JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Measurement of Bovine Somatotropin (bST) and Insulin-like Growth Factor-1 (IGF-1) in Bovine Milk Using an Electrochemiluminescent Assay

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Bovine somatotropin (bST) and insulin-like growth factor-1 (IGF-1) are peptide hormones that are involved in the regulation of milk production in dairy cows. Because these hormones are present at extremely low concentration in fresh and processed bovine milk, a highly sensitive and specific electrochemiluminescent immunoassay (ECLIA) has been developed to better estimate the concentration of these hormones in milk. The assay employs an imager, a capture antibody bound to a carbon electrode, and a detection antibody coupled to a ruthenium label. In the presence of tripropylamine and an electric pulse, ruthenium generates light proportional to the amount of antigen bound, and the light is captured as signal by a charge-coupled device (CCD) camera. Using bovine milk as the starting matrix, 99.69% of bST and 104.79% of IGF-1 were recoverable. The limit of detection (LOD) was <5 pg/mL for bST and <1 pg/mL for IGF-1. The limit of quantification (LOQ) was <14 pg/mL for bST in milk and <2 pg/mL of IGF-1. The assay is highly specific and shows <0.2% cross-reactivity with other peptide hormones found in bovine milk such as insulin and IGF-2. These data indicate this new, ECLIA is highly sensitive and specific for estimating the concentration of bST or IGF-1 in milk.

KEYWORDS: Bovine somatotropin; insulin-like growth factor-1; electrochemiluminescent immunoassay

INTRODUCTION

Bovine somatotropin (bST) is a hormone that increases the fraction of nutrients directed toward milk production in cows (*1*) and is found at higher concentration in the blood of cows genetically selected for milk yield (*2, 3*). When administered parenterally, bST also raises the circulating concentration of insulin-like growth factor-1 (IGF-1) (*4*). Together, these peptide hormones are involved in bovine mammary gland development (*5, 6*) and lactation performance (*1*). In 1993, a recombinant form of somatotropin (rbST, POSILAC, Monsanto Corp.) was approved by the FDA for increasing the production of salable milk from dairy cows. Throughout its product history, numerous health and scientific organizations, including the Food and Drug Administration, the National Institute of Health, the Inspector General, and the Department of Health and Human Resources concluded there is no substantive difference in the milk or meat from rbST-supplemented animals (*7, 8*). Yet some critics continue to question what impact this particular technology may have on milk composition and safety. As this debate over food safety continues, retailers in the United States now insert more claims on food labels that do not relate to composition and are difficult to verify. To provide meaningful information to consumers, highly sensitive assays are required to substantiate these expanded label claims (*9*).

Traditionally, the concentration of IGF-1 or bST was estimated using either radioimmunoassay (RIA) (*4, 10–13*), enzyme-linked immunosorbent assay (ELISA) (*14, 15*), or bioassay methods (*16, 17*). Still, considerable differences in the estimation of bST or IGF-1 concentration persist; possibly due to differences in stage of lactation (*18*), nutritional status of the cow (*19*), or simple assay variation (*20, 21*). Recently, a new method of ELISA was developed that uses an antibody linked to a chemical "label" that emits a measurable signal when electrochemically stimulated (*22, 23*). The procedure (electrochemilumenescent immunoassay or ECLIA) uses a 96-well plate with electrodes imbedded into the plate. A capture antibody is passively bound to a carbon electrode at the base of a well; the test material containing the antigen is added, followed by a second antibody that is linked to a ruthenium(II) sulfo-tris-bipyridine *N*-hydroxysuccinimide ester label. This label, in the presence of tripropylamine and an electric pulse, generates light (the signal) that is proportional to the amount of antigen bound to the capture antibody. Light is captured as signal by an ultralow noise charge-coupled device (CCD) that is linear over nearly a six log-dynamic range. Because only light captured near the detector is measured, there is less background interference, allowing for greater sensitivity while lowering the impact of other matrix components (*23*). We report the development of an ECLIA for measurement of IGF-1 and bST in milk.

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10.1021/jf800696d CCC: $40.75
Published on Web 08/01/2008

MATERIALS AND METHODS

IGF-1 and bST. Two ECLIAs were developed to quantify IGF-1 and bST in bovine milk using a Sector Imager 6000 (Meso Scale Discovery, Gaithersburg, MD). Custom IGF-1 capture and detection antibodies (Monoclonal Cell Line ID 2030909 and 2030891, Harlan Bioproducts for Science, Inc., Indianapolis, IN), rbST capture antibody (Monoclonal Cell Line 2C7.3.1, Washington University Hybridoma Center, St. Louis, MO), and rbST detection antibody (rabbit polyclonal R860, Harlan Bioproducts for Science, Inc.) were purified using protein G affinity chromatography (GammaBind Plus Sepharose, GE Healthcare, Piscataway, NJ). Columns were equilibrated with phosphate-buffered saline, pH 7.4 (PBS, Roche Diagnostics Corp., Indianapolis, IN) prior to the addition of either serum diluted 2-fold with PBS or ascites fluid clarified by centrifugation at 10000*g*. The column was washed with 20 volumes of PBS before elution of the antibody with 0.5 M acetic acid, pH 3.0, into tubes containing a $^1/_{20}$ volume of 2.5 M Tris-HCl, pH 9.0, for neutralization. Fractions containing antibody were pooled and dialyzed overnight at 4 °C with two changes of PBS. Antibodies were quantified using a spectrophotometer (extinction coefficient at 280 nm = 1.44 M^1 cm^{-1}). The column was regenerated after each antibody purification by passing 10 column volumes of regeneration buffer (1 M acetic acid, pH 2.5) over the column followed by 10 column volumes of PBS.

R860 was further purified using an rbST affinity column [7.1 mg of solubilized rbST powder, pH 8 [(M020, Monsanto, St. Louis, MO) coupled to 2 mL of Affi-gel 10 resin (*N*-hydroxysuccinimide ester of a derivitized cross-linked agarose gel bead support, Bio-Rad, Hercules, CA)]. The column was equilibrated with PBS prior to the addition of R860 antisera diluted 2-fold with PBS. The column was then washed with 20 volumes of PBS before elution of the antibody with 0.5 M acetic acid, pH 3.0, into tubes containing a $^1/_{20}$ volume of 2.5 M Tris-HCl, pH 9.0, for neutralization. Antibody fractions were pooled, dialyzed overnight in two changes of PBS at 4 °C, and quantified using a spectrophotometer. The column was regenerated using 10 column volumes of regeneration buffer (1 M acetic acid, pH 2.5) followed by 10 column volumes of PBS.

Sulfo-Tag NHS-Ester [ruthenium(II) trisbipyridine *N*-hydroxysuccinimide ester, Meso Scale Discovery] was reconstituted with cold distilled water and combined with purified antibody (2 mg/mL in PBS) at a ratio of 20:1 for the IGF-1 detection antibody and 12:1 for the rbST detection antibody. After incubation (2 h in the dark with mixing), free Sulfo-Tag was removed using a G-50 Sephadex spin column equilibrated with PBS. Concentration of labeled antibody was determined using a DC Protein assay kit (Bio-Rad), and incorporation of label was determined using the manufacturer's instructions (Meso Scale Discovery).

IGF-1 Binding Protein Extraction and Immunodepleted Milk Preparation. Prior to use in the IGF-1 assay, all milk (test samples and milk used to produce the standard curve) were extracted to remove IGF-1 binding proteins using the following procedure. Milk samples were thawed, mixed, and diluted 1:50 by adding 490 μL pf PBS−0.05% Tween 20 (PBS-T) to 10 μL of milk. IGF-1 binding proteins were removed by acidification (*24*) (100 μL 0.1 M glycine, pH 2.0 to 500 μL of diluted milk) followed by mixing and incubation (18−72 h, 33−41 °C). Samples were then centrifuged for 10 min (3220*g*, 4 °C), and 300 μL of supernatant was transferred to a clean tube without disturbing the pellet or the surface fat. The supernatant was neutralized by adding 30 μL of 200 mM Tris, pH 10.00, resulting in a milk extract with a final dilution of 66-fold.

Prior to IGF-1 standard curve preparation, milk extract was immunodepleted to remove endogenous IGF-1 using the following procedure. Milk extract was passed over an IGF-1 affinity column [10 mg of a mixture of eight custom monoclonal IGF-1 antibodies (cell line ID 2030885, 2030899, 2030891, 2030892, 2030900, 2030901, 2030902, and 2030909; Harlan Bioproducts for Science, Inc.)] coupled to 1 mL of Bio-Rad Affi-gel 10 resin equilibrated with PBS-T. The eluate from the first pass was collected and passed over the column a second time. The eluate from the second pass was collected and passed over the column a third time. The immunodepleted extracted milk (IDP-M, third eluate) was used as the matrix (assay buffer) for preparing standards. The column was regenerated by removing the bound IGF-1 and re-equilibrating the column by passing 10 column volumes of PBS-T over the column followed by 10 column volumes of regeneration buffer (1 M acetic acid, pH 2.5) and then an 10 additional column volumes of PBS-T.

IGF-1 Quantification. IGF-1 standards were prepared in IDP-M that contained no endogenous IGF-1. Because all bovine milk contains endogenous IGF-1, this was removed from milk extract using an IGF-1 affinity column (described above). The standard curve consisted of a zero plus seven nonzero concentrations (8−512 pg/mL) of IGF-1 (Novozymes GroPep, Adelaide, Australia) serially diluted into IDP-M.

To estimate IGF-1 concentration, 25 μL of IGF capture antibody (20 μg/mL in PBS) was added to each well of a 96-well multiarray high-bind plate (Meso-Scale Discovery). The plates were sealed with a Mylar cover and incubated on an orbital shaker (150 rpm, 1 h, ambient temperature). Each plate was washed three times (300 μL of PBS-T) using an automatic 96-well plate washer (Bio-Tek, Winooski, VT), and then 150 μL of room temperature Blocking Buffer C (1× TBS/casein, Bio-Rad) was added to all wells. The plates were again washed as described. After washing, 25 μL of prepared sample or standard was added to the appropriate well. The plates were sealed and incubated on an orbital shaker (325 rpm, 1 h, ambient temperature) followed by washing as described. Twenty-five microliters of IGF-1 detection antibody (1.5 μg/mL) labeled with ruthenium(II) sulfo-tris-bipyridine *N*-hydroxysuccinimide ester was added to each well. The plates were sealed, incubated on an orbital shaker (325 rpm, 1 h, ambient temperature), and then washed as described. Finally, 150 μL of 2× Read Buffer T was added immediately before measurement of the ECLIA signal using an MSD Imager 6000 (Meso Scale Discovery).

bST Quantification. Milk samples were thawed, mixed, and diluted 2-fold with PBS. The standard curve consisted of a zero plus seven nonzero concentrations of rbST (18.8−1200 pg/mL, M020, Monsanto) serially diluted in assay buffer (milk diluted 2-fold with PBS) (2% milk, Prairie Farms, Carlinville, IL).

To estimate bST concentration, 25 μL of bST capture antibody (8 μg/mL in PBS) was added to each well of a 96-well multiarray high-bind plate (Meso-Scale Discovery). The plate was sealed with a Mylar cover and then incubated on an orbital shaker (150 rpm, 1 h, ambient temperature). Each plate was washed three times as described above, and 150 μL of room temperature NAP-Blocker blocking buffer (G-Biosciences/Genotech, St. Louis, MO) was added to each well. The plates were sealed and incubated on an orbital shaker (150 rpm, 1 h, ambient temperature). The plates were again washed as described. After washing, 25 μL of sample or standard was added to the appropriate well. The plates were sealed and incubated on an orbital shaker (325 rpm, 1 h, ambient temperature) followed by washing as described. Twenty-five microliters of rbST detection antibody (4 μg/mL) labeled with a ruthenium(II) trisbipyridine *N*-hydroxysuccinimide ester was added to each well. The plates were sealed, incubated on an orbital shaker (325 rpm, 1 h, ambient temperature), and then washed as described. Finally, 150 μL of 2× Read Buffer T was added to all wells immediately before measurement of the ECLIA signal.

Because the starting medium (milk) for all analytes is a complex matrix, five parameters of assay performance were estimated for IGF-1 and bST. These were assay accuracy (analyte recovery), precision, parallelism, limits of detection and quantification (LOD and LOQ, respectively), and cross-reactivity. In addition, a generalized Hill equation was used to extrapolate analyte concentration. This method was used because multiple equilibrium events occur in a sandwich immunoassay plus the expected concentrations of IGF-1 and bST in bovine milk approach their respective lower limits of detection. A four-parameter logistic model was used to improve accuracy of measurement in the lower range of detection (lower asymptote), and the data were weighted by one over the square of the mean signal to obtain homogeneity of variance. All data used for validation had R^2 values of at least 0.98 for each assay standard curve.

Specificity. Assay specificity denotes the ability to selectively estimate the concentration of a desired analyte, that is, to accurately estimate the concentration of either bST or IGF-1 in an assay buffer when in the presence of other matrix components.

Six hormones potentially present in milk or with sequence homology to factors potentially present in milk were tested for their ability to cross-react with assay antibodies at a concentration higher than typically anticipated for milk: recombinant bovine somatotropin (rbST, 5 ng/mL), pituitary bovine somatotropin (pbST, 5 ng/mL), bovine insulin (50 ng/mL), human pro-insulin (50 ng/mL), human IGF-1 (100 ng/mL), and human IGF-2 (100 ng/mL). Insulin, pro-insulin, and IGF-2 have considerable sequence homology to bovine IGF-1, which is identical to human IGF-1 (*25*). Human IGF-2 and human pro-insulin were tested because they are commercially available and share a high degree of sequence homology with their bovine counterparts (*26–28*). Three assays were used to estimate cross-reactivity with IGF-1 antibodies, and two assays were used to estimate cross-reactivity with bST antibodies. Individual factors were assayed in duplicate within each assay.

To estimate cross-reactivity, the hormone of interest was spiked into the same matrix used for preparation of the standards. This was done so binding of the antibody to the hormone of interest could be directly compared to antibody binding to standards. This method focuses on measurement of cross-reactivity independent of sample processing. The concentration of the hormone tested was adjusted to take into account dilution of samples due to processing. For example, sample processing dilutes the milk 66-fold in the IGF-1 ECLIA, and therefore 1.5 ng/mL of IGF-2 was assayed to test the equivalent of 100 ng/mL of human IGF-2 in milk ($^1/_{66}$ of 100 ng/mL).

Cross-reactivity for each selected milk factor was calculated as

$$\%\ \text{cross-reactivity} = 100 \times (\text{actual/expected})$$

where actual = observed concentration of the factor and expected = expected concentration of the factor.

Parallelism (Linearity). By convention, parallelism is the ability of an assay to produce results that are proportional to the concentration of the analyte within the assay matrix. For validation of the IGF-1 assay, two separate milk samples (determined to be above the LOQ of the assay) were diluted with assay buffer using the following dilution factors: 66, 132, 198, 264, 330, and 396. For validation of the bST assay, a single milk sample (determined to be above the LOQ of the assay) was diluted with assay buffer using the following dilution factors: 2, 4, 6, 8, and 10. A single assay was used to estimate assay parallelism for each analyte. Analyte concentration for each diluted sample was estimated using duplicate wells within each assay.

Parallelism was assessed by PROC MIXED in SAS (*29*), where the slope of the sample analyte (pg/mL) value versus the dilution factor was estimated. The assay was considered to be linear across the concentration range (parallelism) if the slope was not different from zero at the 5% level of probability.

Accuracy. Assay accuracy (analyte recovery) was estimated by dividing two milk samples into aliquots and then "spiking" them to produce a basal (0 spike), low, or high analyte concentration. IGF-1 was spiked into milk prior to extraction, and bST was spiked into milk prior to dilution (1:1). Spiked aliquots were duplicated in each of 9 (IGF-1) or 10 (bST) separate assay runs. The original milk samples were assayed to determine the endogenous concentration of analyte prior to spiking the samples gravimetrically with the selected amount of IGF-1 or bST (amount selected was to span the range of the assay standard curve). Assay accuracy was calculated for IGF-1 and bST as follows:

$$\text{accuracy}\ (\%) = (\text{observed value/expected value}) \times 100$$

The "observed value" is the sample analyte concentration (pg/mL) estimated in the assay multiplied by the respective assay dilution factor, and the "expected value" is the target analyte (pg/mL) calculated as the mean of the observed basal analyte concentration (pg/mL) plus the spike concentration multiplied by the specific gravity of milk (1.03). Accuracy for IGF-1 and bST was calculated for each individual well, and PROC MIXED in SAS (*29*) was used to calculate a least squares mean (LSM) for each spiked sample as well as an LSM for all samples.

Precision. Depending on sample availability, assay precision was estimated using results from 10 (IGF-1) or 12 (bST) assays. Data from all basal and spiked milk samples run in duplicate within each assay were analyzed for within-assay variation, across-assay variation, and the corresponding coefficient of variation (CV) using PROC VARCOMP in SAS (*29*).

Limit of Detection (LOD) and Limit of Quantification (LOQ). By convention, the LOD is defined as the lowest concentration of analyte that can be detected. In this paper, the LOD for IGF-1 or bST in milk is further defined as the median analyte concentration at 3 times the standard error (SE) above the lower asymptote. By convention, the LOQ is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable accuracy. In this paper, the LOQ for IGF-1 or bST in milk is further defined as the median analyte concentration at 10 times the SE above the lower asymptote. Ten assays were used to estimate the LOD and LOQ for IGF-1. Twelve assays were used to estimate the LOD and LOQ for bST. For each assay, the LOD and LOQ were calculated as

$$\text{LOD} = c \times \text{abs}[a - (a + 3 \times \text{SE})/(a + 3 \times \text{SE} - d)]^{**}(1/b)$$

$$\text{LOQ} = c \times \text{abs}[a - (a + 10 \times \text{SE})/(a + 10 \times \text{SE} - d)]^{**}(1/b)$$

where a = the lower asymptote, b = Hill coefficient, $c = \text{ED}_{50}$ (effective dose, 50%), and d = the upper asymptote.

RESULTS AND DISCUSSION

Since 1994, a recombinant form of bST (rbST, POSILAC, Monsanto Co.) has been commercially available in the United States as a management tool for increasing milk production of dairy cows. Nearly since its inception, attempts have been made to distinguish rbST from pituitary bST (pbST) (*15, 30*) in supplemented cows. However, any attempt to distinguish rbST from pbST is difficult because "extremely reduced differences in primary sequence are involved" (*15*). What is referred to commonly as pbST is actually a mixture of four molecular variants, two with 190 amino acids and two with 191 amino acids (*31*). The rbST commercially available in the United States differs from one of the 191 amino acid variants by a single amino acid. Attempts to identify milk from rbST-supplemented cows have also been made by monitoring the concentration of IGF-1 in milk (*13*), but the natural variation of IGF-1 concentration in bovine milk (*18*) coupled with the small increase that may or may not occur during supplementation (*8*) makes any large-scale use of this method inaccurate.

Traditionally, the concentrations of IGF-1 and bST in bovine milk or blood were estimated using a variety of analytical procedures including RIA (*4, 10–13*), ELISA (*15*), biosensor technology (*14*), or bioassay (*16*). Examples of the reported sensitivities of these assays for bST range from 50 pg/mL in serum (*15*) to 500 pg/mL in milk (*16*). The sensitivity for IGF-1 ranges from 200 pg/mL in serum (*24*) to 0.3 ng/mL in milk (*18*). When estimates of the concentrations of these hormones in a complex matrix such as milk are attempted, the matrix itself may have a confounding effect. Electrochemiluminescence (ECL) is a technology reported to be relatively insensitive to matrix effects, plus it provides a method for detecting analytes at low concentration (*23*). By coupling this immunobased technique at the lower range of analyte detection with a four-parameter standard curve and weighting data by $1/\text{mean}^2$ (*32–34*), the accuracy of our two assays was further increased.

Our laboratory initially employed the ELISA technique to estimate IGF-1 in milk and an RIA to detect bST in milk. Both assays used a simple, two-parameter standard curve and data weighted by 1/mean to estimate concentration. The ELISA and RIA also used capture and detection antibodies that, due to availability, could not be tested in the new ECLIA. Although the sensitivity of the earlier assays (IGF-1, LOD = 17 pg/mL, LOQ = 50 pg/mL; bST, LOD = 500 pg/mL, LOQ = 1000 pg/mL; data

Table 1. For Assay Specificity, Proteins Were Added to Assay Buffer at the Indicated Concentration;[a] Test Materials Were Assayed Using the Standard Protocol and Evaluated for Cross-Reactivity for both IGF-1 and bST

	% cross-reactivity	
protein	IGF-1	bST
rbST (5 ng/mL)	0.60	
pbST (5 ng/mL)	0.48	123.49
bovine insulin (50 ng/mL)	0.04	0.18
human pro-insulin (50ng/mL)	0.02	0.05
IGF-1		0.16
IGF-2	0.03	0.18

[a] Estimated concentration in milk is in parentheses.

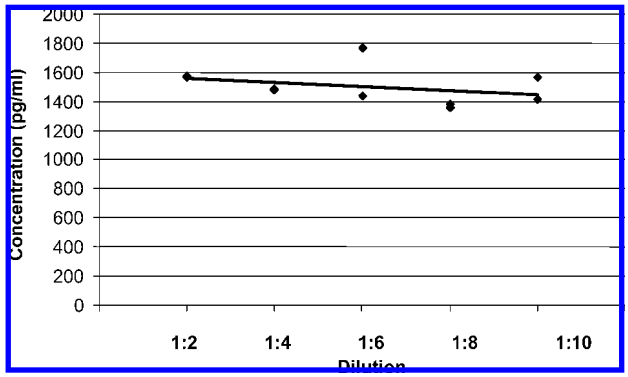

Figure 1. For assay parallelism, bST was spiked into a single milk sample and serially diluted. The sample was assayed and the analyte concentration corrected for dilution. The corrected concentration was plotted against dilution factor, and no significant deflection in slope from 0 was detected (slope = −14.15, $P > 0.25$).

not shown) was adequate for many types of milk samples, an improved, more sensitive assay was sought.

A recent survey of 293 homogenized and pasteurized milk samples obtained from retail outlets (*9*) found that >98% of those samples had a bST concentration that was below the 500 pg/mL LOD of our previous RIA. Even with the ECLIA improvements, approximately 60% of those samples had a bST concentration below our current LOD of 5 pg/mL. The extremely low concentration of bST in processed milk from that study (*9*) is in contrast to higher estimates for the concentration of bST in milk [range of 0.58−4.2 ng/mL (*11, 16*)] reported for fresh, unprocessed or freshly frozen milk samples. However, these differences can be reconciled by the finding (*16*) that 90% of immunoreactive bST may be destroyed by heat treatment. Thus, studies that investigate pasteurization-sensitive peptides in processed milk require highly sensitive assay techniques. In contrast to bST, the reported concentrations of IGF-1 for all 293 retail milk samples in that study (*9*) were above the LOD of our current assay.

Prior to estimation of the assay attributes directly related to analyte concentration (assay accuracy, precision, LOD, and LOQ), the potential impact of matrix components was evaluated by determining assay specificity (**Table 1**) and parallelism (**Figures 1** and **2**) for both IGF-1 and bST. Specificity was determined for bST and IGF-1 by estimating cross-reactivity of five proteins with potential homology to bST and/or IGF-1.

As shown in **Table 1**, all of the analytes tested, including IGF-2 and insulin, had <1% cross-reactivity in the IGF-1 assay. This indicates the current antibody and assay combination is highly selective for IGF-1. Likewise, insulin, pro-insulin, IGF-1, and IGF-2 had <1% cross-reactivity in the bST assay. As expected, pbST had substantial cross-reactivity with rbST. This is not surprising because this purified preparation contains the four native variants

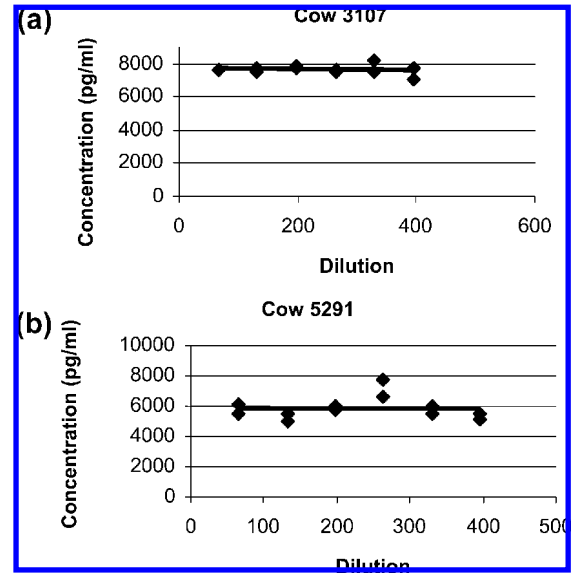

Figure 2. For assay parallelism, two milk samples from two individual cows with relatively high concentrations of IGF-1 were serially diluted with IDP-M. The samples were assayed, and the analyte concentration was corrected for dilution. The corrected concentration was plotted against dilution factor. No significant deflection in slope from 0 was detected for either sample (cow 3107, slope = −0.278, $P > 0.80$; cow 5291, slope = 0.080, $P > 0.90$).

Table 2. Assay Accuracy, Assay Precision (Inter- and Intra-assay Variation), Lower Limit of Detection (LOD), and Lower Limit of Quantification (LOQ) for bST and IGF-1 in Milk Using ECLIA

		assay precision			
analyte	accuracy	inter (%CV)	intra (%CV)	LOD (pg/mL)	LOQ (pg/mL)
bST	99.69	15.80	4.08	4.045	13.996
IGF-1	104.79	20.87	3.80	0.3342	1.114

of bovine somatotropin (*31*). The observed cross-reactivity being >100% may be due to the purity of the pbST preparation in relation to the recombinant bST.

Parallelism was evaluated by estimating the concentration of bST in a single milk sample over five different dilutions (**Figure 1**) and the concentration of IGF-1 in samples from two separate cows over six different dilutions (**Figure 2**). A significant deflection in slope from zero was not detected for samples from either assay when dilution was plotted against luminescence (after correcting for the dilution factor). Again, this indicates assay parallelism and suggests that there are no interfering components within the milk matrix.

Assay accuracy was estimated as the percent of IGF-1 or bST recovered from duplicate spiked milk samples with known concentration of analyte. Nine assays were used to determine accuracy for IGF-1, and 10 assays were used for bST (**Table 2**). For IGF-1 and bST, 99−105% of spiked analyte was recoverable using the described assay procedures. Assay precision (inter- and intra-assay variation) was determined from 10 IGF-1 and 12 bST assays (**Table 2**). LOD and LOQ describe the overall sensitivity of an assay and define the lower limits for assay utility. The LOD and LOQ for bST and IGF-1 in milk are also presented in **Table 2**.

The high percentages of recovery of both IGF-1 and bST demonstrate that the current extraction protocol and assay procedure provide an acceptable method for measuring these analytes in a complex matrix such as milk. However, the interassay CV for both IGF and bST analytes (approximately 15−20%) needs to be

considered when experiments are designed to compare values among treatment groups. Assays should be designed as blocking factors in the experiment. For noncontrolled experiments, caution should be exercised when values are compared among assays. Significant differences would need to exceed the variability found between assays. If these precautions are taken, the low intra-assay variation (~4%) indicates the current assay is acceptable for detecting subtle treatment differences among samples, especially if test samples are allocated to the same assay so that critical comparisons are made within an assay. More importantly, the LOD and LOQ for our current ECLIA are approximately 17 and 25 times lower for IGF-1 compared to our original ELISA, and the LOD and LOQ for our current ECLIA are approximately 100 and 70 times lower for bST compared to our original RIA. This greater degree of sensitivity improves our ability to estimate the concentration of these analytes in milk.

We conclude that the ECLIA, used in conjunction with the four-parameter logistic model weighted by one over the square of the mean signal, provides a highly sensitive method for estimating the concentration of bST and IGF-1 in a complex matrix such as milk.

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Received for review March 6, 2008. Revised manuscript received June 6, 2008. Accepted June 24, 2008.

JF800696D