is captured by one polyclonal or monoclonal antibody and subsequently detected with a second monoclonal or polyclonal antibody. However, when the biomarker in question is the antigen for a therapeutic monoclonal antibody, the circulating biomarker can become bound to the therapeutic antibody rendering it more difficult if not impossible to quantify accurately.

There are currently over 20 FDA approved monoclonal antibodies with numerous others in clinical trials. These antibodies can be present at levels measured in 100's of μg/ml [8]. Quantitation of the antigen bound by these antibodies can provide critical information on the therapeutic effects of the antibody treatment as well as dosage levels in subsequent treatments. For example, several monoclonal antibodies to amyloid-beta are currently in development for potential treatment of Alzheimer disease. Measurement of amyloid-beta in the presence of high levels of circulating therapeutic anti-amyloid beta monoclonal antibody would be important in tracking therapeutic effects [9]. Therefore, a simplified approach is needed to enable routine biomarker quantification when both free and antibody bound forms of a biomarker are present in the circulation. Such an approach would permit one to monitor temporal changes in circulating levels of a target biomarker following treatment with a therapeutic antibody. Changes in total biomarker

Abbreviations: MoAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; NHS, normal human sera; CV, coefficient of variation.

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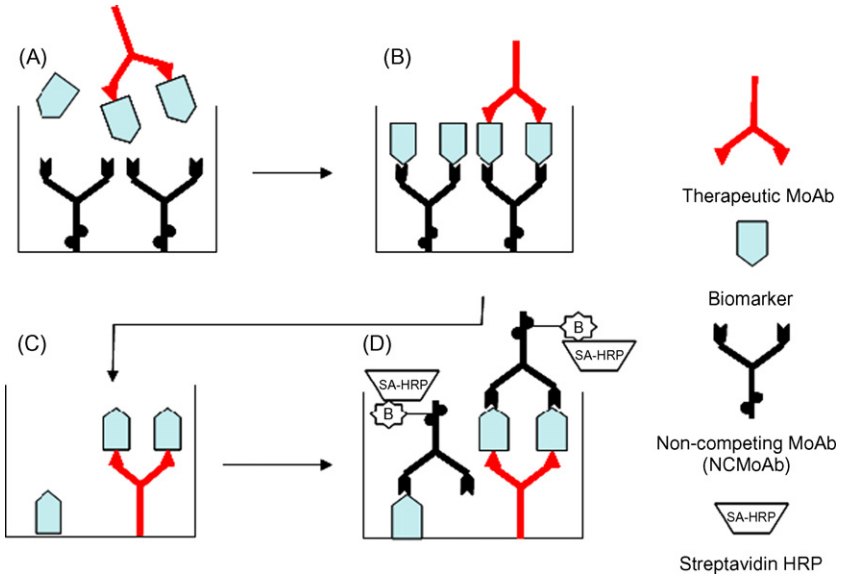

Fig. 1. Biomarker assay. Samples containing biomarker and therapeutic anti-biomarker are added to non-competing anti-biomarker MoAb (NCMoAb) coated plate (A). Captured free and bound biomarker (B) is eluted with acid, transferred to a fresh plate and allowed to bind (C). Bound biomarker is detected with biotinylated NCMoAb and developed with Streptavidin–HRP/TMB (D).

concentrations can only be correctly assessed, if both free and bound biomarker forms are accessible in the assay.

We describe here a novel immunoassay format to determine the total concentration (bound plus free) of a circulating biomarker in the presence of a therapeutic antibody to that biomarker. The assay is based on acid elution techniques originally developed for measurement of anti-drug antibodies in the presence of free drug [10–11]. Serum samples containing free biomarker and potential anti-biomarker therapeutic monoclonal antibody bound biomarker are incubated on a microtiter plate coated with a monoclonal antibody that does not compete with binding of the anti-biomarker therapeutic monoclonal antibody. This allows for capture of both free and therapeutic antibody bound biomarker. Captured biomarker is subsequently eluted with acid and transferred to a second microtiter plate where it is neutralized and allowed to non-specifically bind. This bound biomarker can then be detected using a biotinylated version of the same non-competing monoclonal antibody used in the original capture step. This format has been shown to allow quantitation of ng/ml levels of biomarkers in the presence of several hundred μg/ml of anti-biomarker therapeutic monoclonal antibodies in multiple biomarker systems.

2. Experimental

2.1. Materials

Non-competing monoclonal antibodies for biomarkers, therapeutic monoclonal antibodies (LY-A and LY-B) as well as recombinant biomarker standards for biomarkers A (approximately 20 kDa monomeric protein) and B (approximately 25 kDa homodimer) were developed at Lilly Research Laboratories (Indianapolis, IN, USA). Sodium carb-bicarb coating buffer (BupH carbonate–bicarbonate), Ezlinked™ Sulfo-NHS-LC Biotin and Streptavidin–Horseradish Peroxidase (HRP) (1 mg/ml) were obtained from Pierce (Rockford, IL, USA). 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate was purchased from BioFx (Owings Mills, MD, USA). The ELx405 plate washer was purchased from Bio-Tek (Winooski, VT, USA). Clear polystyrene 96-well microtiter plates were acquired from Nunc (Rochester, NY, USA).

2.2. Biotinylation of non-therapeutic anti-biomarker MoAb

Sulfo-NHS-LC Biotin was added to a 2 mg/ml solution of non-therapeutic anti-biomarker MoAb in 25 mM sodium phosphate/150 mM sodium chloride, pH 7.4 (PBS) to yield a 20-fold molar excess of biotin. The reaction was mixed by vortexing and allowed to incubate for 1 h at ambient temperature. The mixture was dialyzed against PBS for 2 h at ambient temperatures followed by overnight dialysis at 4 °C to remove free biotin. Protein concentrations were determined using a BCA protein quantification kit from Pierce. Biotinylated antibody was stored at −20 °C in 50% glycerol at a concentration of 1 mg/ml.

2.3. ELISA reagents

All reagents, except the biomarker standard, were stored at 4 °C. Assay buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA and 5 mM EGTA) and dilution buffer (assay buffer with bovine serum albumin and therapeutic monoclonal antibody) were made in house. Coating buffer consisted of BupH Carbonate–Bicarbonate Buffer (Pierce). ELISA plate wash buffer was tris-buffered saline (TBS) with 0.05% Tween-20 (TBST). Blocking buffer was Blocker casein in TBS (Pierce, 10% casein in TBS). Acetic acid (300 mM), 1 M tris base, pH 9.5 and stop buffer (2 M phosphoric acid) were made in house. A vial of recombinant biomarker standard in phosphate-buffered saline (PBS) was used to make stock solutions and spiked recovery samples. Biomarker standard was diluted with dilution buffer to make appropriate stock standard solutions. These standards were stored in aliquots at −20 °C.

2.4. ELISA procedure

A schematic representation of the assay is shown in Fig. 1. Polystyrene 96-well microtiter plates were coated with 5 μg/ml of capture MoAb in coating buffer (100 μl/well). After incubation for 16–24 h at 4 °C, plates were washed three times with wash buffer. Standards and samples diluted with dilution buffer were added to the plate (100 μl/well) and allowed to incubate at ambi-

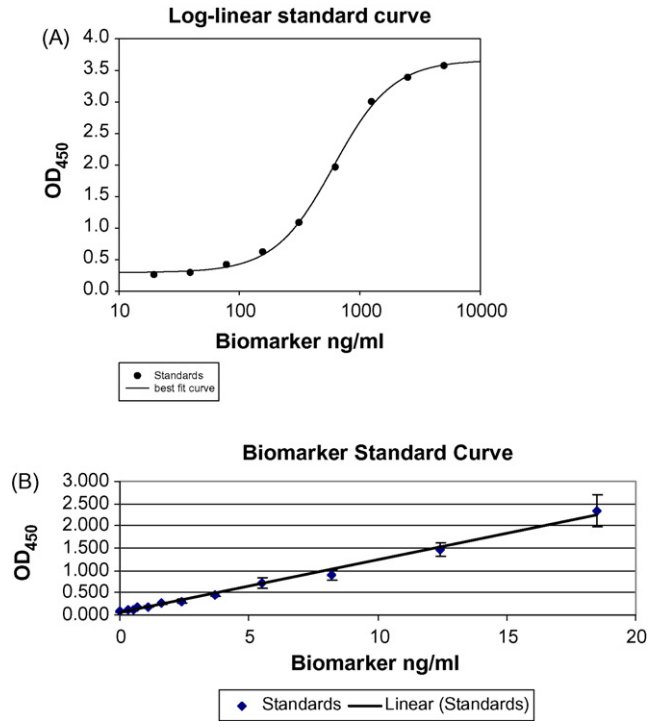

Fig. 2. Standard curves. Biomarker A standards were serially 2-fold diluted from 5000 to 20 pg/ml and run in the normal assay format. Resulting data was fit using a four-parameter curve fit (A). A second biomarker (B) standard was serially 2/3-fold diluted from 18.5 to 0.3 ng/ml. Resulting data was fit using linear regression (B).

ent temperature for one hour. Plates were washed three times and bound biomarker eluted with 65 μl of 300 mM acetic acid added to each well and incubated for 10 min. After incubation, 50 μl of the acid eluted material was transferred to a second, clean polystyrene microtiter plate containing 50 μl of 1 M tris base, pH 9.5. Neutralized samples were allowed to bind for 1 h at ambient temperature. Plates were then washed three times with wash buffer, blocked with blocker casein (200 μl/well) for 1 h at ambient temperature. Plates were washed three times with wash buffer. Biotinylated non-competing MoAb diluted 1:8000 in assay buffer was added to the plate (100 μl/well) and allowed to incubate for 1 h at ambient temperature. After washing the plate three times with wash buffer, Streptavidin–HRP at an appropriate dilution was added to the plate (100 μl/well) and allowed to incubate for 1 h. The plate was washed for a final three times and 100 μl of TMB substrate was added to each well. Plates were allowed to develop for 30 min at ambient temperature, stopped by addition of 100 μl of stop buffer and read at 450 nm.

A data analysis software program (Sigma Plot, Systat Software Inc., San Jose, CA, USA) was used to fit the ELISA binding data by either a four-parameter logistic model algorithm or linear regression. Biomarker concentrations were estimated by interpolation using a standard curve of recombinant biomarker standard in dilution buffer over an appropriate concentration range.

2.5. Assay precision and accuracy validation

Inter-assay precision and accuracy were assessed by measuring biomarker in control samples, which were prepared by adding biomarker standard to normal human serum (NHS) samples at concentrations of 2, 5, 10, 25, and 50 ng/ml. Validation experiments were conducted by measuring each concentration in quintuplet using three separate pools of NHS.

Table 1
Standard curves

	Biomarker assigned value (ng/ml)	Biomarker observed average (ng/ml)	S.D. (ng/ml)
Std 1	0.3	0.4	0.12
Std 2	0.5	0.5	0.06
Std 3	0.7	0.7	0.11
Std 4	1.1	1.1	0.05
Std 5	1.6	1.5	0.07
Std 6	2.4	2.3	0.09
Std 7	3.7	3.5	0.18
Std 8	5.5	5.6	0.44
Std 9	8.2	8.2	0.37
Std 10	12.4	12.2	0.35
Std 11	18.5	18.7	0.42

Standards were back fit from 5 separate standard curves after linear regression for biomarker B. Results are expressed as mean and standard deviation (S.D.).

2.6. MoAb drug tolerance

Assay tolerance was validated by measuring biomarker levels in the presence of therapeutic MoAb directed toward the biomarker in two separate experiments. The first experiment spiked NHS samples with biomarker levels of 2, 5, 10, 25, and 50 μg/ml and therapeutic MoAb levels of 12.5, 25, 50, 100, and 200 μg/ml. These experiments were run using three separate NHS samples in duplicate. The second experiment spiked therapeutic MoAb into NHS at levels of 62.5, 125, 250, 500 and 1000 μg/ml. These samples were assayed over 3 separate runs.

3. Results

3.1. Standard curve assessment

Standard curve assessment was shown to be dependent on the biomarker, the non-competing monoclonal antibody and the range requirements dictated by expected levels of the biomarker in question. A four-parameter model for fitting the ELISA calibration curve was shown to be suitable for biomarker A/monoclonal antibody anti-A pair tested over a range of biomarker standards from 5 to 5000 pg/ml (Fig. 2A). Back fitted standards showed excellent fit to the standard curve. Biomarker B requiring a much smaller range of normal values demonstrated a good fit for the Biomarker B/monoclonal antibody anti-B combination using linear regression over a range of 0.3–18.5 ng/ml (Fig. 2B). Biomarker B standards evaluated by plotting evaluated results against their target values (Table 1) showed excellent correlations with a slope of 1.003 and an intercept of −0.03 ng/ml.

3.2. Precision and recovery

Intra-assay precision was assessed by running biomarker B samples repeatedly ($N = 20$) within a single run and showed CVs ranging from 6 to 11% (Table 2). Inter-assay precision was assessed by run-

Table 2
Precision—serum samples were run 20 times each to assess intra-assay precision for biomarker B

	Mean (ng/ml)	S.D.	%CV	*N*
Sample 1	6.4	1.22	5.9	20
Sample 2	7.9	0.67	10.4	20
Sample 3	20.9	0.87	11.0	20

Table 3
Recovery—samples were spiked with biomarker A at levels from 2 to 50 ng/ml into 3 separate normal human sera pools and in quintuplet

Assessment of spiked recovery experiment					
Spiked amount (ng/ml)	Recovered (ng/ml) (mean)	Expected (ng/ml)	Difference (%)	S.D.	CV (%)
0	1.35	1.35	100[a]	0.12	9.07
2	2.84	3.35	85	0.78	27.45
5	7.44	6.35	117	1.95	26.16
10	11.47	11.35	101	1.80	15.67
25	26.32	26.35	99	2.10	7.99
50	45.25	51.35	88	3.81	8.42

[a] Expected values were derived from endogenous levels detected in serum.

ning the same samples across 5 runs and was shown to range from 3 to 12%. Accuracy was assessed by spiking biomarker A standard into normal human serum (NHS) samples containing endogenous levels of biomarker. When spiked with biomarker standard levels of 2–50 ng/ml, recoveries were shown to range from 85 to 117% of expected values (Table 3).

3.3. MoAb drug tolerance

The tolerance of the assay for excess therapeutic MoAb was evaluated by creating control samples that simulated a therapeutic MoAb treated patient. Samples were spiked with controlled amounts of biomarker A from 2 to 50 ng/ml as well as 0 to 200 μg/ml of therapeutic MoAb to biomarker A. Results in Fig. 3 show excellent recoveries within ±20% of the expected of biomarker levels even in the presence of 200 μg/ml of potentially interfering therapeutic MoAb. Biomarker B was evaluated for therapeutic MoAb levels of up to1000 μg/ml to simulate samples from a patient treated with high levels of MoAb within a relatively short time after dosing. Results shown in Fig. 4 show excellent recoveries of biomarker levels across the range from 62.5 to 250 μg/ml of therapeutic MoAb. Higher levels of MoAb show slight decreases in recoveries with 92 and 86% at 500 and 1000 μg/ml of therapeutic MoAb, respectively, showing good tolerance to extremely high levels of therapeutic drug.

3.4. Assay sensitivity

The limit of detection for biomarker B was shown to be approximately 2 ng/ml. This compared favorably with a normal range of biomarker B in 50 normal human sera of 2.2–18.5 ng/ml with a median value of 8.2 ng/ml.

4. Discussion

We describe here a novel technique for quantifying biomarkers in the presence of high concentrations of therapeutic MoAbs reactive with those same biomarkers. Therapeutic monoclonal antibodies are often dosed at high levels and have long half-lives resulting in high blood levels over significant time periods. There are many reports of various types of antibodies interfering with immunoassays and biomarker assays are no exception. Acid elution techniques have been recently developed to address the issue of anti-drug antibody detection in the presence of free monoclonal therapeutic MoAb [10–11]. We have adapted those techniques to the detection of biomarkers in the presence of high concentrations' of therapeutic MoAb.

Detection and quantification of a circulating protein biomarker is commonly performed by sandwich ELISA using a polyclonal or monoclonal antibody for capture and a second antibody for detection. When a therapeutic antibody is administered it is by function going to bind to its intended target. If the target is a circulating biomarker, steric hindrance and blocking of epitopes can cause inconclusive results, especially for smaller biomarkers with limited epitopes. A typical sandwich ELISA would require binding of two non-competing antibodies in addition to the therapeutic antibody to the biomarker at the same time to achieve detection in the presence of significant therapeutic blood levels.

The approach that we used to avoid potential steric hindrance on the biomarker was to use a single MoAb that was directed toward an epitope of the protein that was not in competition with that of the therapeutic MoAb which could therefore bind to both therapeutic bound and unbound biomarkers. After subsequent acid elution the same antibody used for capture was used in a labeled form as the detecting antibody allowing for assay development utilizing a single non-competing MoAb. This also obviates the need for a second non-competing antibody. Since the therapeutic and capture/detection antibody theoretically never interact, any relative affinity differences between the two antibodies should not impact the assay.

Standard curves using this format showed excellent quantitative properties. The range of quantitation required for a particular biomarker can depend on the range of expected values. We have demonstrated here that for relatively narrow ranges, linear regression is adequate for quantitation. However, if a broader range is

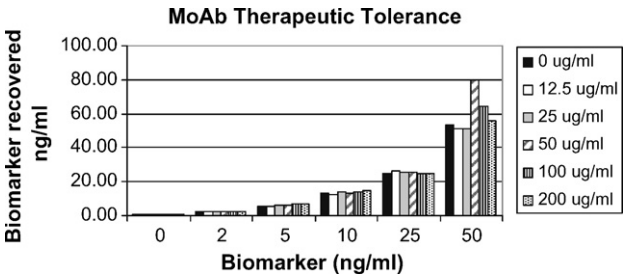

Fig. 3. Therapeutic MoAb tolerance. Biomarker A was spiked into normal human serum at levels of 2–50 ng/ml and these samples were spiked with 12.5–200 μg/ml therapeutic MoAb and run in the normal assay format. Results are expressed in ng/ml of biomarker recovered.

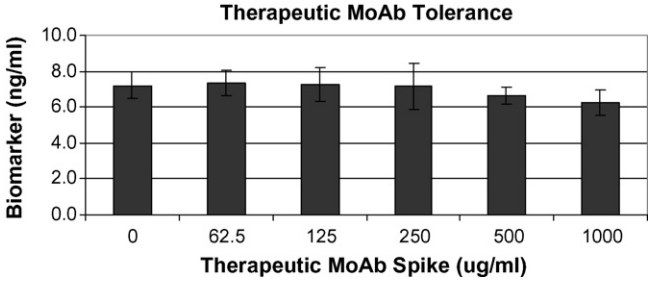

Fig. 4. High level therapeutic MoAb tolerance. Normal human serum was spiked with 62.5–1000 μg/ml therapeutic MoAb and analyzed for biomarker A in duplicate over 4 runs. Results are expressed as means with error bars to indicate standard deviations.

required, a four-parameter fit gives excellent results. If biomarker levels exceed the dynamic range of the existing standard curve, we have demonstrated excellent dilutional linearity to accommodate broader concentration ranges (data not shown).

The format described here requires extra manipulations relative to a standard sandwich ELISA involving the capture, elution and transfer of the biomarker to a second plate. These extra steps in the assay could have an adverse impact on the precision. Results shown here however indicate that the assay has excellent precision with values 3–12% CVs for normal assay values.

Spiked recoveries across a range of biomarker concentrations and spiked therapeutic MoAb concentrations showed excellent recoveries. Spiked recoveries demonstrate that the biomarkers in use here are capable of binding to the non-competing monoclonal antibody in the first plate, being eluted with acid, binding quantitatively to the blank second well after neutralization and being detected by the labeled version of the non-competing antibody. Samples that have extremely high biomarker levels could result in reduced reactivities due to saturation of the capture antibody or saturation of the binding surface on the second plate. As with any quantitative assay, care should be taken to avoid quantitating results outside of those that have been validated through spiked recovery studies. High biomarker levels can be diluted to give signals that fall within the standard curve, alleviating concerns about high levels of biomarker saturating the system at any of the binding steps. Tolerance to drug levels up to 1 mg/ml would indicate the capability to follow biomarkers throughout a clinical trial, regardless of dosing (single or multiple) and timing of sample acquisition. This could provide valuable information to the trial that would otherwise be unattainable due to drug interference.

Waldrop et al have described a technique that also utilizes an antibody capture and elution step to analyze anti-glutamic acid decarboxylase 65 (GAD65) in the presence of interfering autoantibodies [12]. Their assay utilizes a capture MoAb to a site on the GAD65 that is not normally bound by autoantibodies. Samples were captured by MoAb covalently bound to magnetic beads followed by elution using 6 M guanidine HCl and transfer to high-binding filter plates followed by detection with polyclonal anti-GAD antibody and a secondary anti-IgG antibody.

We have shown that covalent attachment of the capture antibody is not required for antibodies coated onto standard polystyrene plates. Elution treatment with 300 mM acetic acid had no detectable effect on coated antibody levels (data not shown). We have not found any system to date that required more stringent denaturation conditions than provided for by the acetic acid elution to accomplish release of the bound antigen although this should be confirmed for each individual antibody systems.

Similarly, coating the eluted protein onto standard microtiter plates has proved to be adequate in the systems used. Additionally, the use of directly labeled detection antibody avoids the extra wash steps required when using a secondary detection antibody.

Radioimmunoassays (RIA) often use single antibodies in conjunction with labeled antigens to establish sensitive assays and could be considered if a second non-competing antibody was available. However, the use of radio-labeled material entails numerous difficulties including frequent labeling due to decay and waste disposal issues. The use of common ELISA techniques abrogates many of these concerns while providing for a sensitive assay format.

The assay described here utilizes standard 96-well formats and normal ELISA plate technologies that most laboratories could run without special equipment or supplies. This allows for greater freedom in development and possible assay outsourcing as well as a format readily adaptable to higher throughput techniques and possible automation.

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