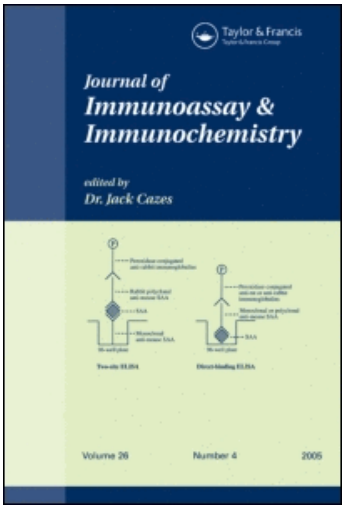

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AN IMMUNOLIGAND ASSAY FOR QUANTITATION OF PROCESS SPECIFIC *ESCHERICHIA COLI* HOST CELL CONTAMINANT PROTEINS IN A RECOMBINANT BOVINE SOMATOTROPIN

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ABSTRACT

We have developed a Threshold™ System immunoligand assay for the quantitation of residual, process-specific, *Escherichia coli* host cell contaminant proteins (HCP) in somavubove (a normal sequence recombinant bovine somatotropin). The assay has a dynamic range of 2 to 160 ng/mL, with a limit of quantitation of 2 ng/mL. Daily analytical precision (CV) for six replicates of the designated somavubove laboratory standard is 3.0%. Cumulative analytical precision for multiple assay runs of this standard (a total of 69 laboratory standard replicates) is 8.4%. A conservative alert limit of 100 ng/mL has been assigned in order to assure analytical precision for each assay sample under conditions of stoichiometric antibody excess. Although the assay was designed for use as a profile-release specification assay, it has also been used to validate removal of HCP by the proprietary somavubove purification process. This use is consistent with regulatory guidelines related to "well characterized" recombinant biopharmaceutical proteins.

(Key Words: bovine somatotropin, host cell contaminant protein, immunoligand assay, biopharmaceutical, proprietary immunoreagent).

INTRODUCTION

Pharmacia and Upjohn, Inc. , (Kalamazoo, Michigan, USA) normal-sequence recombinant bovine somatotropin (somavubove; SBV) is a parenterally administered, recombinant, veterinary biopharmaceutical for use in lactation enhancement in dairy cattle. Residual host cell contaminant protein (content is considered to be a critical indicator of product quality, purification process efficiency, and purification process consistency for SBV, as well as for all other recombinant proteins. Regulatory authorities recognize that there is no acceptable, generic, analytical method for quantitation of process specific host cell contaminant proteins (HCP). A practical alternative is to develop process-specific analytical methods. The current biopharmaceutical industry consensus methodology for development and validation of process-specific, multi-analyte, HCP immunoassays is based upon the null cell, mock purification model (1). We have used this approach, inclusive of the elicitation and purification of proprietary immunoreagents, in order to develop a process-specific, immunoligand assay (ILA) for the quantitation of residual *Escherichia coli (E. coli)* HCP in SBV.

MATERIALS AND METHODS

Preparation of Proprietary Process-Specific *E. coli* HCP Immunogen:

The null cell (non-expressing) strain used for HCP immunogen preparation consisted of a version of the proprietary SBV *E. coli* expression cell strain whose plasmid had been cured of the bovine somatotropin gene, and hence, could not express SBV protein. The null cell strain was fermented at pilot scale (i.e. at less

than full SBV manufacturing scale), and the harvested and lysed cells were subjected to sequential steps of the proprietary SBV protein purification process to a point at which, under normal manufacturing conditions, the biopharmaceutical protein product would consistently have been more than 99% pure. This procedure yielded 560 mg of complex, process-specific HCP for use as a proprietary immunogen, as an analytical reagent, and as a lifetime supply of antigen for use in generation of the assay standard curve. These HCP were evaluated by two dimensional (2-D) electrophoresis consisting of: first dimension isoelectric focusing (IEF), and second dimension gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)(2). The resulting silver-stained 2-D SDS-PAGE gels revealed a heterogeneous mixture of more than 100 distinct protein spots at widely differing relative concentrations, ranging in isoelectric point from pI 4.6 to pI 6.0, and ranging in molecular weight from 22 kilodaltons (Kd) to greater than 100 Kd. All protein concentration determinations for this material utilized a generic bicinchoninic acid assay (BCA; Pierce, Rockford, IL, USA) method compatible with that described in reference (3).

Elicitation of Specific Polyclonal Rabbit Anti-HCP Antibody:

The practical goal was to elicit a high-affinity, polyclonal, immunoreagent which could be empirically demonstrated to specifically detect "most" of the HCP in the complex immunogen mixture (1). Specific polyclonal anti-HCP immunoreagent antibody was elicited in a group of five specific pathogen free Elite New Zealand White rabbits. At nominal three week boost-bleed intervals, the animals were immunized with 100 μg doses of purified HCP (emulsified in adjuvant), by injection into the flank. The primary immunization employed 0.5 mL of Complete

Freund's Adjuvant (Sigma, St. Louis, MO, USA) mixed with 0.5 mL (100 μg) of complete HCP immunogen. The secondary immunization employed 0.5 mL of Incomplete Freund's Adjuvant (Sigma) mixed with 0.5 mL (100 μg) of complete HCP immunogen. The third, and all subsequent, "tertiary" immunizations employed 0.5 mL (100 μg) HCP mixed with 0.5 mL of an MDP-based adjuvant mixture consisting of: 1 mL squalene (Sigma), 40 μL Tween 80 (Sigma), 5 mg MDP (muramyl-dipeptide) (Sigma) in 2.5 mL 1X DPBS (Irvine Scientific, Santa Ana, CA, USA), 500 μL Pluronic L121 (BASF, Parsippany, NJ, USA) and 960 μL 1X DPBS (Irvine Scientific). Among the tertiary immunizations, three successive rounds of passive immunization using whole HCP immunogen were completed, followed by five rounds of cascade immunization using sequentially antigen depleted HCP, obtained as described below. At each bleed interval approximately 50 mL of blood was obtained from each animal by standard phlebotomy of the medial ear artery. After the animals were empirically determined to have reached maximum responsiveness (see below) they were repeatedly boosted with complete HCP immunogen emulsified in MDP-based adjuvant until a total of approximately 2.5 grams of purified process specific anti-HCP IgG had been purified, pooled, qualified in comparison with the original qualified batch, and then stored at -80°C. This qualified reagent supply represents in excess of 50 years worth of immunoreagent at the normal projected rate of analytical expenditure.

Subpurification of HCP Immunogen:

Sequentially enhanced immune responsiveness to minor HCP species (by either masking or depleting the immunodominant HCP epitopes in the prepared immunogen) was accomplished in each of the tertiary boost cycles by passive

immunization *in vivo* (4) and/or iterative antigen selection *in vitro* (5). Passive immunizations involved diluting purified immune IgG (obtained from the immediate preceding rabbit bleed) 1:2 with HCP stock. This mixture was incubated at room temperature for 1 hour, prior to administration of a 1.0 mL dose. Iterative immunoaffinity antigen selection was accomplished by percolating a portion of the original HCP immunogen stock over a freshly prepared rabbit anti-HCP IgG Affi-Gel Hz Hydrazide chromatographic matrix according to the manufacturer's recommendations (6; BioRad, Hercules, CA, USA). The major antigenic HCP which had already been recognized by the rabbits were immunospecifically retained by the column matrix, and the "major antigen depleted" minor HCP species exiting the column were collected and used for the next scheduled HCP immunization dose. Iterative administration of these progressively more specific minor protein immunogens to the rabbits at each sequential boost (the later tertiary immunizations) is an example of "cascade" immunization (5).

Immunoreagent IgG Purification:

Immunoreagent IgG was purified from rabbit serum at each bleed interval by semi-preparative Protein-G affinity chromatography (7; PerSeptive Biosystems, Framingham, MA, USA). Evaluation of progressive chronological immunoreagent IgG batch "acceptability" was monitored by classical methods of 2-D electrophoresis (2) and immunoblotting (8). After seven months of cascade immunization, approximately 95% of the proteins observed in silver-stained 2-D gels were also detected by comparative immunoblotting of duplicate gels with rabbit anti-HCP IgG. After this point, further immunization of the rabbits did not result in detection of additional protein spots, although some previously detected minor

protein spots demonstrated increased immunostaining intensity. At this "practical endpoint" the immunoreagent IgG was designated by the analytical developers to be empirically qualified (1) for use in immunoligand assay (ILA) development. As described previously, in excess of a fifty year supply of qualified reagent was eventually accumulated.

Generic Immunoligand Assay (ILA) Format and Description of Threshold™ System Technology:

We routinely employ a "sandwich" ILA format (9) which is compatible with the Molecular Devices Corporation (MDC, Sunnyvale, CA, USA) Threshold™ System (10,11). A dilution of a bulk drug sample which contains contaminant proteins is first incubated with equal amounts of fluorescein-labeled and biotin-labeled "contaminant specific" antibody in solution-phase. Streptavidin capture reagent is added to each sample and the solution is transferred to filtration units. Streptavidin-antibody-antigen-antibody complexes are captured upon a biotin coated filter stick via vacuum filtration, followed by washing. Urease-labeled anti-fluorescein detection antibody is added and binds to fluoroscein-labeled antibody. After washing, the filter "stick" which contains the complete membrane immobilized streptavidin-antibody-antigen-antibody-enzyme "sandwich" is inserted into a Threshold™ System reader which contains a urea substrate buffer. The enzyme-substrate reaction initiates a concentration dependent change in pH which is detected by a sensitive potentiometric silicon sensor in the reader. The elicited μV/sec signal from each filter stick position is relayed to a computer for standard curve generation and data analysis (12). Several curve regression analysis formats are available for the Threshold™ System. For this specific HCP ILA, quadratic regression analysis performed best.

Immunoligand Assay Labeling Kits and anti-HCP IgG Hapten Labeling:

We used an Immunoligand Assay Labeling Kit (MDC) (13), and N-N-dimethylformamide (Sigma) to haptenate aliquots of proprietary rabbit anti-HCP IgG with either fluorescein or biotin according to the manufacturer's specifications. Labeled IgG was purified over PD-10 columns (Pharmacia, Uppsala, Sweden) in 1X DPBS (Irvine Scientific). Labeled IgG preparations were monitored for molar incorporation of hapten at 362 nm for biotin and 490 nm for fluorescein. Corrected total protein was monitored at 280 nm in accordance with the manufacturer's instructions. Observed molar incorporations of hapten for these two labeled working reagent IgG stocks, calculated according to the formulae provided, were 3.8 (IgG*f) and 5.9 (IgG*b), respectively. The labeled antibody preparations are stable when stored at -80^{0}C in 1X DPBS buffer.

Immunoligand Assay Kit and Sample Preparation:

Reagents and buffers are those specified in the Immunoligand Assay Kit and applicable manufacturer's protocols (14), unless otherwise noted. In this instance, we used purified streptavidin capture reagent (Pierce) and bovine serum albumin free assay buffer (BSAFAB) as described in (15). The assay is performed upon a sterile bulk drug aliquot which is taken from an in-process bulk solution. The sample is lyophilized. Each lyophilized SBV sample and SBV standard is equilibrated at 31% humidity overnight in order to minimize the impact of water content variability upon the accuracy and precision of the assay. Weighed 1 mg samples are reconstituted to 1 mg/mL in BSAFAB buffer. Samples are further diluted 1:20 (50 μg/mL) prior to assay. However, SBV at concentrations as high as 0.5 mg/mL may be analyzed for HCP without assay interference.

HCP Standard Curve Parameters and Preparation:

HCP standard curve point replicates are dilutions, made from the previously described purified process-specific HCP stock, into a background of BSAFAB with the addition of 0.05 mg/mL bovine serum albumin (BSA Fraction V) (Miles, Kankakee, IL, USA). This protein background mimics the protein load of routine dilutions of SBV samples. An HCP standard curve generated in BSA had been previously demonstrated to be indistinguishable from an HCP standard curve which had been generated in an equivalent concentration of pituitary bovine somatotropin. Standard curve dilution replicates are treated the same as SBV samples (described in the format below). HCP standard curve points are: 160, 80, 60, 40, 20, 10, 2 and 0 ng/mL, respectively.

HCP ILA Format:

The HCP ILA is performed for the detection and quantitation of HCP in stabilized in-process pre-formulation bulk SBV samples. The assay utilizes a calibrated MDC Threshold™ System, ILA technology and a dedicated ILA cleanable reader. All new ILA kit lots are qualified using an ILA check-out assay prior to use with this assay. Each daily run contains two standard curves, and six replicates of the SBV laboratory standard (an early HCP-containing somavubove batch), and SBV samples. The assay format is as follows: The 1X BSAFAB working stock is prepared and sterile filtered with a 0.2 μm filter (Corning, Corning, NY, USA). Sufficient rabbit anti-HCP IgG*Hapten Conjugate working stock is prepared in 1X BSAFAB to provide both 200 ng of biotin-labeled antibody and 200 ng of fluorescein-labeled antibody per test sample. The HCP standard curve is

prepared in 1X BSAFAB, then 1 mL of 0.25 mg/mL BSA in 1X BSAFAB is added to each tube. Standard 1:20 dilutions of the SBV standard and all samples are made, then all standards and samples are dispensed at 100 µL into 12x75 mm tubes (Falcon, Lincoln Park, NJ, USA) in a Threshold Array sample rack (MDC). 100 µL of the 1:50 Rabbit anti-HCP IgG*Hapten Conjugate working stock is added to all tubes. The tubes are covered with Parafilm (American National Can™, Greenwich, CT, USA), then incubated at 37°C for three hours in a rotating waterbath. During the incubation, all remaining buffers are prepared, and the Threshold System is initialized. When the incubation period is finished, 1.0 mL affinity purified streptavidin capture reagent (Pierce) is added to all tubes using an Eppendorf Easy Step™ (Continental Laboratory Products, Inc., San Diego, CA, USA) repeater pipette with a 50 mL Combitip (Brinkman Instruments, Inc., Westbury, NY, USA). The entire tube contents are then transferred to the threshold filter units using a threshold array pipette and tips. Following low vacuum filtration, 2.0 mL of 1X wash buffer is added to every filter stick position using an Eppendorf Easy Step™ repeater pipette with a 50 mL Combitip. Following high vacuum filtration, 1.0 mL of 1:10 enzyme working stock is added to every well using an Eppendorf repeater pipette with a 50 mL Combitip. Following low vacuum filtration, 2.0 mL of 1X wash buffer is added to every well using the Easy Step™ Eppendorf repeater pipette with a 50 mL Combitip. Following high vacuum filtration, the filter units are disconnected from the manifolds and the sticks are placed into beakers of 1X Wash with the blank sticks. The filter sticks are read in the order of: blank, 4 assay sticks, blank, 4 assay sticks, blank, etc.

RESULTS

General Assay Acceptance Criteria:

Blank stick μV/sec signals must be within ± 10.0 μV/sec in order to accept the data. If any of the blank sticks' positions are outside of ± 10.0 μV/sec, then the reader must be cleaned according to the manufacturer's instructions. Mean signal rates (μV/sec) from duplicate standard curve values are calculated and entered into the computer as stick S. The quadratic standard curve linearity error term (e) must be < 0.1, and the data points should closely fit the regression line. The data group report is checked for any CV > ± 10% using a Q-test to determine if the replicates contain an outlier. If any mean sample value obtained (ng/mL) is greater than a specified conservative 100 ng/mL alert limit, then the sample is reassayed after diluting further. Total HCP "contamination" (ng/mL) is corrected for total protein content (mg/mL) in each assay sample, and expressed in parts per million (ppm).

Dynamic Range, Linearity Error Term, and Limit of Quantitation:

Actual specifications for HCP content result from the interaction of a number of factors. They are unique to each proprietary biopharmaceutical and are, therefore, evaluated by regulatory authorities on a case-by-case basis (1). What is more important than an HCP specification *per se* is to demonstrate analytical capability which is suitable to their quantitation in the given product. Generally speaking, it is desirable to be able to quantitate HCP at levels less than 10 parts-per-million.

A typical Threshold™ System HCP ILA standard curve readout may be seen in Figure 1. The dynamic range of the HCP assay standards is 2 ng/mL to 160 ng/mL. Minimum ILA limit of detection for the Threshold™ instrument is defined as the

concentration of antigen which gives a "net 50 μV/sec signal above background, if the background rate is lower than 150 μV/sec" (12). We met this criterion and using our specified assay conditions we obtained a theoretical limit of detection of 0.16 ng/mL. However, we imposed a second criterion, namely that there must be acceptable spike recovery at the specified limit. A 2 ng spike into the carrier protein is recovered within the accepted 80-120% limit. A 1 ng spike cannot quite meet this requirement. Thus, we have conservatively set the limit of quantitation at 2 ng/mL (greater than 10-fold higher than the theoretical limit), and have conservatively set the useful limit of detection at 1 ng/mL (greater than 5-fold higher than the theoretical limit.). This 1 ng/mL limit of detection would correspond to 2 parts-per-million (ppm) for a 0.5 mg/mL SBV assay sample. Quadratic regression analysis ($y = a+bx+cx^2$) of the standard curve shown in Figure 1 gives an acceptable linearity error term of 0.029 (i.e. < 0.1).

Spike-Recovery, Sensitivity and Linearity of Dilution:

Spike-recovery of 2, 5, 10, 20 or 40 ng HCP spikes into bulk drug samples was analytically acceptable (i.e. 80-120%) as long as the sum of the HCP spike signal and the endogenous HCP signal fell within the normal standard curve dynamic range. These spikes demonstrated appropriate linearity of response (i.e. additive signal) within the normal dynamic range. Analytical sensitivity (i.e. discrimination of small increments along the standard curve), approaches 2 ng at the lower end of the standard curve. Analytical results obtained from serial dilutions of either bulk drug samples, or SBV laboratory standard, whose signal fell within the assay dynamic range, showed linearity of dilution.

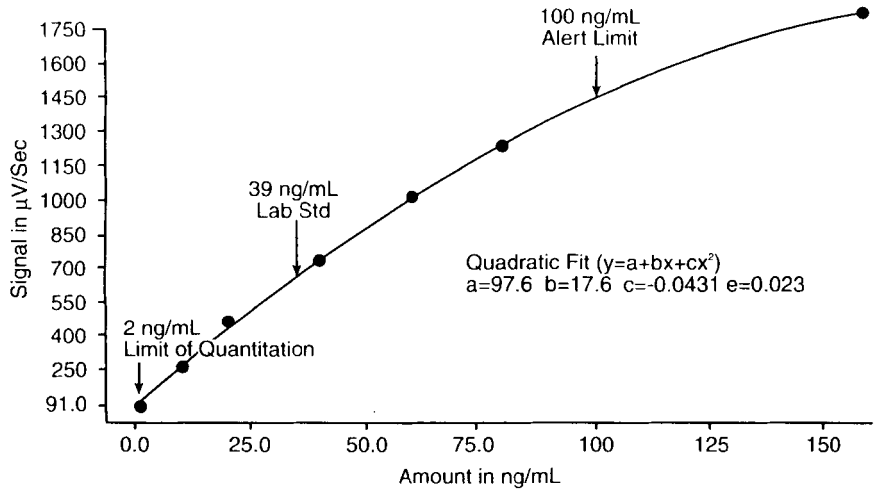

FIGURE 1. A representative Threshold™ HCP immunoligand assay (ILA) standard curve readout (ng/mL HCP concentration versus µV/sec signal) for purified process specific *E. coli* HCP standards of 160, 80, 60, 40, 20, 10, 2, and 0 ng/mL, respectively. The generated line is a quadratic fit ($y = a + bx + cx^2$) of the data points. The data points closely fit the regression line, and the error term (e = 0.023) is well below the 0.1 limit. Limit of quantitation (2 ng/mL), cumulative mean somavubove lab standard HCP value (39 ng/mL), and assigned conservative analytical stoichiometry alert limit (100 ng/mL) are also indicated on the curve.

Accuracy:

Accuracy, in a complex multi-analyte immunoassay, is assigned relative to the characterization of the analytical reagents (1). This rationale presumes that: 1] the immunogen represents the "most probable" contaminant HCP for a specific purification process, 2] that an accurate total protein value has been assigned to the immunogen stock (i.e. assay positive control), and 3] that the elicited proprietary anti-HCP immunoreagent has been empirically qualified to detect "most" of the potential immunogen proteins. Accuracy is, thus, assured if analytical samples are assayed under conditions of stoichiometric antibody excess (16), using

qualified reagents. Accuracy (and precision) may be routinely monitored by demonstration of consistent assay performance using a qualified laboratory standard. Repetetive analytical determinations using positive control laboratory standards are essential for effective system suitability monitoring.

Precision:

Precision of assay performance has been monitored by repetetive HCP measurement of the designated SBV laboratory standard, at the routine sample dilution of 50 μg/mL. For example, a typical within-day measurement of six replicates of this standard gave a mean HCP value of 41 ng/mL (CV = 3.0%). Cumulative measurement of this standard for seven separate assays (69 total sample replicates) over a period of one month gave a mean HCP value of 39 ng/mL (CV = 8.4%).

Stoichiometric Antibody Excess and Analytical Alert Limit:

Accurate determination of HCP content for a given sample depends upon the specific anti-HCP immunoreagent being in "stoichiometric antibody excess" (16) throughout the dynamic range of the assay. This is not usually a problem with routine analytical batch profile samples which are prepared with a standard dilution, since they are expected to consistently fall well within the standard curve dynamic range (usually at the low end). However, spiked additions of HCP to the upper end of the standard curve have demonstrated that spike-recovery efficiency (and precision of measurement) will begin to deteriorate somewhat (i.e. fall outside the acceptable 80-120% spike-recovery range) as the additive HCP content approaches or exceeds antibody-antigen equivalence at the highest standard curve

point concentration. Therefore, as a precaution, we have set a very conservative analytical alert limit of 100 ng/mL for this assay. We prefer to maintain a dynamic range of 2-160 ng in order to catch the odd high sample. However, an analytical sample which gives an initial signal of greater than 100 ng/mL (though uncommon) will alert the analyst to prepare further dilutions of the sample for re-assay. In this way analytical accuracy and precision is assured for the occasional atypical sample.

System Suitability Indicators:

Effective use of this assay requires that system suitability be maintained. All calibration requirements of the Threshold™ System instrument and readers are maintained under current Good Manufacturing Practices (cGMP) conditions. For each performance of the HCP ILA assay the reader μV/sec signal on the Y-axis, the slope of the quadratic standard curve fit, the closeness of individual standard curve point fit to the curve, and the linearity error term are closely monitored for historical consistency. The consistency of accuracy and precision measurements for the SBV laboratory standard is assessed and recorded for each assay run.

DISCUSSION

If immunoreagents are readily available, analytical timeframes for development of individual immunoligand assays (in conjunction with the use of related commercially available labeling kits, assay kits, and manufacturer's written protocols) can be quite rapid for an investigator with extensive experience in use of the Threshold™ System. In the case of the HCP ILA described above, *as soon as*

qualified reagents became available, the fundamental assay parameters which gave a reasonable standard curve and good limit of quantitation were worked out in a week of experimentation. Spike-recovery studies and other general validation parameters were evaluated in a few additional weeks.

However, by definition, neither immunogen proteins nor specific immunoreagent antibody preparations are readily available for the development of process-specific host cell protein assays. In this instance, the labor and time intensive (and expensive) planning, manufacture, preparative isolation, and characterization of HCP immunogen proteins, followed by the labor and time intensive (and expensive) elicitation, purification, and characterization of specific anti-HCP immunoreagent antibody stocks, followed by the relatively short period of actual "assay development", spanned a total of 18 months.

The HCP ILA was developed and validated in a manner consistent with regulatory guidelines relevant to biopharmaceutical assay analytical performance characteristics (17), in order to serve as a bulk drug, profile-release, specification assay. However, the most valuable application of the assay has been to analytically support purification process validation (e.g. clearance) studies. This assay has been successfully employed to demonstrate consistent removal of host cell proteins by the full scale, proprietary, SBV biopharmaceutical purification process. This emphasis upon process validation and product consistency, rather than mere final product profiling and comparison with specifications, is consistent with recent published draft guidelines and related industry discussions, which concern the regulatory approval of "well characterized" recombinant biopharmaceutical proteins (18).

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