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Novel approaches using alkaline or acid/guanidine treatment to eliminate therapeutic antibody interference in the measurement of total target ligand

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ABSTRACT

Measurement of the total target ligand can help to provide pharmacokinetic (PK) and pharmacodynamic (PD) informations. However, the presence of monocloncal antibody therapeutics (ThAs) interferes with ELISA determinations of the total target proteins. The interferences can cause over- or under-estimation of the target protein analysis. The nature of interferences was dependent upon the ThA, target protein, antibody reagents and assay conditions of the ELISA. We have developed novel alkaline and acid/guanidine treatment approaches to dissociate the protein binding and preferentially denature the ThA. The neutralized target proteins can be determined by ELISA. These methods provide reproducible measurements of total target protein without ThA interference. Serum samples, standards and QCs containing target protein and ThA were treated with alkaline buffer (pH > 13) containing casein or acid/guanidine buffer (pH < 1). Total target proteins for two different ThA systems were successfully measured and interferences were completely eliminated by the treatments. These methods were successfully applied to analysis in pre-clinical serum samples.

1. Introduction

A monoclonal antibody therapeutic (ThA) is often developed to bind to a target protein/peptide receptor to illicit downstream biological effects. The target can be a receptor at the cell surface or a soluble ligand in circulation. Generally, immunoglobulins (IgGs) have significantly longer half-lives than other circulating proteins due to the protection from degradation by the neonatal Fc receptor (FcRn) [1]. The binding of target protein to IgG will likely decrease its catabolism resulting in accumulation of the bound form [2,3]. However, the free drug, not the complex form represents the bioactive ThA. To select the proper dosing for maintaining the efficacious level, it is important to determine the free ThA concentrations. Measuring the free ThA has many limitations due to lack of inhibitory anti-idiotypic reagents as well as the uncertainty of complex dissociation due to sample dilution and long incubation time resulting in an over-estimation of the free level. However, the free ThA level can be estimated with data of the total ligand and total ThA [4,5]. Therefore, reliable methods of total target ligand and ThA are needed for the estimation of the efficacious level of a ThA.

The widely used methods for quantification of target proteins are immunoassays (typically ELISA) because of their superior specificity and sensitivity [6–8]. However, ThA will interfere with immunoassay [9] either by binding to an epitope that overlaps with that of the assay reagent resulting in under-estimation or by forming complexes that enhances the readout signals resulting in over-estimation. Since the relative concentrations of antibody and target protein can vary over a wide range, data adjustment with correction factors by preparing the corresponding mixtures is not feasible.

We have developed an option to measure total target protein in the presence of ThA. The ThA–target complex was dissociated under conditions that selectively and irreversibly denatured the ThA wherein the target protein would retain immunoreactivity upon renaturation to be measured by ELISA.

IgGs are relatively sensitive to pH and temperature changes that may cause conformational and structural changes [10–14]. At extreme pHs (<1 or >13) these changes can be irreversible for the ThA to regain binding activity to the target protein even after restoring the pH to neutral [11]. However, the target protein may be more resistant to pH variations. Factors such as isoelectric points of the protein, molecular weight, structural conformation and the number of disulfide bonds may play a role in its stability at different pHs and refolding to the native state.

Abbreviations: AP, alkaline phosphatase; ELISA, enzyme-linked immunosorbent assay; HRP, horse radish peroxidase; PBS, phosphate buffered saline; QCs, quality control samples; RLU, relative luminescence unit; Stds, standards; ThA, therapeutic antibody.

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Acid dissociation of protein complexes such as that of ThA with anti-ThA antibody or target ligands, as well as target with proximal proteins has been reported [15–22]. Acid treatment to dissociate complexes has been commonly used as a method for purification. For example, the bound protein was eluted from a solid phase immunoabsorbent by acid [16]. Acid dissociation has also been used for ELISA measurement of anti-ThA antibodies [20]. The dissociation conditions of these applications have been chosen to be non-denaturing and reversible. If the pH is substantially lower (pH < 2.5), it may result in irreversible denaturation of the antibody and/or antigen. In addition, many serum proteins at very low pH become insoluble even after neutralization, resulting in samples that cannot be pipetted. However, at 8 M guanidine and low pH (pH < 1) most IgGs and serum proteins are instantly denatured while being soluble and remained soluble upon neutralization (unpublished observations).

It was reported that placental alkaline phosphatase (AP) was stable at pH 10.7 and alkaline elution could be used for isolation from antibody affinity columns [16]. Isolation of antibodies from placental AP columns was also feasible, however, there was rapid denaturation of specific antibodies under the conditions where the enzyme was stable (unpublished observations). The data suggest that alkaline conditions can be used to dissociate complexes and irreversibly denature ThA to measure total ligand that is relatively stable in alkaline.

In this work, we investigated the use of alkaline and guanidine/acid to dissociate ThA–target complex prior to ELISA and demonstrated the successful proof of principle and method development for two target proteins (protein-X and -Y). Standards (Stds), study samples and quality control samples (QCs) were all treated under the same conditions. In both cases, treatment of the samples at extreme pH (>13 or <1) preferentially denatured ThA while the target proteins remained immunoreactive to be analyzed.

2. Experimental

2.1. Chemicals and reagents

Fully human monoclonal ThA, monoclonal antibody reagents, and purified target proteins were produced at Amgen, Inc. (Thousand Oaks, CA). Casein, bovine serum albumin (BSA), horse radish peroxidase (HRP), sodium hydroxide, acetic acid, sulfuric acid, potassium phosphate, Tween 20, sodium chloride, imidazole, guanidine and glycine were purchased from Sigma (Saint Louis, MO). Dulbecco's phosphate buffered saline (PBS) was obtained from Invitrogen (Carlsbad, CA). Streptavidin alkaline phosphatase (AP) conjugate was acquired from Jackson Immuno Research Laboratories, Inc. (West Grove, PA). 96 well plates were purchased from Corning (Corning, NY). The Femto super signal chemiluminescence substrate for HRP was obtained from Thermo Fisher (Rockford, IL). CDP-Star Ready-to-Use with Emerald-II Enhancer was purchased from Applied Biosystems (Foster City, CA). 20×KPL wash buffer was obtained from KPL, Inc. (Gaithersburg, Maryland). I-Block assay buffer was prepared at Amgen, Inc. (Thousand Oaks, CA).

2.2. Instruments

Alkaline or acid/guanidine treatment of standards, QCs and samples was performed using a Tecan/TEMO liquid handling system (Tecan, Durham NC). The precise timing of the alkaline or acid/guanidine treatment of Stds, QCs and samples was accomplished by a 96 tip head of the Tecan/TEMO liquid handling system. The chemiluminescent signal of the ELISA was measured by a SpectraMax 384Plus Luminometer (Molecular Devices, Sunnyvale, CA).

2.3. Preparation of alkaline and acid/guanidine treatment buffers

The alkaline treatment buffer was prepared by heating a mixture of 1 l of 1 M NaOH/0.5 M glycine buffer (pH estimated to be >13) to the boiling point in a 2-l glass bottle. After removed from the heater 100 g of casein was immediately added to the solution and mixed well. The alkaline buffer was stored at room temperature for 3 days. Solid particulates were removed from the buffer by centrifugation followed by filtration. The acid/guanidine buffer was prepared by making an 8 M guanidine solution in 1 M acetic acid and 0.1 M sulfuric acid (pH estimated to be <1).

2.4. Alkaline and acid/guanidine treatment procedures

2.4.1. Alkaline treatment protocol

The Stds, QCs and samples were first diluted 1:4 in I-Block buffer and 80 μl of these diluted samples were transferred to a 96 well plate. An equal volume of the alkaline treatment buffer was added and thoroughly mixed. Immediately 100 μl of each sample was transferred to another 96 well plate. After 30 min incubation, 100 μl of neutralizing buffer (1 M potassium phosphate buffer, pH 6.0) was added and mixed. The alkaline treated and neutralized samples were directly loaded into an ELISA plate.

2.4.2. Acid/guanidine treatment protocol

The Stds, QCs and samples were diluted 1:10 in a buffer of 1× PBS, 1 M NaCl, 0.5% Tween 20 and 10 mg/ml BSA and 80 μl of these diluted samples were transferred to a 96 well plate. An equal volume of the acid/guanidine treatment buffer was added and thoroughly mixed. Immediately 80 μl of each sample was transferred to another 96 well plate and incubated for 10 min. Then 100 μl of neutralizing buffer (1 M potassium phosphate buffer, pH 7.8) was added and mixed well. The acid/guanidine treated and neutralized samples were directly loaded into an ELISA plate.

2.5. ELISA method

2.5.1. ELISA method for target ligand

The schematic diagram of ELISA for total target ligand is shown in Fig. 1a. Half-area 96 well black plates (Corning 3694) were coated with capture antibody (1–2 μg/ml) in 1× PBS and incubated overnight at 4 °C. The plates were washed with 2 mM imidazole buffered saline with 0.02% Tween 20 (1×KPL buffer), and blocked overnight by I-Block buffer at 4 °C. After washing the plate, 50 μl of the treated samples were added and incubated for 1.5–2 h. The ELISA plate was washed with 1×KPL and 50 μl of 200 ng/ml HRP-conjugated detection antibody was added and incubated for 1.5 h. After washing the plate, 75 μl of the chemiluminescent Femto substrate was added and the signal was read by a SpectraMax 384Plus Luminometer.

2.5.2. Duplex ELISA method for complex

The protocol of duplex ELISA method (Fig. 1b) was similar as described in Section 2.5.1 except that 50 μl of 100–200 ng/ml of AP-conjugate of the ThA detection antibody along with 100–200 ng/ml HRP-conjugate of the detection antibody for the target ligand were added to the plate and incubated for 1.5 h. After washing the plate, 75 μl of the chemiluminescent Femto substrate was added and the signal was read by a SpectraMax 384Plus Luminometer. Then the plate was washed and 50 μl of CDP-Star with Emerald-II Enhancer chemiluminescent substrate for AP added and incubated for another 30 min. The chemiluminescent signal for AP activity was measured by a SpectraMax 384Plus Luminometer.

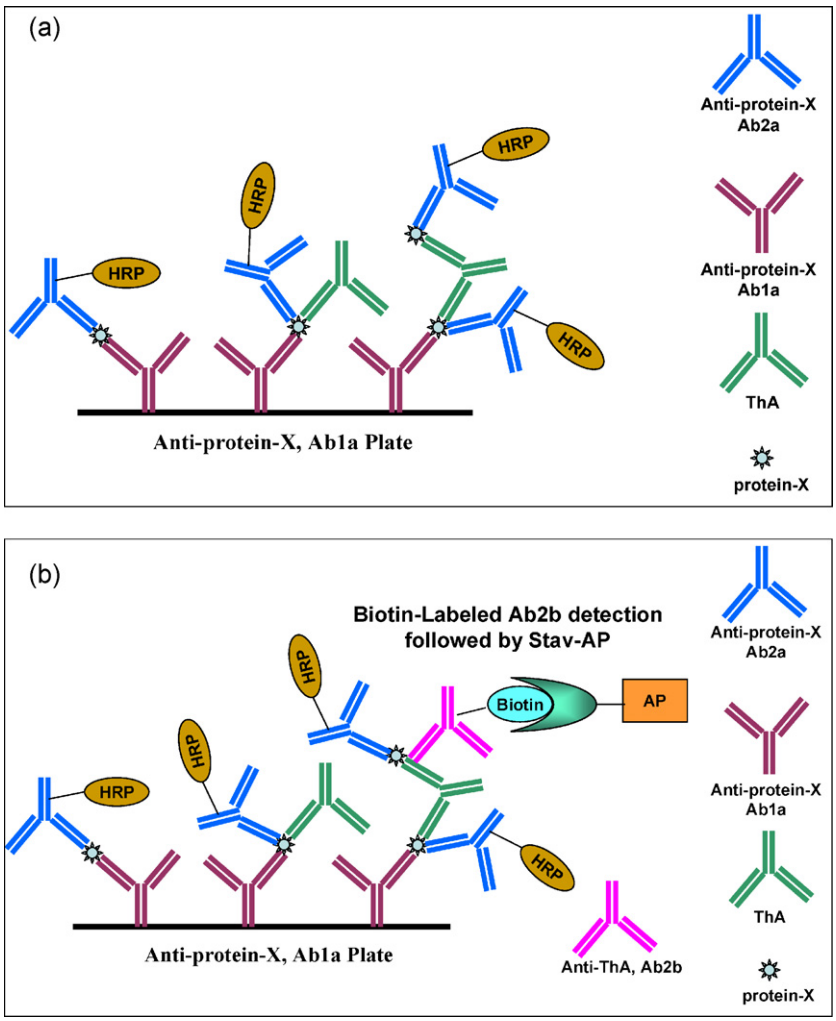

Fig. 1. (a) Schematic diagram of ELISA to measure the target protein. The ligand was measured by chemiluminescent reading through HRP-conjugated detection antibody. (b) Schematic diagram of duplex ELISA to measure ThA and its complex with target ligand. The ligand was measured by chemiluminescent reading through HRP-conjugated detection antibody. The ligand/therapeutic complex measurement was measured by alkaline phosphatase conjugated to anti-ligand detection antibody and using a chemiluminescent substrate.

3. Results and discussions

3.1. Target assay interfered by therapeutic antibody

The data of total target protein in biological samples from PK studies provide proof of in vivo binding and help PK/PD modeling for dose selection. Generally, the assay for target protein is straightforward in the absence of ThA, as in samples from pre-dose or after the washout period. Upon dosing the ThA concentrations are usually much higher than those of the target and will cause negative or positive interference with the target assay. Negative interference (inhibition) may occur if binding sites of the capturing and/or detecting antibody overlap with those of the ThA. If the capture and detection antibodies have non-overlapping binding epitopes with those of the ThA, the results may be no inhibition or enhancement, depending upon the molar ratio of ThA to target protein. A positive bias may be caused by the enhanced signal from the complex of the target ligands being bound to both arms of the IgG (bivalent complex).

In addition to reagent binding epitopes, assay conditions such as coating density, sample dilution and incubation time can also be manipulated to shift the ThA–target binding equilibrium for the measurement of the free or total forms. Therefore, it is important to determine the effect of ThA on the target ligand assay under the defined assay conditions.

For protein-X, we initially developed an assay using two antibodies with two epitopes that were distinct from that of ThA binding. In this method, direct interference from ThA was not expected. However, as shown in Fig. 2a, when the assay of target protein-X was performed with ThA added at concentrations from 40 to 1300 nM there were signal enhancements of up to 300%, depending on the concentrations of target protein and ThA. The signal enhancement indicated an over-estimation of up to 10-fold of the actual target protein level in comparison to Stds without ThA added (Fig. 2b).

The highest interference was observed at molar ratios where the bivalent complex was highest (Fig. 2a). However, it is unlikely that the signal enhancement of bivalent complex can fully account for the observed increase of more than 2-fold. Protein-X is a basic protein that tends to bind to various serum proteins in addition to IgG. Thus, other binding proteins could also contribute to the positive bias. Dissociation of protein complexes would be a logical approach to develop a reliable total ligand method (see Section 3.2).

For protein-Y assay, the capture antibody binding sites overlapped with those of ThA, while the labeled detection antibody had a distinct epitope from that of ThA. The presence of ThA in the sam-

Table 1
Effect of NaOH and time of alkaline treatment on immunoreactivity of ThA and protein-X after neutralization.

Time (min)	% Remaining of ThA–X in NaOH				% Remaining of protein-X in NaOH			
	1 M	0.67 M	0.5 M	0.4 M	1 M	0.67 M	0.5 M	0.4 M
0	100	100	100	100	100	100	100	100
5	2.5	40.3	66.1	63.7	90	97	89	100
10	0	5.0	24.6	39.3	73	82	88	100
20	0	0.4	2.1	5.3	43	69	86	98
30	0	0.1	0.4	0.4	19	46	74	90
60	0	0.7	1.1	1.6	1	4	20	42

The percentage remaining immunoreactivity was determined by duplex ELISA of the ThA against X and protein-X after treated for different time periods at 37 °C with different concentrations of NaOH.

ple interfered with the assay as shown in Fig. 3a. More than 95% inhibition of the luminescent signal was observed at 260 nM of ThA.

With alkaline or acid/guanidine denaturation, the denatured proteins remained soluble after neutralization, without the problem of insolubility of acid alone. Therefore, the alkaline and acid/guanidine treatment of samples were further tested for possible differential denaturation of ThA, releasing the relatively stable antigens for ELISA analysis. We tested if ThA interferences could be eliminated by these approaches.

3.2. Alkaline treatment to measure total protein-X in serum samples

The alkaline treatment conditions were tested at 1.0, 0.67, 0.50 and 0.40 M of NaOH in 0.5 M glycine with 10% casein as the suitable protein carrier at high pH (unpublished observations). The pH was estimated to be >13. The alkaline treated samples were assayed for protein-X and its corresponding ThA using the duplex ELISA. Table 1 shows that more than 97% ThA was inactivated by alkaline buffer containing 1.0 M NaOH at 37 °C for 5 min, while only 10% of protein-X immunoreactivity was lost compared to the control samples without ThA. Slower inactivation rates were observed at lower concentrations of NaOH.

To examine temperature effect on inactivation of ThA and protein-X, the alkaline treatment was performed at 37 °C and at room temperature. Table 2 shows their denaturation kinetics. For ThA, denaturation was >97% at 37 °C and 66% in 5 min at room temperature, and >97% in 30 min at both temperatures. Protein-X is more stable than ThA. In 5 min alkaline treatment, more than 90 and 95% protein-X remained immunoreactive at 37 °C and room temperature, respectively. A 30 min alkaline treatment denatured 35% protein-X at room temperature as compared to 81% at 37 °C.

It is necessary to have precise timing and reagent addition to control dissociation and to preferentially denature ThA rather than protein-X. Consequently, a 96 tip head of Tecan/TEMO liquid handling system was used to meet this requirement. A 30 min treatment at room temperature effectively inactivated ThA and completely eliminated assay interference.

Protein-X in samples containing ThA was successfully assayed by the alkaline treatment method with no interference from ThA.

Table 2
Temperature effect of alkaline treatment on immunoreactivity of ThA and protein-X.

% Remaining of ThA and protein-X				
Time (min)	ThA (37 °C)	ThA (RT)	Protein-X (37 °C)	Protein-X (RT)
0	100	100	100	100
5	2.5	66	90	95
10	0.0	59	73	92
30	0.0	3	19	67

The percentage remaining immunoreactivity of the ThA against X and protein-X for various alkaline treatment times at 37 °C and room temperature (RT) was determined by the duplex ELISA after neutralization.

Table 3 shows the consistent recovery of QCs in the presence of 0–625 nM ThA. The QC recovery was regressed against Stds that underwent the same treatment. The treatment recovery was consistent at about 65% for the majority of the Stds when compared to

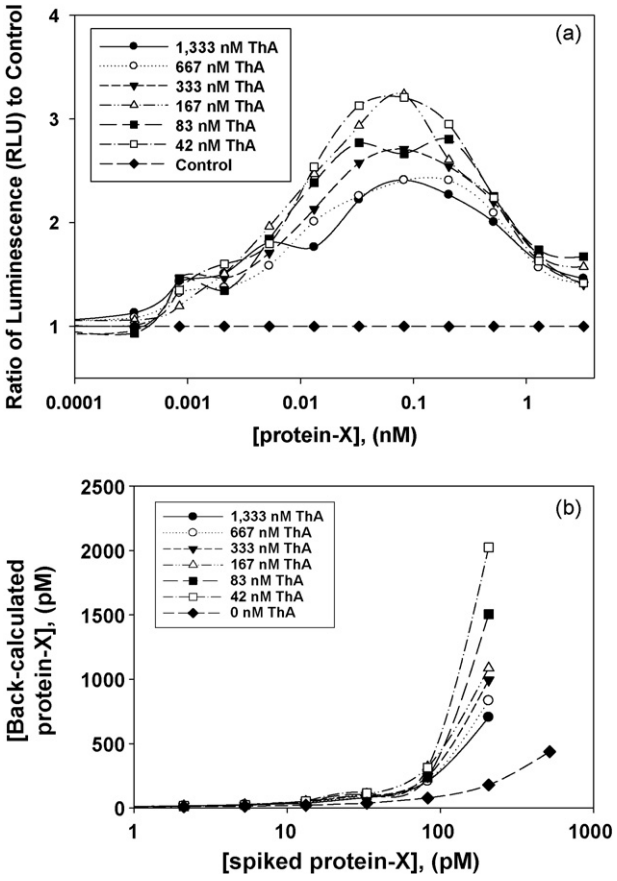

Fig. 2. (a) The effect of ThA on protein-X immunoreactivity. The percentage change in RLU was calculated against the reference points of samples without ThA. Protein-X Stds were serially diluted 2.5-fold from 3200 to 0.2 pM with the presence of 0, 42, 83, 167, 333, 667, and 1333 nM of ThA in I-Block buffer. (b) The effect of ThA on protein-X ELISA determination. The back calculated concentrations were performed using standard curve without ThA. Other conditions were the same as in (a).

Table 3
The recovery of protein-X in quality control samples containing 0–625 nM ThA of X.

Protein-X	% Recovery				
	ThA 0 nM	ThA 0.6 nM	ThA 6.2 nM	ThA 62.5 nM	ThA 625 nM
2.4 nM	100	93	87	93	114
1.9 nM	100	109	94	108	109
0.2 nM	100	113	98	96	97
0.03 nM	100	109	104	112	94

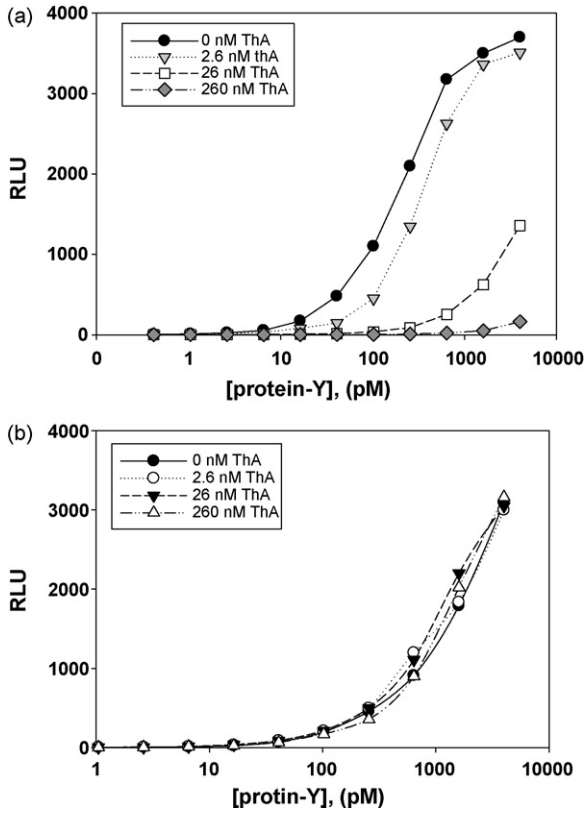

Fig. 3. (a) The interference of ThA on protein-Y ELISA determination. Stds were serially diluted 2.5-fold from 3700 to 5 pM with the presence of 0, 2.6, 26, and 260 nM of ThA in mouse serum samples. (b) The elimination of ThA interference on protein-Y ELISA by acid/guanidine treatment. Stds were treated for 10 min with acid/guanidine buffer followed by 1:1.25 neutralization with 1 M phosphate buffer ph 7.8. Other conditions were the same as in (a).

the untreated. The QCs were acceptable within 15% of the nominal value.

The alkaline treatment method was applied to measure total protein-X in mouse PK study samples. In this study 0.0, 1.0, 2.5, 5.0, 10.0, and 25.0 mg/kg of ThA against X was subcutaneously administered. The sample results of protein-X are shown in Fig. 4. Protein-X level increased from 5 pM at pre-dose to as high as 600 pM at C_{max} which was about 35 days after dosing. These results demonstrate the capability of the alkaline inactivation to accurately measure total protein-X in the presence of ThA in PK samples. The data show

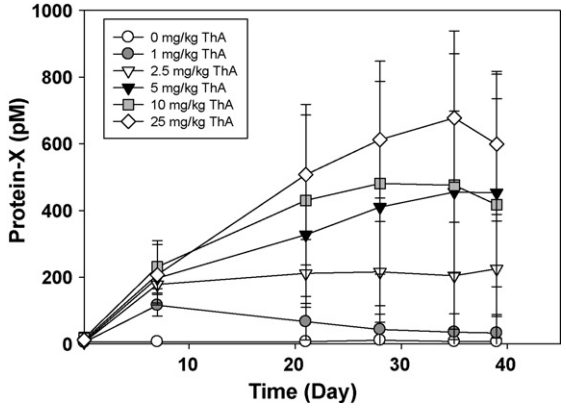

Fig. 4. Dose-dependent accumulation of total protein-X in mouse PK samples. 0.0, 1.0, 2.5, 5.0, 10.0, and 25.0 mg/kg of ThA was subcutaneously administered. Stds, QCs, and samples were treated for 30 min with alkaline method.

that the total levels increased with increased doses, which may be due to accumulation caused by the binding of the target protein to ThA.

Depending upon the ThA and ligand pair, the optimal alkaline treatment time may vary. The alkaline inactivation for ThA and protein-X requires 30 min while for other ThA/ligand systems, a shorter time may be sufficient to completely eliminate ThA interference.

3.3. Acid/guanidine treatment to measure total target protein-Y in serum samples

The effectiveness of alkaline inactivation depends on the ThA and target ligand system and this had to be examined empirically because general differentiating molecular parameters such as size, structure and isoelectric point have not been systematically investigated. The alkaline method could fail such as the case of protein-Y. As shown in Fig. 3a, the presence of ThA significantly inhibited protein-Y assay. More than 95% signal inhibition of protein-Y was observed at 260 nM ThA. When alkaline treatment was applied to total protein-Y assay, a 90 min incubation was needed to completely inactivate ThA, with protein-Y losing more than 85% of immunoreactivity. This loss led to very poor assay sensitivity and rejection of the method.

We attempted 8 M urea as an alternative since it has been used to disrupt protein/protein interaction and complexes [23–25]. The 8 M urea treatment resulted an approximately 40% complex dissociation and failed to completely eliminate ThA interference.

Guanidine is a stronger denaturant than urea for protein complex dissociation. In addition, acid dissociation has been used to dissociate protein binding and commonly used for elution of protein in affinity purifications. We tested the effect of acid and guanidine as well as combining both acid and guanidine. Treatment with acid or guanidine alone had minimal effect in eliminating the interference. However, treatment with 8 M guanidine, 1 M acetic acid, and 0.1 M sulfuric acid dissociated 85–90% protein-Y/ThA complex. As shown in Fig. 3b, ThA interference in total protein-Y assay was completely eliminated by a 10 min acid/guanidine treatment.

The immunoreactivity of protein-Y after the acid/guanidine treatment appeared to be lower than that of the control without the treatment. This may be due to the residual amount of ~1.8 M guanidine after neutralization. To examine whether the acid treatment and/or the presence of guanidine in the treated samples affect the protein-Y assay sensitivity, we tested the assay with and without acid treatment in the absence of ThA. Acid alone did not affect the immunoreactivity of protein-Y. However, when the samples was placed in 1.8 M guanidine, about 30% drop in the chemiluminescent signal was observed similar to that of the samples treated with acid/guanine. Thus guanidine contributed to the loss of immunoreactivity of protein-Y. In spite of this reduction, the chemiluminescent signal was reproducible and the CV was less than 10% and recovery was within 15% of nominal values. Furthermore, the assay sensitivity was not affected significantly. The signal-to-background ratios at 1 pM (detection limit) of protein-Y assay without and with acid/guanidine treatment were 3.0 and 2.4, respectively.

We have applied the acid/guanidine pretreatment to study samples from mice administered with 5 mg/kg ThA intravenously. As shown in Fig. 5, protein-Y levels increased from 21 pM at pre-dose to as high as 3500 pM at C_{max} time (168 h). The increase was about 160-fold, followed by returning back to the baseline levels. These results demonstrate the applicability of acid/guanidine treatment to measure total protein-Y in PK serum samples in spite of the presence of ThA.

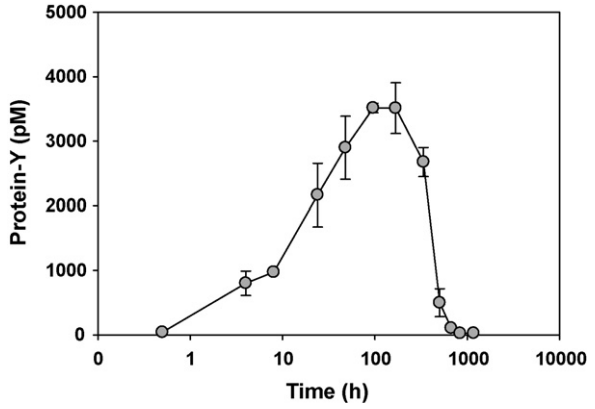

Fig. 5. Accumulation of total protein-Y in mouse PK samples upon intravenous administration of 5 mg/kg of ThA.

4. Conclusions

The presence of ThA posts challenges on the determination of target proteins in post-dosed samples. Determination of the total target protein is more reliable than that of the free because of reagent limitation and the uncertainty of complex dissociation affected by sample dilution and incubation time. The presence of ThA would interfere with the measurement of total target ligand, resulting in over- or under-estimation. Binding reagents and assay conditions can be designed and controlled to deliver a total target assay. An alternative novel approach is to add a pretreatment step to selectively inactivate ThA with consistent remaining immunoreactivity of the target protein for ELISA determination. Using protein-X and -Y as examples, we demonstrated that alkaline or acid/guanidine treatment can dissociate protein binding and irreversibly denature ThA while the target ligand regained immunoreactivity upon renaturation. We applied these methods to measure total target protein-X and -Y in serum in the presence of their respective ThA. The data contributed to the understanding of the interaction of the ThA with its target protein and PK/PD relationships.

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