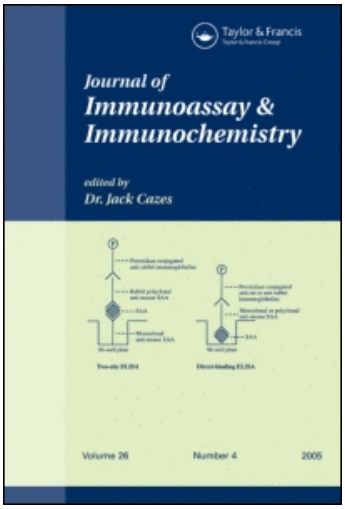

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INFLUENCE OF BOVINE LH TRACER QUALITY ON LEVELS OF LH IN GnRH-TREATED COWS

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ABSTRACT

Chromatography of ^{125}I-bovine LH (LER-1716-2 and USDA-I-1) by means of anion exchange high performance liquid chromatography (HPLC) revealed two main peaks of radioactivity regardless as to whether or not the tracer was initially purified on cellulose CF11. The content of radioactivity in the first peak tended to increase as the storage time of the bLH preparation, either before or after iodination, increased. The first peak of radioacti-

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vity after HPLC fractionation either with or without cellulose adsorption consisted of material with low binding ability to bLH antiserum (6.9%±0.5 and 13.0%±1.0, respectively) and high binding ablity to ovine LHα antiserum (51.0%±2.7 and 35.2%±3.6, respectively). The average ratio of α-subunit immuno- reactivity to ^{125}I-bLH immunoreactivity in this material was 7.4±0.1 and 2.7±0.2, respectively ($P<0.001$). Peaks in ^{125}I-bLH radioactivity and ^{125}I-bLH immunoreactivity had different elution times. Radioimmunoassays with tracers obtained from fractions derived from the first radioactive peak after HPLC chromatography (i.e. ^{125}I-bLH-LER-1716-2) both with and without cellulose adsorption, yielded significantly lower mean plasma LH levels in GnRH-treated cows compared with the control tracer routinly purified only on cellulose CF11 (e.g. 5.7 vs. 8.2 μg/; 4.6 vs. 8.2 μg/l). Plasma LH levels in GnRH-treated cows were significantly ($P<0.001$) lower as measured by radioimmunoassay utilizing ^{125}I-USDA-bLH-I-1 tracers than by radioimmunoassays utilizing ^{125}I-bLH-LER-1716-2 tracers (i.e. either $Y = 0.17 + 0.75X$ or $Y = 1.18 + 0.60X$).
(KEY WORDS: lutropin, HPLC, radioimmunoassay)

INTRODUCTION

The microheterogeneity occurring in ovine (o) LH was probably first suggested by Jutisz and Squire (1). Since then a number of studies concerning the existence of isoforms of LH in domestic species have been published (2-6). For human (h) LH the number of reported components has varied from 3 to 14 depending on the separation method and standard preparation utilized (7-12). The highest number of hLH forms was revealed when employing anion exchange fast protein liquid chromatography. The existence of even more forms cannot be excluded particularly in light of the findings of Wide (13), who reported 20 or more forms of hLH in pituitary extracts. Such heterogeneity can influence the reliability of radioimmunoassays (14-17).

In earlier reports we used anion exchange and reversed phase high performance liquid chromatography (HPLC) to study the behaviour of bovine (b) LH and ^{125}I-oLH, respectively (18-19). Here we report on the influence of various fractions of ^{125}I-bLH, obtained from anion exchange HPLC on assay performance when used as tracers in a radioimmunoassay of bLH.

MATERIALS AND METHODS

Hormones

Highly purified bLH-LER-1716-2, USDA-bLH-I-1 (AFP-4011-B), α-subunit of oLH (WRR-1-α), and β-subunit of oLH (WRR-2-β) were of immunochemical grade. Highly purified NIAMDD-bLH-4, NIADDK-bTSH-11 and NIAMDD-oFSH-RP1 were of biological grade. Small portions of the respective preparation weighed, on a Cahn electrobalance, were dissolved in 50 mM phosphate buffer, pH 7.5, to reach a concentration of 100 μg/ml. Part of this solution was frozen (-20°C) in 25 μl aliquots, stored and used for iodination. The rest was diluted 10-fold with 50 mM phosphate buffer, pH 7.5, containing 0.5% bovine serum albumin (BSA), frozen (-20°C) in 100 μl aliquots, stored and used for construction of standard curves.

Radioiodination

Iodinations (carrier-free ^{125}I Amersham International plc, Buckinghamshire, England) of bLH-LER-1716-2, USDA-bLH-I-1, oLHα

(WRR-1-α) and oLHβ (WRR-2-β) were performed by the Chloramine-T (20), based on the procedure described by Stupnicki and Madej (21), except that 8 μg of Chloramine-T per 2 μg of glycoprotein and an exposure time of 40 sec were used. The iodinated material was immediately separated from ^{125}I by chromatography on a Sephadex G25M column (1.5 x 5.6 cm). The specific activities of ^{125}I-bLH, ^{125}I-oLHα, and ^{125}I-oLHβ achieved were 20-50 μCi/μg. The radioiodinated hormones were stored at 4^{o}C. For routine assay, ^{125}I-bLH was first adsorbed by cellulose (Whatman CF11) (21,22). The radioiodinated hormone was then eluted with 10 mM sodium phosphate buffer, pH 7.5, containing 6% BSA. Radioactivity was quantified in a gamma counter (Searle Analytic Inc.,IL, USA).

HPLC

Anion exchange HPLC was carried out according to the method described by Hallin and Madej (18). Briefly, HPLC was performed on a Waters Associates Chromatographic system, consisting of two M 6000 solvent delivery systems, a U6K injector and an M 660 solvent programmer (Waters Associates, Milford, MA, USA). All reagents used were of analytical grade. A PAK DEAE 5PW anion exchange column (7.5 mm x 7.5 cm) was also obtained from Waters Associates. The HPLC solvent system consisted of buffer A (20 mM Tris-HCl, pH 6.5) and buffer B (20 mM Tris-HCl, 500 mM sodium chloride, pH 6.5). Typically, 0.2-0.9 ml samples containing about

10 μCi (370 kBq) of ^{125}I-bLH either with or without cellulose adsorption, were loaded onto a DEAE column. The samples were eluted with a linear gradient running for 20 min from 98%/2% of buffers A/B to 100% of buffer B with a flow rate of 2 ml/min. The gradient started immediately following injection. Every 30 sec a 1 ml fraction was collected for gamma counting and subsequent analysis. All HPLC experiments were carried out at ambient temperature (22°C).

Radioimmunoassays

The antiserum against bovine LH (a-bLH/R7; 21) was used at final dilution of 1:60,000. Bovine LH (NIAMDD-bLH-4) was used as standard. Overnight incubation, including a 2 h delay in the addition of ^{125}I-bLH, was carried out at room temperature. The separation of free and antibody-bound hormone was carried out by adding a second antibody coupled to Micro Sepharose beads (Pharmacia AB, Uppsala, Sweden). A 0.5 ml suspension was dispensed into each tube, and after incubation at room temperature for 30 min the contents of the tubes were centrifuged for 20 min at 1500 x g. The coefficient of variation for bLH concentrations in the range of 1.5 to 20.0 μg/l was below 8.0%. The mean bLH concentration in 3 pool samples and the coefficients of variation, estimated in 13 different assays were 3.0 (C.V. 6.7%), 5.0 (C.V. 8.0%) and 21.3 (C.V. 8.2%) μg/l, respectively.

The system to measure α-subunit immunoreactivity in bovine plasma consisted of oLHα (WRR-1-α), as tracer and reference preparation, and oLHα antiserum (NIAMDD-anti-oLHα-1) at a final dilution of 1:120,000. Additionally, 1.6 ng of bLH was pipetted into each tube to diminish the bLH influence in the bLHα system. The oLHα antiserum bound 18.2%±0.3 SEM (n=8) of the ^{125}I-oLHα, and the standard curve ranged from 0.05 to 3.2 ng. Dilutions of bovine plasma were parallel with the oLHα standard. The sensitivity of the assay, calculated from the precision profile, was 0.027±0.003 SEM ng/tube (n=8) (0.27 μg/l for 100 μl samples) and the amount of oLHα needed to cause 50% inhibition was 0.328±0.010 SEM ng/tube. The intra-assay coefficient of variation was 18.1%. The mean concentration of bLHα in 3 pool samples of bovine origin and their coefficients of variation, estimated in 8 different assays, were 1.2 (C.V. 16.7%), 2.9 (C.V. 13.8%), and 8.4 (C.V. 13.1%) μg/l. The analysis of cross-reactivity of the oLHα antiserum indicated that bLH and bTSH levels up to 2 μg/l and bFSH level up to 32 μg/l did not influence the assay system. At higher concentrations cross-reactivities were 9.0% for bLH, 5.3% for bTSH and <0.1% for bFSH. The incubation and separation of free and antibody-bound hormone were performed as described above for the bLH assay. All assays were performed in 50 mM sodium phosphate buffer, pH 7.5, containing 0.45% sodium chloride, 10 mM EDTA, 0.001% merthiolate and 0.2% BSA (assay buffer).

After anion exchange chromatography, the amount of radioactivity was measured in 0.05 ml of each fraction. The eluted fractions were then diluted with assay buffer to obtain 4000 cpm/0.1 ml. These solutions were incubated overnight at room temperature with antiserum against either α- or β-subunit of oLH (NIAMDD-anti-oLHα-1 and NIAMDD-anti-oLHβ-1, respectively), both at a final dilution of 1:90,000. At this dilution the ^{125}I-oLHα did not bind to the oLHβ antiserum and ^{125}I-oLHβ did not bind to the oLHα antiserum. The oLHα antiserum bound about 58% of the ^{125}I-oLHα. Each fraction was also tested for its ability to bind to bLH antiserum (final dilution 1:60,000). The non-specific binding was also estimated. At this dilution the ^{125}I-oLHα did not bind to bLH antiserum.

It should be noted that all comparisions of various iodinated tracers were properly designed and performed always in duplicate in a single assay.

Animals and Sampling Procedure

Three primiparous dairy cows, crosses between Swedish Friesian and Swedish Red and White breeds, were given 100 μg of GnRH (Nialutin, NOVO AS, Copenhagen, Denmark) i.v. during proestrus. Blood samples (10 ml) were collected at 15-60 min intervals in heparinized glass tubes from a cannula inserted into the jugular vein at least one day before treatment. The blood

samples were immediately centrifuged and the obtained plasma was kept at -20°C until assayed.

Statistical Analysis

Differences between tracers were analysed by Kruska-Wallis' test, van der Waerden's test, Student's t-test, Duncan's multiple range test, available from the Statistical Analysis System (23).

RESULTS

When either ^{125}I-bLH-LER-1716-2 (Fig. 1) or ^{125}I-bLH-I-1 was fractionated by anion exchange HPLC, two main peaks of radioactivity appeared regardless as to whether or not the tracer was initially purified on cellulose CF11. The first sharp peak of radioactivity appeared up to 2 min after injection (at about 50 mM NaCl) and the second, a broad one, had a retention time of up to 12 min (up to 300 mM NaCl). Cellulose adsorption and subsequent HPLC of ^{125}I-bLH-LER-1716-2 recovered an average of 80.0± 3.5% (n=4) of the radioactivity, when the cellulose step was omitted the recovery was 46.4±1.6% (n=4). Changes in the percentage of radioactivity in the first peak tended to be depended on the length of storage of the bLH preparations both before and after iodination. The radioactivity in the first peak

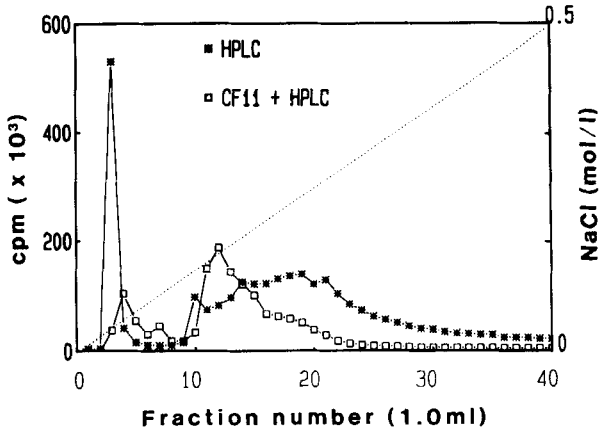

FIGURE 1. Anion exchange HPLC of radioiodinated bovine LH (LER-1716-2) on a Waters Protein PAK DEAE 5PW column (7.5 x 75 mm) with (□) and without (*) cellulose adsorption on Whatman CF11 prior to application onto a DEAE column. The samples were eluted with a linear gradient running for 20 min from 20 mM Tris-HCl (pH 6.5) to 20 mM Tris-HCl, 500 mM sodium chloride (pH 6.5) with a flow rate of 2 ml/min. Sample load was 5 μCi (185 kBq).

increased from 8.7% to 21.4% in the one-week old ^{125}I-bLH-LER-1716-2 preparations where it had been stored before iodination for 26 weeks. The radioactivity in the first peak was about 30% where the tracer had been stored for more than 4 weeks, and this was independent of the storage time of the bLH preparations before iodination.

The elution profile of ^{125}I-bLH radioactivity did not correspond to that of ^{125}I-bLH immunoreactivity (Fig. 2). The fractions derived from the first peak of radioactivity contained lower ^{125}I-bLH immunoreactivity as compared with the same tracer

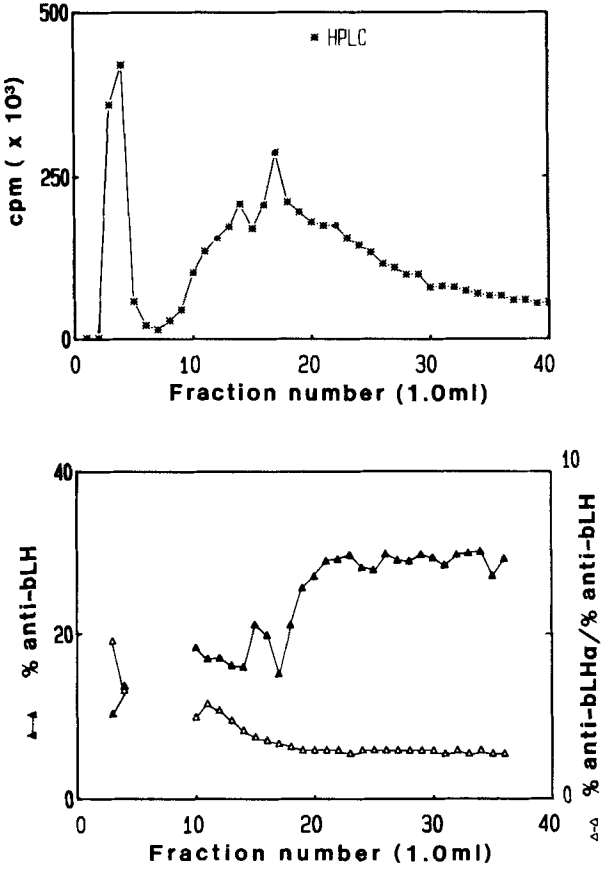

FIGURE 2. Anion exchange HPLC of radioiodinated bovine LH (LER-1716-2) without cellulose adsorption (see Fig. 1) - radioactivity profile (*); bLH immunoreactivity expressed as binding to bLH antiserum (▲) and ratio of α-subunit immunoreactivity (expressed as binding to oLHα antiserum) to bLH immunoreactivity (Δ).

without HPLC fractionation (i.e. 13.7% vs. 20.8%). The relation between ^{125}I-bLH immunoreactivity and the content of radioactivity in the second broad peak seemed to be negative. The plateau in ^{125}I-bLH immunoreactivity reached at fraction no. 21 (retention time 10.5 min) corresponded to the start of the gradual decrease in radioactivity. The ratio of α-subunit immunoreactivity to ^{125}I-bLH immunoreactivity decreased from 4.8 in fraction no. 3 to 1.5 in fraction no. 21 at the plateau of ^{125}I-bLH immunoreactivity (Fig 2).

When ^{125}I-bLH was subjected to HPLC fractionation after cellulose adsorption, the ^{125}I-bLH immunoreactivity was very low at the maximum radioactivity of the second peak but began to increase concomitantly with a decrease in radioactivity (Fig.3). A very high ratio (20.6) of α-subunit immunoreactivity to ^{125}I-bLH immunoreactivity was found for fraction no. 3. Thereafter the ratio continuously decreased and reached a value of 1.5 in fraction no. 22, which coincided with the highest recorded ^{125}I-bLH immunoreactivity (Fig. 3).

The changes in β-subunit immunoreactivity of the tested fractions were parallel to those in ^{125}I-bLH immunoreactivity (data not shown). Overall, it was found that the first peak of radioactivity after HPLC fractionation, either with or without cellulose adsorption consisted of material with low binding ability to the bLH antiserum (6.9%±0.5, n = 3 and 13.0%±1.0, n=4, respectively) and high binding ability the oLHα antiserum

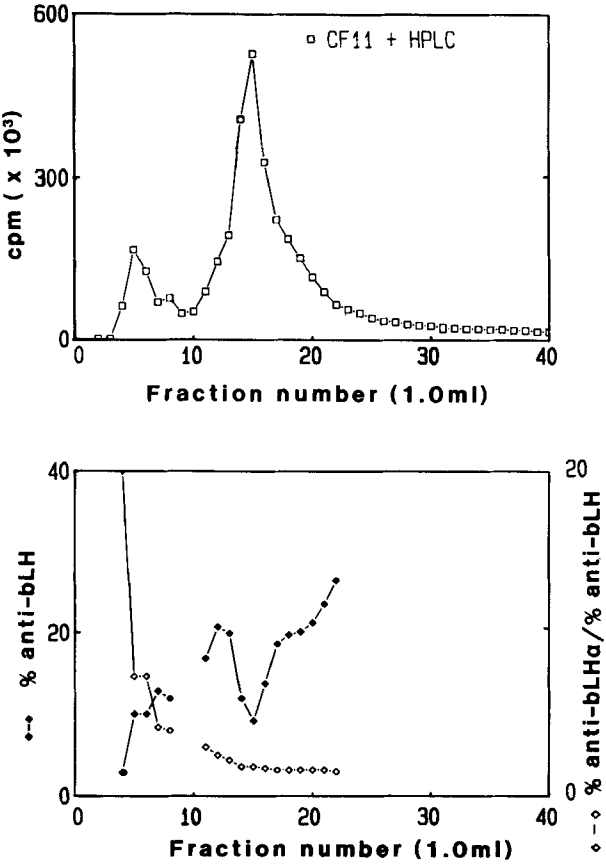

FIGURE 3. Anion exchange HPLC of radioiodinated bovine LH (LER-1716-2) with cellulose adsorption on Whatman CF11 prior to application onto a DEAE column (see Fig. 1) - radioactivity profile (□); bLH immunoreactivity expressed as binding to bLH antiserum (♦) and ratio of α-subunit immunoreactivity (expressed as binding to oLHα antiserum) to bLH immunoreactivity (◇).

(51.0%±2.7, n=3 and 35.2%±3.6, n = 4, respectively). Hence the average ratio of α-subunit immunoreactivity to ^{125}I-bLH immunoreactivity in the first radioactive peak was 7.4±0.1 with and 2.7±0.2 without cellulose adsorption ($P<0.001$).

In a preliminary study the fractions derived from the first radioactive peak after HPLC chromatography of ^{125}I-bLH-LER-1716-2 were used as tracers in a radioimmunoassay and compared with the control tracer (routine purification of ^{125}I-bLH on cellulose CF11). The radioimmunoassay with the former tracer resulted in significantly lower mean plasma LH levels in the GnRH-treated cow no. 1 as compared with the levels obtained with control tracer (3.1 vs. 4.7 μg/l; $P<0.05$).

Radioimmunoassays utilizing different tracers of ^{125}I-bLH-LER-1716-2 were then applied to measure plasma LH levels in cow no. 2 before and after GnRH injection (Figs. 4 and 5). All tracers were tested within one week after iodination, but bLH was stored for either 7 (Fig. 4) or 26 (Fig. 5) weeks before iodination. As can be seen from Figures 4 and 5 the measured LH levels both before and after GnRH administration were substantially influenced by the tracer preparation used in the radioimmunoassay. Tracers made up of fractions from the first radioactive peak after HPLC, with (CF11 + HPLC) or without (HPLC) previous cellulose adsorption, yielded mean concentrations of LH significantly ($P<0.05$) lower than those obtained with the control tracer (CF11) (e.g. 5.7 vs. 8.2 μg/l; 4.6 vs. 8.2 μg/l). The bLH

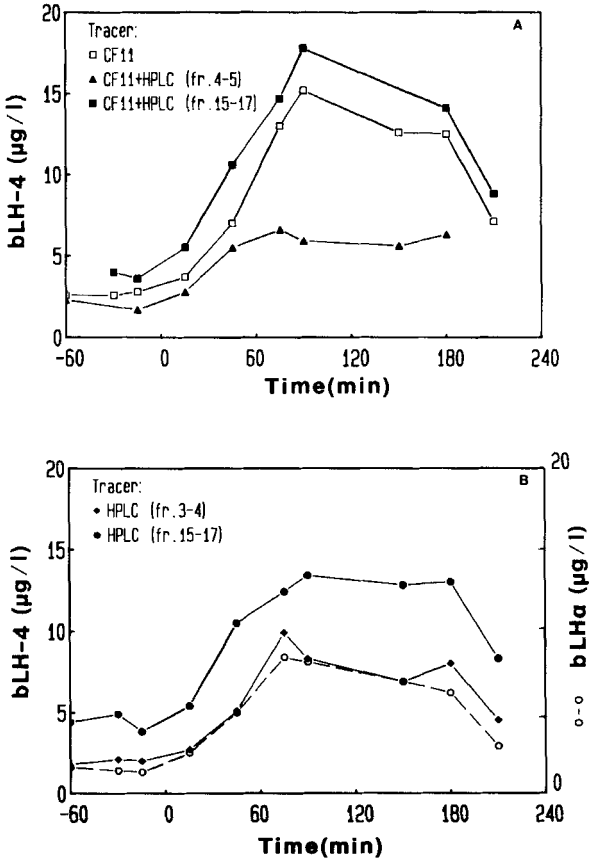

FIGURE 4. Peripheral blood plasma concentrations of bLH and bLHα (o) in cow no. 2 before and after i.v. administration (at time 0) of 100 µg GnRH. Bovine LH levels were measured by radioimmunoassays utilizing different ^{125}I-bLH-LER-1716-2 tracers: A) control tracer purified by cellulose adsorption only (□), by cellulose adsorption + HPLC (fractions no. 4-5: ▲, fractions no. 15-17: ■); and B) purified by HPLC only (fractions no. 3-4: ♦, fractions no. 15-17: ●).
Note: bLH was stored for 7 weeks before iodination and was tested within one week after iodination.

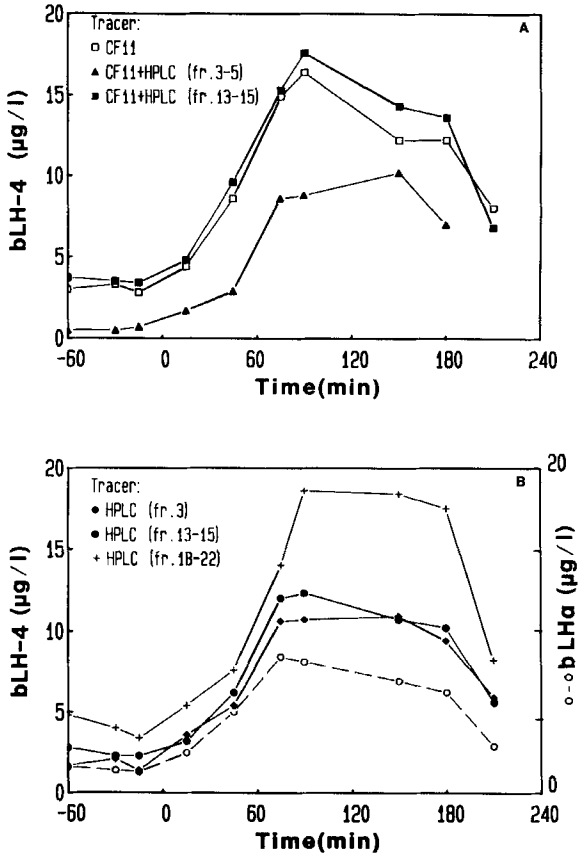

FIGURE 5. Peripheral blood plasma concentration of bLH and bLHα (o) in cow no. 2 before and after i.v. administration (at time 0) of 100 µg GnRH. Bovine LH levels were measured by radioimmunoassays utilizing different ^{125}I-bLH-LER-1716-2 tracers: A) control tracer purified by cellulose adsorption only (□), by cellulose adsorption + HPLC (fractions no. 3-5: ▲, fractions no. 13-15: ■); and B) purified by HPLC only (fraction no. 3: ♦, fracions no. 13-15: ●, fractions no. 18-22: +). Note. bLH was stored for 26 weeks before iodination and was tested within one week after iodination.

TABLE 1

Average (± SEM) plasma LH levels (μg/l) in two bovine samples assayed using five different plasma dilutions (from 100 μl to 6.25 μl).

	Treatment of tracer	Plasma sample no. 1	Plasma sample no. 2
^{125}I-bLH-LER-1716-2	CF11	16.3±1.2^{a}	23.0±1.4^{a}
	HPLC (1st peak)	8.6±0.7^{c}	12.8±1.6^{d}
	HPLC (2nd peak)	7.2±0.6^{c}	14.9±0.4^{dc}
	CF11+HPLC (2nd peak)	16.1±2.0^{a}	19.7±1.8^{ab}
^{125}I-bLH-I-1	CF11	12.2±0.7^{b}	17.7±0.6^{bc}
	HPLC (1st peak)	9.6±1.3^{bc}	15.9±1.7^{dc}
	HPLC (2nd peak)	6.9±0.3^{c}	12.9±0.6^{d}
	CF11+HPLC (2nd peak)	8.0±0.6^{c}	12.4±0.6^{d}

a,b,c,d Means within the same column with different superscripts differ ($P<0.05$)

levels obtained using these tracers from the first radioactive peak were similar to the levels of the bLHα which averaged 4.4 μg/l (Figs. 4B and 5B).

As seen in Table 1 the use of ^{125}I-LH-LER-1716-2, obtained either by cellulose adsorption (CF11) or cellulose adsorption and

HPLC fractionation (CF11+HPLC), led to significantly higher concentrations of bLH than the use of the corresponding fractions of tracer ^{125}I-bLH-I-1.

The difference between the two radioiodinated standard preparations disappeared in radioimmunoassays using HPLC tracers without previous cellulose adsorption and derived either from the first or second peak of radioactivity. The tracer from the first HPLC peak of ^{125}I-bLH-I-1 did not result in significantly lower LH concentrations as did the corresponding tracer from ^{125}I-bLH-LER-1716-2 when compared with the respective CF11 tracers (Table 1).

Overall, bLH concentrations were lower when measured by radioimmunoassay utilizing control ^{125}I-bLH-I-1 than when measured by control ^{125}I-bLH-LER-1716-2 ($Y = 0.17 + 0.75X$) despite a high and significant correlation ($r = 0.99$, $n = 25$, $P<0.001$). For tracers obtained from fractions of the second radioactive peak after HPLC with cellulose adsorption, the difference between the two radioiodinated standard preparations (i.e. bLH-I-1 vs. bLH-LER-1716-2) was even larger ($Y = 1.18 + 0.60X$, $r = 0.87$, $n = 25$).

DISCUSSION

Our results reveal that ^{125}I-bLH-LER-1716-2 and ^{125}I-bLH-I-1 are heterogenous when fractionated by means of anion exchange

HPLC. The chromatographic profile obtained resembles the profile of hLH reported by Stockell Hartree et al. (12). These authors found that about 11% of the hLH was not adsorbed by the anion exchange column (Mono Q) and that the lowest and highest receptor binding activity appeared in the fractions derived from the ascending and descending part, respectively, of the second broad peak. The pH used in their study (pH 7.8) was, however, higher than that used in the present study (pH 6.5). Josic et al. (24) suggested that anion exchange column (i.e. Mono Q) is very sensitive to the pH of the mobile phase and under slightly acidic conditions (e.g. pH 6.5) it is a suitable tool for protein separation. Additionally, Scopes (25) reported that due to the Donnan effect, the pH in the microenvironment of the matrix was usually about 1 unit higher than that in the surrounding buffer. Accordingly, a pH of 6.5 applied here probably increased to about 7.5 in the matrix of the column.

Stanton et al. (26) found that anion exchange chromatography at pH 9.0 yielded different components from crude bTSH preparation but not from pure ^{125}I-bTSH. In contrast, recent results from gel permeation and HPLC by the same group (27,28) indicate that pure ^{125}I-bTSH preparation obtained by mild lactoperoxidase radioiodination exists as the multiple tracer components. The differences between the chromatographic pattern of unlabelled bLH-4 (18) and ^{125}I-bLH-LER-1716-2 as well as ^{125}I-bLH-I-1 (present study) when subjected to the same

chromatographic procedure suggest that differences exist between bLH preparations - i.e., those for biological studies vs. those for iodination, or between unlabelled and labelled hormones. It is tempting to assume that the former alternative is more probable, particularly in the light of the findings of Takai et al. (29,30), who concluded that the different profiles of bioactivity and radioactivity depend more on variability as a function of the hormone preparation subjected to radioiodination, than on the iodination per se. In addition, Keel and Grotjan (31) reported that three highly purified oLH preparations differed in the percentage of acidic and basic forms. Even by means of gel filtration on Sephadex G-100 there was a slight difference in the elution volume between unlabelled and radioiodinated hLH (14,32). In contrast, Sharp and Pierce (33) found that bLH radioiodinated twice was indistinguishable from native bLH after rechromatography on Sephadex G-100 and polyacrylamide gel electrophoresis. However, some of the radioactivity represented either free α-subunit or a small population of dissociated subunits.

The significant increase in the ratio of α-subunit immunoreactivity to ^{125}I-bLH immunoreactivity after cellulose fractionation accords with the findings of Marana et al. (34). These authors concluded that a large amount of ^{125}I-hFSH was formed by fractionation on cellulose. However, this process did not occur in the case of ^{125}I-hLH (15) and ^{125}I-cynomolgus LH

(35). Moreover, Khan et al. (35) reported that cellulose adsorption was an effective method for purifying radioiodinated cynomolgus LH.

In conclusion, the anion exchange HPLC purification of radioligand in combination with a test for immunoreactivity may be particularly advantageous in studies of LH microheterogeneity, especially for those investigating the cause of discrepancies between concentrations obtained by different radioimmunoassay procedures (e.g. 36). Further, the importance of appropriate purification of hFSH radioligand to improve sensitivity and specificity of both radioimmunoassay and receptor assay has recently been reported (37).

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