Measuring Concentrations of Insulin-Like Growth Factor-I with an Enzyme-Linked Immunosorbent Antibody Assay in Plasma Samples from Holstein Cows

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ABSTRACT

This study describes the use of a commercial nonextraction ELISA to quantify total concentrations of insulin-like growth factor-I (IGF-I) in plasma samples from Holstein cows fed pasture-based diets varying in dry matter and metabolizable energy density. The assays were conducted using the protocols provided by the manufacturer. The ELISA was verified for linearity, accuracy in measuring IGF-I from spiked plasma samples, and precision involving variation within and between assays. Validation also involved comparing results of the ELISA against an established RIA after defatted plasma samples were subjected to acid gel HPLC to dissociate and separate IGF and IGF binding protein complexes. Validation results had low coefficients of variation (CV; intraassay CV of less than 6% and interassay CV of less than 8%) and a high recovery percentage of IGF-I (79%) from samples spiked with unbound IGF-I. The coefficients of determination between the ELISA and the RIA reference assay were 0.90 in 2 separate assays. Associations between the RIA and the ELISA were higher, and the limits of agreement at 95% confidence intervals were narrower compared with those between the RIA and a modified ELISA procedure in which IGF binding protein complexes were extracted using HPLC. The samples were obtained from cows sampled over a period of 5 wk. These results demonstrated that the DSL-10-2800 nonextraction IGF-I ELISA was acceptably specific and sufficiently sensitive to be used to measure the extent and patterns of change in the plasma concentrations of IGF-I in samples from lactating Holstein cows.

Key words: insulin-like growth factor-1, dairy cow, enzyme-linked immunosorbent antibody assay, plasma

INTRODUCTION

Insulin-like growth factor-I is a single-chain polypeptide that stimulates cell growth, cell development, and differentiation (Jones and Clemmons, 1995; Rosen and Pollak, 1999). It is present mostly as a complex with 6 specific IGF binding proteins (**IGFBP**, IGFBP-1 to -6) in biological fluids and cell culture medium. The IGFBP influence the rate of clearance of IGF-I as well as modulating its biological actions (Jones and Clemmons, 1995; Rechler and Clemmons, 1998).

Various assays have been used for the quantification of total IGF-I in biological samples. The most frequently used are RIA that may be complicated by IGFBP interference unless complete dissociation and extraction of IGF from the IGFBP are achieved before assaying the samples (Holly and Cwyfan-Hughes, 1994). Size exclusion chromatography under acid conditions produces dissociation allowing extraction of IGF from IGFBP, but it is tedious for routine use with a large numbers of samples (Holly and Cwyfan-Hughes, 1994; Frystyk, 2004). Alternative separation techniques involving reverse-phase chromatography (Frey et al., 1994); acid-ethanol extraction (Daughaday et al., 1980) with its adaptations, acid-ethanol acetone (Gutierrez et al., 1997), acid-ethanol cryoprecipitation (Breier et al., 1991), and formic acid acetone (Bowsher et al., 1991) extractions; or the glycyl-glycine extraction (Plaut et al., 1991) have been developed. Although these are rapid procedures allowing for screening of many samples, they can sometimes lack general validity because of inadequate inactivation of IGFBP interference (Holly and Cwyfan Hughes, 1994). Additional validation is needed for the specific sample under investigation and when assay conditions may have altered (Blum and Breier, 1994). The criteria for confirming the sensitivity and specificity of these alternative assays was outlined by Bang (1995).

The interference by IGFBP may also be diminished by nonextraction methods involving the blocking of

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IGFBP binding sites through addition of a large excess of an IGF not being measured [e.g., excess IGF-2 in an IGF-I RIA (Blum and Breier, 1994)]. They may simply make the interference less apparent (Holly and Cwyfan-Hughes, 1994).

A range of commercial kits that are immunodiagnostic sandwich assays have been developed for measuring IGF-I concentrations in human blood samples. These kits are rapid, highly specific, and sensitive and are more convenient for high throughput analyses (Clemmons, 2001). They include a nonextraction IGF-I ELISA (Diagnostic Systems Laboratories, Webster, TX), the nonextraction IGF-I immunoradiometric assay, and the OCTEIA IGF-I immunoenzymometric assay (Immunodiagnostic Systems Ltd., Tyne and Wear, UK) that have been adapted and evaluated for use with blood samples from horses (De Kock et al., 2001), pigs, and cattle (Dunshea et al., 2002). The human diagnostic DSL-10-2800 IGF-I ELISA has been validated recently for measuring total IGF-I concentrations in porcine plasma (Primegro Laboratories, Adelaide, Australia). Porcine and bovine IGF-I are identical and have similarities in plasma IGFBP profiles (Sara and Hall, 1990). Nonetheless, Bang (1995) and associates were emphatic in concluding that assay validation would apply only to samples similar to those included in the validation and for measurement of total IGF concentrations in biological fluids from the particular class of animal within a species. The purpose of the present study was to assess the potential for using the DSL-10-2800 IGF-I ELISA to measure concentrations of IGF-I in plasma from Holstein cows that had pasture as their main source of forage.

MATERIALS AND METHODS

Animals and Experimental Design

The study consisted of 2 parts: first, the data collection phase that was conducted at the Department of Primary Industry's Ellinbank Dairy Research Station, Victoria, Australia (38 14' 36.4"S; 145 56' 09.5"E), during August and September 2000, and then the assay development and evaluation phase in the laboratory of Primegro Ltd. The initial part received approval from the Ethics Committee of the Department of Primary Industries, Ellinbank. Multiparous Holstein cows ($n = 32$) that had calved during July 2000 and were in the fourth or fifth week of lactation when enrolled were randomly assigned to 4 groups receiving different rations. These rations were formulated to provide high (H) or low (L) DM intake with H or L ME density for 5 wk. The first 3 wk were considered to be an adaptation period. The treatments were LDM/LME: 16.6 kg of DM and 174 MJ of ME; HDM/LME: 17.3 kg of DM and 181.1

MJ of ME; LDM/HME: 15.4 kg of DM and 183.1 MJ of ME; and HDM/HME: 17.9 kg of DM and 213.3 MJ of ME, with the first letter indicating DM and the second ME. The cows were milked at 0600 and 1500 h daily before being individually offered their allocated ration at 0900 and 1600 h, respectively, for 5 h. They were then held as a single group in a bare holding yard. The diets comprised freshly cut ryegrass-clover pasture, meadow hay, and cereal (barley) grain pellets to achieve the 2 different levels of DM and ME. Water was available between the feeding and milking times.

Blood Sampling Procedure

A blood sample was taken from a coccygeal vein of each cow at weekly intervals for the first 3 wk and then daily for the final 2 wk. The sampling was at 0700 h after a morning milking and was into a 10-mL heparinized Vacutainer tube (BD Vacutainer Systems, Plymouth, UK). Each blood sample was briefly stored on ice and then centrifuged ($1,600 \times g$ for 15 min at 4°C) within 10 min of collection. Plasma was harvested and stored at -20°C until assayed for IGF-I.

Extraction of IGF-I from IGFBP in Plasma

Before RIA, the IGF-I in a sample of plasma was dissociated and separated from IGFBP by acidification and size-exclusion HPLC (Owens et al., 1994). Plasma (90 μ L) was diluted in 150 μ L of 0.8 *M* acetic acid, 0.2 *M* trimethylamine, 2.0 mL/L of Tween-20 (BDH, Kilsyth, Victoria, Australia) with the pH adjusted to 2.5, and 360 μ L of Milli-Q water (Millipore, North Ryde, New South Wales, Australia). Acidified plasma was defatted by mixing with an equal volume of freon at room temperature and was centrifuged ($10,000 \times g$) in an EBA 12 microfuge (Andreas Hettich GmbH and Co, Tuttlingen, Germany). The aqueous phase (500 μ L) was recovered and ultrafiltered by centrifugation through 0.22- μ m cellulose acetate (Microspin Centrifuge Filters, catalog no. 2485, Alltech, Dandenong South Victoria, Australia). Filtered, defatted, and acidified bovine plasma (200 μ L) was subjected to size-exclusion HPLC (Waters ProteinPak 125, Waters Chromatography, Millipore) using 0.2 *M* acetic acid, 0.05 *M* trimethylamine, and 0.05% Tween-20, pH 2.5, at a flow rate of 1 mL/min. In addition, [125 I]-IGF-I was eluted with a peak at 9.5 min. Retention time varied less than 5%. Four fractions were collected from each plasma sample: acid-dissociated IGFBP (retention times 6 to 8 min), acid-dissociated IGF (retention times 8.5 to 10.5 min), an intermediate fraction (8 to 8.5 min), and a tail fraction (10.5 to 11 min). Fractions were collected into polypropylene tubes that were capped and stored at 4°C until

assayed. One quality control (QC) sample (plasma from cow 9670) was also chromatographed during each chromatography session and assayed using the RIA. The mean IGF-I concentration of the cow plasma QC was 70 ng/mL with a CV of 5.6%.

IGF-I RIA

Triplicate aliquots (50 μ L) of acid IGF-I fractions from extracted plasma were neutralized with 30 μ L of 0.4 M Tris base and added directly to the IGF-I RIA. Acid column mobile phase (50 μ L) and neutralizing agent were also added to the standards, reference, and blank in the RIA. The IGF-I RIA was performed as described by Owens et al. (1994). Human IGF-I (GroPep Pty. Ltd., Adelaide, Australia) was used as a standard and for preparation of ligand tracer by the chloramine-T method (specific activity 60 Ci/g of IGF-I). Rabbit antihuman IGF-I (GroPep Pty. Ltd.) was used as the primary antibody at a final dilution of 1/80,000. Goat antirabbit IgG (GroPep Pty. Ltd.) was used to precipitate the primary antibody IGF-I complexes. Radioactivity in the precipitate was measured with a gamma scintillation spectrophotometer (Wallac Wizard 1470 Automatic, Wallac Oy, Turku, Finland), and data reduction was accomplished using the Ria Calc WIZ program (Wallac Oy).

Plasma IGF-I ELISA

Total IGF-I concentrations in plasma were measured using a commercial kit, DSL-10-2800 ACTIVE Nonextraction IGF-I ELISA (Diagnostic Systems Laboratories Inc.). This is a highly specific, enzymatically amplified 2-step sandwich-type immunoassay with no cross reactivity with IGF-2 or rat IGF-I and no interference from IGFBP as stated in the specifications of the manufacturer. The assay utilized 2 monoclonal IGF-I antibodies that were each directed against 2 specific sites on the IGF-I molecule. Procedures for the assays were followed as specified by the manufacturer. Briefly, the IGF-I kit standards consisted of 0, 10, 45, 250, and 500 ng/mL of IGF-I (synthetic) in a BSA buffer with a nonmercury preservative. In addition, there were 2 IGF-I controls (150 and 325 ng/mL) in a protein-based BSA buffer with a nonmercury preservative. Plasma samples (20 μ L) that had been thawed overnight were first aliquoted into disposable 12 $\times$ 75 mm polypropylene culture tubes and then pretreated for 30 min with a releasing reagent (Sample Buffer I) to denature IGFBP. Then they were mixed with a neutralizing diluent (Sample Buffer II).

In the assay, 20 μ L of each standard, control, and prediluted unknown plasma samples (test samples)

was aliquoted in duplicate into microtitration wells coated with an anti-IGF-1 antibody (monoclonal IGF-I antibody), followed by the addition of 100 μ L of assay buffer to each well using a semiautomatic dispenser. The wells were incubated with rapid shaking on an orbital microplate shaker (Ratek Instruments, Victoria, Australia) for 2 h at room temperature ($\sim$ 25°C). The wells were aspirated after incubation and washed 5 times with a wash solution using an automatic plate washer (MeterTech Inc., Taiwan, China). The wells were treated after washing with 100 μ L of a second anti-IGF-1 detection antibody (monoclonal IGF-I antibody) labeled with horseradish peroxidase enzyme and incubated for 30 min on an orbital microplate shaker at room temperature followed by washing (as above). After this second incubation step and washing, the wells were incubated for 10 min with shaking at room temperature with 100 μ L of tetramethylbenzidine chromogen solution containing a solution of tetramethylbenzidine in buffer with hydrogen peroxide and avoiding exposure to direct sunlight. An acidic stopping solution containing 0.2 M sulfuric acid (100 μ L) was added to stop the reaction. The absorbance of the resulting yellow acid dye solution in the wells was measured within 10 min of the reaction being stopped using a Multiskan Ex microplate reader (Labsystems, Vantaa, Finland) set at 450 nm with a reference filter at 620 nm. The absorbance measured was directly proportional to the concentration of IGF-I.

Validation for IGF-I ELISA

The DSL-10-2800 ELISA for total IGF-I concentration was validated for linearity, accuracy in measuring IGF-I in spiked plasma samples, and for precision measured as variation within and between assays. In addition, the ELISA was validated against the accepted standard method, namely, RIA after acid gel HPLC dissociation and extraction.

The accuracy of the ELISA in measuring IGF-I concentrations was assessed by estimating recovery of IGF-I from plasma samples with known high ($n = 4$) or low ($n = 4$) IGF-I concentrations that were each spiked with 100 ng/mL of recombinant human IGF-I. The spiking process involved the addition of 10 μ L of a stock solution of IGF-I to 90 μ L of Dulbecco's PBS. The diluted IGF-I (5 μ L) was then added to 500 μ L of a plasma sample. Each plasma sample (20 μ L) was then analyzed using the ELISA procedure. Recovery of IGF-I from each spiked sample of plasma was calculated using the formula:

$$\begin{aligned} \% \text{ recovery} = & \\ & \frac{\text{Observed concentration of IGF-I in a spiked sample}}{\text{Expected concentration of IGF-I in that sample}} \\ & \times 100. \end{aligned}$$

Interassay CV were calculated using the QC samples throughout the assay. Intraassay CV were calculated using the QC samples within each assay.

The effectiveness of the ELISA in eliminating IGFBP interference was evaluated by comparing IGF-I values in plasma ($n = 32$ cows with 1 sample/cow) measured with the ELISA against the concentration of IGF-I measured by RIA in the same defatted plasma samples extracted by HPLC under acid conditions. The association between RIA and the ELISA for changes in IGF-I over the 5-wk sampling period was assessed in plasma samples from 8 selected cows (the cows with the maximum and minimum IGF-I concentrations in each of the 4 dietary groups of 8 cows).

A modified ELISA procedure (**ELISA-m**) was also investigated in 24 samples. In the ELISA-m procedure, plasma samples from which IGFBP had already been extracted using HPLC were assayed directly in the ELISA, excluding the steps for dissociation and removal of IGFBPs in the standard ELISA procedure (addition of Sample Buffers I and II).

The effects of freezing and thawing on concentrations of IGF-I were measured in a single plasma sample from each of 6 cows and ranging from an initial concentration measured using the ELISA of 19.5 to 109.6 ng/mL. The samples were frozen and thawed once, 3 times, and 6 times and IGF-I concentrations were analyzed by ELISA after each of these freezing and thawing cycles. The changes in the concentrations of IGF-I in these samples were expressed as a percentage of the initial concentration measured between 1 and 3, 3 and 6, as well as 1 and 6 freeze and thaws.

Statistical Analyses

Statistical analyses were performed mainly using the statistical program SPSS Version 11.5 for Windows (SPSS Inc., Chicago, IL). Linear regression analyses were used to assess the relationships among the 3 procedures, RIA, ELISA, and ELISA-m, for measured IGF-I concentrations using cluster robust standard errors to account for multiple observations per cow as incorporated (Williams, 2000). The limit of agreement (**LOA**) among the 3 assays (RIA, ELISA, and ELISA-m) for measured IGF-I were also estimated. The 95% LOA were determined from the mean difference ± 2 SD of the differences, taking into account multiple measurements per animal (Bland and Altman, 1986, 1999). The

95% LOA provided an interval within which 95% of differences between single measurements by any 2 methods being tested were expected to lie. Data on the effect of freezing and thawing on plasma IGF-I were base-10 logarithm transformed before analysis using SPSS to take account of the range in concentrations and using percentage change rather than absolute change in concentration as the measured outcome.

RESULTS

Recovery of ^{125}I from Plasma by HPLC

The separation of IGFBP from IGF was confirmed by IGF-I RIA in each of the 4 selected fractions that were collected over 3 chromatography sessions. Mean recovery of [^{125}I]-labeled IGF-I was $98 \pm 5.0\%$ (mean $\pm$ SEM; $n = 3$).

Precision of the RIA

The observed intra- and interassay CV for the plasma IGF-I RIA were 4.3 and 10.4%. The 50% displacement was 382.7 ng/mL.

Recovery Rates of IGF-I from Spiked Plasma Using the ELISA

The mean recovery of the IGF-I from the spiked plasma samples was 79%, ranging from 66.2 to 90.0% (Table 1). The coefficient of determination between the observed and expected IGF-I concentrations was 0.94 ($P < 0.001$). The mean recovery of IGF-I from plasma samples with above-average concentrations was $78.0 \pm 4.6\%$ (mean $\pm$ SEM; $n = 4$) and was similar (t -test; $P = 0.29$) to the value obtained for plasma samples with below-average concentrations ($80.0 \pm 5.0\%$).

Precision of the ELISA for Measuring IGF-I in Plasma

The intra- and interassay CV for plasma were 3.2 to 5.0% and 4.2 to 7.6%, respectively ($n = 2$ per assay, 4 assays), for the 2 QC sera supplied and 3.4 and 5.6% ($n = 2$ per assay, 4 assays) for 1 cattle QC (Table 2). The minimum detection limit was calculated from serial dilutions of plasma samples with known IGF-I concentrations. The minimum detection limit for the ELISA was 10 ng/mL in plasma.

Elimination of IGFBP Interference

The effectiveness of the steps within the ELISA in eliminating IGFBP interference was evaluated by comparing IGF-I concentrations in single plasma samples

Table 1. Percentage recoveries of IGF-I concentrations in spiked samples of plasma measured using the ELISA

Cow ID	Trt ¹	IGF-1 level	Unspk ² (ng/mL)	Expected ³ (ng/mL)	Observed ⁴ (ng/mL)	% Recovery ⁵
9457	HDM/LME	Low	13.8	113.8	75.3	66.2
9246	HDM/LME	High	23.5	123.5	86.5	70.0
9103	LDM/LME	Low	12.6	112.6	90.0	80.0
9729	LDM/LME	High	24.9	124.9	87.4	70.0
9510	LDM/HME	Low	47.1	147.1	132.4	90.0
9521	LDM/HME	High	63.5	163.5	140.4	85.9
9474	HDM/HME	Low	28.3	128.3	107.6	83.9
9560	HDM/HME	High	98.6	198.6	171.4	86.3

¹Trt = treatment. HDM = high DM; LME = low ME; LDM = low DM; HME = high ME.

²IGF-I concentrations in unspiked plasma.

³Expected IGF-I concentrations in plasma after spiking with 100 ng/mL of recombinant IGF-I.

⁴Measured IGF-I concentrations after spiking.

⁵% Recovery = ratio of IGF-I expected in sample and IGF-I measured $\times$ 100.

from 32 cows measured by the ELISA and RIA at d 21 (the beginning of the data collection period after cows had adapted to their diets). There was a strong association between the 2 methods ($R^2 = 0.90$; $P < 0.001$; Figure 1), a slope of 1.05 (that did not differ from unity; $P = 0.96$), and a y-intercept value of -4.43 that was not different from zero ($P = 0.24$).

Comparison Among Three Assay Procedures for Changes in Plasma IGF-I for 5 wk in 8 Selected Cows

The linear regression analysis comparing changes in IGF-I concentrations over time using the RIA or the ELISA had a coefficient of determination of 0.90 ($P < 0.001$; Figure 2; $n = 48$). The slope of 1.03 did not differ from unity ($P = 0.55$), and the y-intercept of 2.64 did not differ from zero ($P = 0.45$). The linear relationship between the RIA and the ELISA-m had a coefficient of determination of 0.48 ($P < 0.068$; Figure 3; $n = 24$). Between ELISA and ELISA-m, the coefficient of determination was 0.69 ($P < 0.038$; Figure 4; $n = 24$). The y-intercepts for the linear regression between the RIA and ELISA-m and the ELISA and ELISA-m (20.66 and 18.11, respectively) did not differ from zero ($P = 0.36$ and $P = 0.30$ respectively). The patterns of change in IGF-I concentrations over the 5 wk observed with either

RIA or the ELISA were similar, as illustrated with results for the HDM/HME cow 9474 and the LDM/HME cow 9746 (Figure 5).

Results from linear regression analyses computed from the difference and average values of plasma concentrations of IGF-I between any 2 types of assay and the 95% LOA are presented in Table 3. The 95% LOA was less between the RIA and ELISA (-24.57 to 16.07 ; Figure 6) than those between RIA and ELISA-m (-54.84 to 15.44) and between the ELISA and ELISA-m (-41.19 to 12.41). The slopes of the difference vs. the average for the linear regression lines between RIA and ELISA (-0.08) and also between the ELISA and ELISA-m (-0.11) were not significantly different from zero ($P = 0.08$ and $P = 0.38$ respectively), whereas that between RIA and ELISA-m (-0.41) was different from zero ($P = 0.03$), indicating that the difference between the result obtained with these 2 assays would vary with the concentration of IGF-I in the sample.

Effect of Freezing and Thawing Cycles on Plasma IGF-I Concentrations

Repeated freezing and thawing cycles reduced plasma IGF-I concentrations in each of the selected samples (Figure 7). The reductions in estimated plasma IGF-I concentrations (i.e., 100% availability) were 7.3

Table 2. ELISA precision and repeatability: intra- and interassay CV

QC ¹	Total no. of determinations (n)	Mean $\pm$ SD expected (ng/mL)	Mean $\pm$ SD measured (ng/mL)	Intraassay CV (%)	Interassay CV (%)
DSL QC 1	16	150 $\pm$ 45	174 $\pm$ 13.3	5.0	7.6
DSL QC II	16	325 $\pm$ 80	375 $\pm$ 15.9	3.2	4.2
Cow 9560 (plasma)	16	70.8 $\pm$ 8	66.1 $\pm$ 3.7	3.4	5.6

¹QC = quality control.

Figure 1. Linear regression analysis for IGF-I concentrations in plasma measured using an RIA or an ELISA (DSL-10-2800, Diagnostic Systems Laboratories Inc., Webster, TX) at d 21.

$\pm 1.08\%$ ($P = 0.006$), $13.5 \pm 1.96\%$ ($P < 0.001$), and $19.6 \pm 2.02\%$ ($P < 0.001$) between 1 and 3, 3 and 6, and 1 and 6 cycles, respectively.

DISCUSSION

The quantification of IGF-I in biological samples has been constrained by difficulties in adequately dissociat-

Figure 2. Linear regression analysis for IGF-I concentrations in plasma measured with the RIA and the ELISA (DSL-10-2800, Diagnostic Systems Laboratories Inc., Webster, TX) in 6 samples from each of 8 cows (all data combined).

Figure 3. Linear regression analysis for IGF-I concentrations in plasma measured with the RIA and the modified ELISA procedure (ELISA-m) in 6 samples from each of 4 cows (all data combined).

ing or removing the influence of IGFBP (Gutierrez et al., 1997). This led to completing this preliminary study using an ELISA for the measurement of IGF-I concentrations in plasma samples from lactating Holstein cows. The antihuman antibodies from the commercial ELISA kit used should have had 100% cross-reactivity with bovine IGF-I, because the sequence of AA of bovine IGF-I are identical to that for human IGF-I (Honegger and Humbel, 1986; Tavakkol et al., 1988).

The high mean recovery of IGF-I from spiked plasma (79%; Table 1) from the sampled cows was an indication of the capacity of the ELISA to accurately measure IGF-I concentrations in these samples. There was a strong association between the expected and observed (measured) IGF-I concentrations in plasma ($R^2 = 0.94$; Table 1) over the complete range of concentrations among these samples. The ELISA demonstrated high precision for each of the 3 quality controls in plasma, because

Figure 4. Linear regression analysis for IGF-I concentrations in plasma measured with ELISA (DSL-10-2800, Diagnostic Systems Laboratories Inc., Webster, TX) and the modified ELISA procedure (ELISA-m) in 6 samples from each of 4 cows (all data combined).

Figure 5. Two plots of IGF-I concentrations over time for 2 cows (9474 and 9746) on a high DM/high ME (HDM/HME) or low DM/high ME (LDM/HME) diet, respectively, showing results using ELISA (◆) and RIA (■) assays.

Figure 6. Linear regression analysis for plasma IGF-I concentrations for the difference of RIA – ELISA and the average of RIA + ELISA with the 95% limits of agreement (LOA) for 6 weekly samples from each of 8 cows (all data combined).

the intraassay CV percentage was less than 6% and the interassay CV percentage was less than 8% (Table 2).

Plasma IGF-I values obtained with the standard ELISA procedure were similar to the measured values in the RIA for the 32 cows at d 21 (Figure 1). The coefficient of determination of 0.90 between the 2 assay methods indicated that the ELISA was as effective as the RIA in providing quantitative results for IGF-I in the samples of bovine plasma that were tested. There was a stronger association between the RIA and ELISA compared with RIA and the modified ELISA-m procedure for changes in IGF-I concentrations measured over 5 wk in the samples selected from the 8 cows in the 4 dietary groups (Figures 2 to 4). Also, the 95% limits of agreement between the RIA and ELISA were narrower when compared with the RIA and the modified ELISA-m procedure (Table 3).

Sample preparation and storage have been reported to affect free IGF-I concentrations in serum from horses

Table 3. Linear regression equations ($\pm$ SE) computed from the difference (y) and average (x) values of plasma concentrations of IGF-I measured using the RIA, the ELISA or the modified ELISA procedure (ELISA-m), and their 95% limits of agreement (LOA)

RIA vs. ELISA	RIA vs. ELISA-m	ELISA vs. ELISA-m
$-0.08(0.05)x + 0.54(3.04)$	$-0.41(0.18)x + 0.88(9.43)$	$-0.11(0.13)x - 8.37(7.29)$
$R^2 = 0.06$ NS ¹	$R^2 = 0.20^*$	$R^2 = 0.03$ NS
LOA = -24.57 to 16.07	LOA = -54.84 to 15.44	LOA = -41.19 to 12.41
n = 48	n = 24	n = 24

¹NS = not significant ($P > 0.05$).

* $P < 0.05$.

Figure 7. Percentage change in IGF-I concentrations after plasma samples were repeatedly frozen and thawed (mean $\pm$ SEM; $n = 6$).

(De Kock et al., 2001). These authors observed repeatable results when serum samples were stored frozen at -20°C and thawed just before they were assayed. Extended storage at 4 to 8°C and repeated thawing of samples increased free IGF-I concentrations in the serum samples from these horses possibly due to breakdown of IGF-BP through proteolysis or denaturation and subsequent release of bound IGF-I. However, this release of bound IGF-I did not affect total IGF-I concentrations. In the current study, total IGF-I concentrations in bovine plasma decreased with an increase in the number of freeze-thaw cycles (Figure 7). The DSL-9400 IGF-I immunoradiometric assay used by De Kock et al. (2001) measured free IGF-I, whereas the ELISA used in the present study only measured total IGF-I concentrations.

No published research has been found using the DSL-10-2800 IGF-I ELISA to quantify total IGF-I concentrations in the blood plasma samples from dairy cows. Similar immunodiagnostic assay commercial kits were reported to effectively measure free IGF-I concentrations in follicular fluid from dairy cows (Beg et al., 2001) or total IGF-I in plasma samples from pigs and dairy cattle (Norup, 1996; Dunshea et al., 2002). These included the OCTEIA IGF-I immunoenzymometric, the DSL-10-9400, and the DSL-2800 immunoradiometric assay kits.

CONCLUSIONS

The nonextraction ELISA was as effective as the RIA with acid gel chromatography-treated samples in reducing the IGF-BP interference and providing quantitative results for concentrations of IGF-I in plasma samples from lactating Holstein cows. Recoveries of IGF-I from spiked plasma samples by the ELISA procedure

were acceptable over the range of concentrations in the original samples. Intraassay and interassay CV were low, indicating acceptable accuracy and repeatability. These results have demonstrated that the DSL-10-2800 nonextraction IGF-I ELISA is specific and sufficiently sensitive to be used to determine the extent and patterns of change in concentrations of IGF-I in plasma samples obtained from lactating cows. The ELISA procedure was comparatively rapid, only requiring routine dilution steps. It was less expensive and easier to perform and used no radioactive chemicals.

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