Domestic Animal Endocrinology 48 (2014) 145–157

Contents lists available at ScienceDirect

Domestic Animal Endocrinology

journal homepage: www.domesticanimalendo.com

# A novel monoclonal antibody-based enzyme-linked immunosorbent assay to determine luteinizing hormone in bovine plasma

V. Borromeo [a,*], A. Berrini [a], F. De Grandi [a], F. Cremonesi [b], N. Fiandanese [a], P. Pocar [a], C. Secchi [a]

[a] Dipartimento di Scienze Veterinarie e Sanità Pubblica, Università degli Studi, Milano, Italy
[b] Dipartimento di Scienze Veterinarie per la Salute, la Produzione Animale e la Sicurezza Alimentare, Università degli Studi, Milano, Italy

ARTICLE INFO

*Article history:*
Received 24 January 2014
Received in revised form 25 March 2014
Accepted 30 March 2014

*Keywords:*
Luteinizing hormone
Bovine
Monoclonal antibody
Immunoaffinity chromatography
ELISA

ABSTRACT

The development of a novel enzyme-linked immunosorbent assay (ELISA) for determining luteinizing hormone (LH) in bovine plasma is described. Anti-bovine LH (bLH) monoclonal antibodies (mAbs) were produced and characterized. One mAb recognizing the bLH β subunit was used for immunoaffinity purification of substantial amounts of biologically active bLH from pituitary glands. The purified bLH in combination with 2 anti-bLH β subunit mAbs was used to develop a sandwich ELISA, which satisfied all the criteria required to investigate LH secretory patterns in the bovine species. The ELISA standard curve was linear over the range 0.05 to 2.5 ng/mL, and the assay proved suitable for measuring bLH in plasma without any prior treatment of samples. Cross-reactivity and recovery tests confirmed the specificity of the method. The intra- and inter-assay coefficients of variation ranged between 3.41% and 9.40%, and 9.29% and 15.84%, respectively. The analytical specificity of the method was validated in vivo by provocative tests for LH in heifers, using the LH releasing peptide gonadotropin-releasing hormone. In conclusion, the adoption of mAbs for this ELISA for coating the wells and labeling, combined with the easy one-step production of reference bLH, ensures long-term continuity in large-scale measurements of LH in the bovine species.



## 1. Introduction

Luteinizing hormone (LH) is one of the pituitary glycoprotein hormone family, together with follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH). These are all heterodimers composed of 2 noncovalently linked subunits, designated α and β. Within a given animal species the α subunit of each of these glycoprotein hormones has identical amino acid sequences, whereas the β subunits are unique, and determine the hormone biological and immunologic specificity [1–3]. LH is secreted by the pituitary gland in a pulsatile manner in response to gonadotropin-releasing hormone (GnRH) released by the hypothalamus, and is required for the regulation of essential mammalian reproductive processes such as gametogenesis, ovulation, and luteotrophy [4].

The measurement of LH plasma concentrations has played a major role in advancing our knowledge of bovine reproductive physiology, leading to rationalization of a wide range of normal, treatment-induced, and pathogenic phenomena in female and male cattle. Numerous immunoassays have been developed to quantify bovine LH (bLH) in the peripheral circulation. The majority are based on antibodies and standards provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) or the United States Department of

* Corresponding author. Tel.: +39 02 50318125; fax: +39 02 50318123.
*E-mail address:* vitaliano.borromeo@unimi.it (V. Borromeo).

0739-7240/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.domaniend.2014.03.004

Agriculture (USDA) through the National Hormones and Pituitary Program (NHPP, Baltimore, MD) [5–18]. Other assays used in-house produced anti-bLH antibodies [7,19–23]. Only a few bLH immunoassay kits and small amounts of bLH and anti-bLH antisera can be purchased on the market, limiting their widespread and standardized application.

A major problem with the development of a validated LH immunoassay in the bovine species is the availability of pure bLH. The only source of bLH as immunogen and/or standard is the pituitary gland, since no corresponding recombinant protein is available. Biologically active bLH has been obtained from Chinese hamster ovary cells [24,25], but the process did not reach the efficiency required for large-scale production. The use of recombinant human LH is hampered by the fact that anti-bLH antibodies, even with high interspecific cross-reactivity, bind with very low affinity to human LH [19,26]. Furthermore, because LH, TSH, and FSH have similar structures and overlapping charge, their purification by methods based on acid–base separation is problematic and often the LH preparations recovered are contaminated with other hormones.

We previously reported the successful application of an anti-bovine FSH monoclonal antibody (mAb) for the immunoaffinity purification of substantial amounts of pure bovine FSH (bFSH) from the pituitary glands [27]. To our knowledge, no immunoaffinity chromatography purification of bLH has been reported.

The aims of the present study were: (1) to produce a panel of anti-bLH mAb by immunizing with standard bLH obtained from the USDA; (2) to select mAbs suitable for immunoaffinity chromatography purification of bLH from pituitary glands; (3) to combine the mAbs and the purified bLH to develop a enzyme-linked immunosorbent assay (ELISA) for bLH detection; and (4) to test the ability of this assay to monitor bLH plasma levels in adult animals in response to exogenous GnRH.

## 2. Material and methods

### 2.1. Reagents

Bovine LH (USDA-bLH-B-6), FSH (USDA-bFSH-I-2), TSH (USDA-b-TSH-I-1), ovine LH (USDA-oLH-I-2), used as reference standards, were kindly provided by Dr A.F. Parlow through the USDA Animal Hormone Program (Germoplasm and Gamete Physiology Laboratory, Beltsville, MD and Agricultural Research Center, Beltsville, MD, USDA). Human chorionic gonadotropin (hCG, Vetecor 2000 U.I.) was obtained from Serono S.p.A. (Rome, Italy). Horseradish peroxidase (HRP, RZ approximately 3.0), peroxidase-conjugated rabbit anti-mouse immunoglobulins, biotinamidocaproate-N-hydroxysuccinimide ester, avidin (labeled with type IV peroxidase), bovine serum albumin, suitable as diluents in ELISA, and o-phenylenediamine dihydrochloride were purchased from Sigma (Sigma-Aldrich, Milan, Italy). HCl 6 N sequencing grade and high performance liquid chromatography gradient grade acetonitrile were from Merck (Darmstadt, Germany). ELISA microtiter plates were from NUNC (Roskilde, Denmark). All other reagents were of analytical grade.

### 2.2. Monoclonal antibodies

Monoclonal antibodies were produced as previously described [28] using USDA-bLH-B-6 as immunogen. The protocol was approved by the Animal Experimentation Ethics Committee of the State University of Milan. In brief, BALB/c mice were immunized by 2 intramuscular injections of 50 and 25 μg of bLH in 150-μL saline solution containing 12 μg AbISCO-100 adjuvant (ISCONOVA AB, Uppsala, Sweden) with a 3-week interval. They then received a booster intramuscular injection of 25 μg of bLH in saline-AbISCO-adjuvant 4 days before fusion. Splenic lymphocytes were fused with P3X63Ag8U.11 myeloma cells using poly(-ethylene glycol) 1450 (PEG solution 50%, Hybri Max, SIGMA) according to a standard procedure [29]. Hybridomas were screened for their ability to bind bLH in enzyme immunoassay, using a direct ELISA, as previously described [28]. Positive hybridomas were also screened for binding to bFSH and bTSH from USDA. Briefly, 96-well plates were coated with the hormone (0.1 μg/well). After incubation and washing, serial dilutions of hybridoma culture medium or purified mAbs were added. The binding of mAbs to the adsorbed hormone was revealed with HRP-conjugated rabbit anti-mouse immunoglobulins. The antibody class of mAbs was determined by enzyme immunoassay using the mouse-hybridoma subtyping kit (Life Technology, Italy).

Positive clones, producing antibodies belonging to the IgG class, were subcloned by limiting dilution and expanded in culture to obtain antibodies. Monoclonal antibodies were precipitated from the culture supernatants with 50% ammonium sulfate solution, dialyzed against 10 mM phosphate buffer, 0.148 M NaCl, pH 7.2 (PBS) and purified by affinity chromatography on HiTrap r-Protein A columns (GE Healthcare Life Science, Italy). The mAb dissociation constant (Kd) was measured as previously described [28].

According to their characteristics, 3 mAbs (N6H7, N3G8, and D2H1) were selected for further studies. Samples of each (0.5 mg) were conjugated with HRP using the periodate method [30]. mAb N3G8 (0.5 mg) was conjugated with biotin [31], fractionated and stored at −80°C.

### 2.3. Epitope mapping

Bovine LH antigenic epitopes were mapped in relation to one another by a labeled antibody competition test [32]. Serial dilutions of unlabeled mAb were added to bLH-coated plates (0.05 μg/well) to displace a mAb-HRP from the immobilized hormone. The labeled mAb was used at a dilution giving absorbance of about 1.0 in the absence of the competing mAb ($B_0$) under the conditions used for the assay. The percentage of competition was established on the basis of the $EC_{50}$ calculated from each curve [28]. We took the $EC_{50}$ obtained using the same mAb as competing and labeled mAb as 100%.

### 2.4. Immunoaffinity purification of bLH

Ten milligrams of protein A purified mAb N6H7 were coupled to a HiTrap NHS-activated HP column (1 mL; GE Healthcare Life Science, Italy) according to the manufacturer's directions. The derivatized column was stored in PBS.