Turk J Vet Anim Sci
24 (2000) 163–168

Development of an Enzyme Immunoassay for the Determination of Ovine Growth Hormone in Plasma

Behiç SERPEK, Seyfullah HALİLOĞLU
Department of Biochemistry, Faculty of Veterinary Medicine, University of Selçuk, 42031, Konya-TURKEY

Received: 30.07.1999

Abstract: A competitive enzyme immunoassay for the determination of ovine growth hormone (oGH) was developed. The assay is based on (anti-rabbit IgG) goat IgG-coated microtitreplates, anti-oGH from rabbits, biotinyl-oGH and streptavidin-horseradish peroxidase. The assay is done directly with 40 µl of plasma and the calibration graph (90% relative binding at 0.38 ± 0.02 ng ml^{-1} and 50% relative binding at 3.80 ± 0.18 ng ml^{-1}) is prepared in plasma without measurable endogenous oGH. Recovery in different plasma samples with added oGH amounted to 96.78 - 100.03 %, and all variabilities were < 10.44. The assay shows no significant binding of oFSH, oLH, oTSH and oPRL. The assay detects high and low plasma oGH levels within the physiological variation as well as changes in plasma oGH after stimulation with growth hormone releasing factor. Hence, it offers a reliable alternative radioimmunoassay. However, a reference method providing evidence of the identity of exogenous oGH will be required for proving illegal treatment with oGH and for forensic purposes.

Key Words: Enzymatic methods, plasma, ovine growth hormone.

Koyun Kan Plazması Büyüme Hormonu Düzeylerinin Enzim İmmuno Assay Yöntemiyle Belirlenmesi

Özet: Bu çalışma, koyun plazma büyüme hormonu düzeylerinin belirlenmesinde kullanılabilecek bir enzim immuno assay yönteminin geliştirilmesi amacıyla yapılmıştır. Yöntem, keçi IgG anti-tavşan IgG'si ile kaplanmış mikrotitre plaklarında, tavşan IgG anti oGH, biotinle işaretlenmiş oGH ve streptavidin-horseradish peroksidaz kullanılarak yapılmıştır. Assay 40 µl plazma örneklerinin doğrudan mikrotitre plaklarının yuvalarına verilmesiyle yürütülmüş ve standart çözeltiler ölçülmeyecek düzeyde oGH içeren plazma örneklerinde hazırlanmıştır. Assayde 90% ve 50% relatif bağlanma 0.38 ± 0.02 ng ml^{-1} ve 3.80 ± 0.18 ng ml^{-1} bulunurken, farklı plazma örneklerine ilave edilen oGH'nın farklı konsantrasyonlarının yeniden bulunmaları 96.78-100.03%, bu değerler arasındaki varyasyonların 10.44'ten düşük olduğu gözlenmiştir. Assayin oFSH, oLH, oTSH ve oPRL ile çapraz reaksiyonlarının önemli olmadığı, yöntemin growth releasing faktörle stimülasyondan sonra oluşan değişimler de dahil olmak üzere plazma oGH düzeylerinin bilinmesinin gerekli olduğu hallerde güvenle kullanılabilecek alternatif bir yöntem olduğu kanısına varılmıştır. Ancak illegal eksojen oGH kullanımlarının belirlenebilmesi için eksojen oGH'ya özgün bir yönteme de gereksinim duyulmaktadır.

Anahtar Sözcükler: Enzimatik metotlar, plazma, koyun büyüme hormonu.

Introduction

Growing is a complex event controlled by a hormonal system called the somatotrophin line functioning with a number of hormones. Growth hormone (GH), insulin, ketocholamines, thyroid gland hormones, glycocorticoids and steroids are endocrine controllers which are first in the somatotrophin line (1). The somatotrophin line starts from the hyphothalamus to reach peripheral target organs via hypophyses and the liver. This somatotrophic line is mainly controlled by GH (2). GH is a protein hormone built by 191 amino acids (3). The sequence of GH has species specific differences but closely related species show the same physiological activity.

It has been found that an impaired somatotrophic line has a negative effect on growth performance (4, 5). As a result, GH, Growth Hormone Releasing Factor (GRF), and IGF-I have been used for stimulating growth performance (4-6). It has also been thought that there could be a correlation between growth performance and plasma GH level, which could be used to evaluate breeding and production capacity. In addition, it has been found that the basal GH level is higher in species with a high breeding capacity than in species with a lower breeding capacity (7,8).

The important effect of GH on living organisms is that plasma levels can be used to identify the growth

performance of organisms. Furthermore, observations on the basal plasma GH level will provide identification of exogenous GH supplies in organisms. Generally, Radio Immuno Assay (RIA) methods have been used for this purpose, and recently Enzyme Immuno Assay (EIA) methods have been used for detection (9,10). In this study, a simple and optimal EIA method was developed for the identification of sheep plasma GH level.

Materials and Methods

Preparation of biotinyl - oGH Label

500 μg of biotinyl-ε-amino caproic acid N-hydroxysuccinimid ester (Biotin-X-NHS; Sigma, Diesenhofen, Germany) dissolved in 250 μl N-N-dimethyl formamide (Aldrich, 22., 705.6) (Reagent A) was used for coupling with 25 μg of ovine growth hormone (NIDDK-oGH-1-4, kindly provided by the National Hormone and Pituitary Program, Baltimore, Maryland, USA) dissolved in 500 μl 0.01 M $NaHCO_3$ (Reagent B). 100 μl Reagent A and 500 μl Reagent B were mixed and incubated at room temparature with gentle stirring for 4 h. The reaction was stopped by adding 0.5 mg glycine (Serva, Heidelberg, Germany) in 200 μl of 50 mM carbonate buffer (pH 9.98). After overnight incubation at 4 °C, 2 mg of bovine serum albumin (BSA) (Lot Number 05081, Serva - 11930) in 1 ml carbonate buffer was added to the mixture and dialysed three times against carbonate buffer at 0 °C overnight. After dialysis, the conjugate was diluted with assay buffer to achieve a final concentration of 2.5 μg ml^{-1}, pipetted into aliquots and stored at -20 oC until used.

oGH Antibody

NIDDK-oGH-2 rabbit antiserum, which has the same affinity as NIDDK oGH-1-4, was obtained from the National Hormone and Pituitary Program (NIDKK, Baltimore, Maryland, USA). This antiserum undergoes no cross reactions with other hormones (oFSH, oLH, oTSH and oPRL).

Goat IgG- anti rabbit IgG

A Goat IgG- anti rabbit IgG was kindly supplied by Dr Klobosa (Institut für Tierzucht und Tierverhalten, Mariense, Germany).

Enzyme Immunoassay Procedure

First coating: The first coating was achieved by adding 1 μg of goat antirabbit IgG dissolved in 100 μl of coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6) per well of the microtitre plate (Nunc, Rosleilde, Denmark, No. 439454). The plates were subsequently incubated for either 2 h at room temperature or overnight at 0 °C with slight shaking. After incubation, the plates were decanted.

Second Coating: To achieve saturation of the remaining binding sites, 350 μl of assay buffer (40 mM Na_2HPO_4, 150 mM NaCl, pH 7.2, containing 0.1% BSA) was added per well and incubated for 15-45 min at room temperature before subsequent decantation. Non-specific binding (NSB) ranged from 0.081 to 0.125. Coated plates can be stored at -20 °C for up to 6 months.

Washing: Prior to use in the assay, the coated plates were washed twice with 375 μl of 0.05 % Tween 80 per well.

Assay Protocol: Lyophilized pituitary ovine GH (NIDDK-oGH-15, National Hormone and Pituitary Program, Baltimore, Maryland, USA) was dissolved in 0.03 M $NaHCO_3$ in 0.15 M NaCl, pH 10.8, at a concentration of 100 ng ml^{-1}. Standard solution (0.200 - 50 ng ml^{-1} pituitary oGH) was prepared by serial dilution (steps of 1 : 2) in plasma with undetectable levels of endogenous ovine GH (≤ 0.2 ng ml^{-1}). Volumes of standards and unknown plasma samples were diluted in duplicate in 100 μl per well antibody containing (1 : 500.000) assay buffer using a diluter dispenser (Hamilton Microlab 1000). The plates were then incubated with constant gentle shaking for 48 h at 4 °C and subsequently decanted. After decantation, 0.15 ng per well of biotinylated oGH in 100 μl assay buffer was added and incubated for 2 h at 4 °C. Following another decantation, 20 ng per well of streptavidin peroxidase (Böhringer, Mannheim, Germany) in 100 μl of assay buffer was added and incubated for 15 min at 4 °C until decantation.

Substrate Reaction: The plates were washed four times with 375 μl of 0.05 % Tween-80, after which 150 μl of substrates A and B (1 : 1) were added per well [Substrate A, 1.0 g l^{-1}, hydrogen peroxide urea, 18 g l^{-1} $Na_2HPO_4.2H_2O$, 10.3 g l^{-1} citric acid monohydrate, 0.1 ml l^{-1} kathon (Rohm and Haas, Frankfurt, Germany), pH 5.0; Substrate B, 500 mg l^{-1} Tetramethylbenzidine, 40 ml l^{-1} dimethylsülfoxide, 10.3 g l^{-1} citric acid monohydrate, pH 2.4]. The reaction was stopped with 50 μl of 2 M H_2SO_4 per well and the extinction was measured at 450 nm using an eight-channel microtitration plate photometer (Biotek EL 311).

Results

Titration of ovine biotinyl-oGH and anti oGH serum

In order to determine the optimum concentration of antibody NIDDK-oGH-2 and biotinyl-oGH-1-4, for the assay a two-dimensional titre determination was performed. An antibody dilution of 1:10.000-1:640.000 and biotinyl-oGH-1-4 concentrations of 0.15-10 ng per well were tested. The minimum concentrations of antibody and biotinyl-oGH-1-4 concentrations of antibody and biotinyl-oGH-1-4 accepted as suitable for the assay were taken as those sufficient to achieve on optical density (OD_{450}) of ca.1. For the actual assay, with an incubation period of 2 days, an antibody dilution of 1:500.000 and a biotinyl-oGH-1-4 concentration of 0.15 ng per well were chosen.

Assay validation

The influence of incubation temperature on assay kinetics: to determine the optimum incubation time and temperature, microtitre plates were incubated for either 4 or 24 h at 22 °C or for either 24 or 48 h at 4 °C (Figure 1). The relative binding was less in the case of incubation for 4 h and 24 h at 4 °C than with 24 h at 22 °C and 48 h at 4 °C.

Despite the good relative binding with incubation for 24 h at 22 °C, the slope of the calibration graph was inferior with low repeatability and higher standard deviations in the duplicate determinations. For this reason, an incubation period of 48 h at 4 °C was chosen as being optimum for the assay.

Assay Sensitivity

To determine the possible interference of plasma with the assay sensitivity, various amounts of plasma (10, 20, 30, 40 µl) were added to the oGH -15 standard dilutions. The interference of the added amounts of plasma with the calibration graph was observed. Because of this, all standard dilutions were subsequently prepared in plasma, which contained unmeasurable amounts of endogenous oGH (≤ 0.38 ± 0.02 ng ml^{-1}). The samples and standards were standardised to a volume of 40 µl per well to guarantee sufficient assay sensitivity (Figure 2). Using standards prepared in plasma, the recovery rates of various concentrations of pit. oGH-15 (3.13, 6.25, 12.50 ng ml^{-1}) added to unknown samples were determined and acceptable results were obtained (Table 1).

At a volume of 40 µl of standards per well the detection limit of the assay (optical density values significantly different from those at 100 % B / B_0) was found to be 0.38 ± 0.02 ng per ml of pit. oGH-15 (Mean ± S.D., n = 19, R.S.D. = 5.34) and 50 % relative binding (B / B_0) occurred at 3.80 ± 0.18 ng ml-1 pit. oGH-15 (Mean ± S.D., n=19, R.S.D. = 4.36).

Intra- and Inter-assay Variation

Intra- and inter-assay variation was determined using plasma samples with various pit-oGH concentrations (Mean ± S.D.) of 3.39 ± 0.12 (n=11) and 13.45 ± 0.42 (n=10) of pit. oGH, resulting in R.S.D.s of 10.44 and 9.84 %. The interassay variation was 10.26 and 5.03

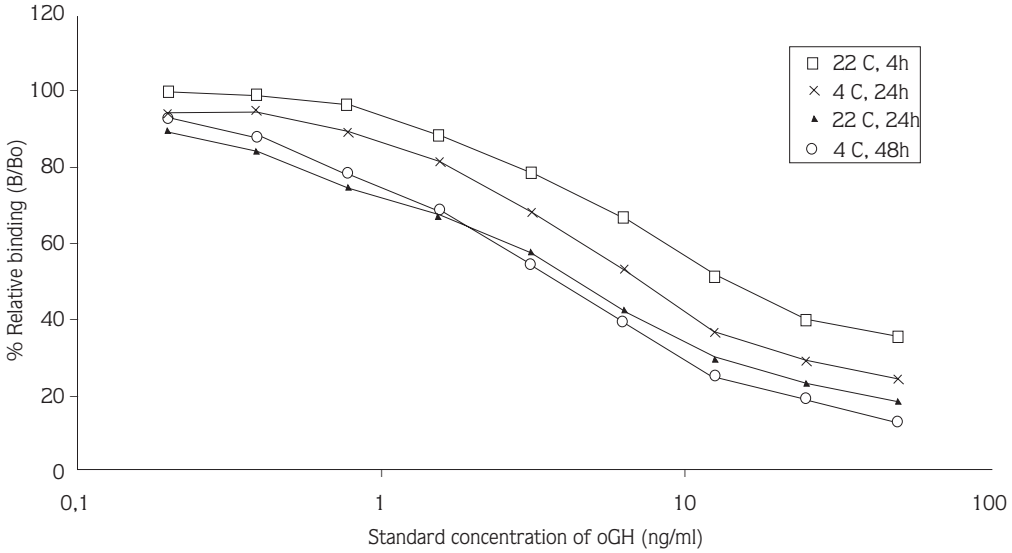

Figure 1. Influence of incubation period and temperature on oGH binding.

Table 1. Recoveries of pituitary-derived GH added to different plasma samples at various concentrations.

Plasma oGH ng/ml	Added Pit oGH-15 ng/ml	Recovery %	Variation %
0.91	3.13	96.78	5.81
0.91	6.25	92.18	7.97
0.91	12.50	100.03	9.84

using plasma samples with concentrations of 6.81 ± 0.11 (n=20) and 12.48 ± 0.11 (n=20) ng ml^{-1} of pit. oGH, respectively.

Cross-reactivity with other hormones

NIDDK-oGH-2 rabbit antiserum, which has the same affinity as NIDDK oGH-1-4, was obtained from the National Hormones and Pituitary Program (NIDKK, Baltimore, Maryland, USA). This antiserum undergoes no cross reactions with other hormones (oFSH, oLH, oTSH and oPRL).

Clinical Validation

Plasma samples were collected every 15 min. from the bleeding vena jugularis of a Merino lamb and an Akkaraman ram lamb by a permanent catheter, and the oGH levels of the samples were analysed as described in materials and methods. Both animals were injected with 8 μl kg^{-1} Human Growth Hormone Releasing Factor (hGRF 1-29, Saxon Biochemicals, Hanover, Germany) and then the samples were collected to observe the responses. The Akkaraman sheep's response to stimulation was smaller and slower than that in the Merino sheep (Figure 3). The Akkaraman lamb exhibited a GH response 20 min after stimulation while the Merino lamb responded only 10 min after stimulation and this implied that the Akkaraman has a slow and weak response to GH stimulation.

Discussion

There has been no information published concerning plasma oGH level analysis using EIA methods. The method developed in this study is the first EIA method to analyse sheep plasma oGH levels. To date, sheep plasma GH levels have been analysed using RIA methods. EIA analyses have been preferred to RIA, because EIA requires less expensive equipment, does not create hazards for the environment or human health and has long-life tracers. Recently, EIA methods have become popular in hormonal detection and EIA methods have been developed for bovine LH, porcine GH (pGH), progesterone, and ostradiol 17β (9-11). It has been reported that EIA methods are optimised by coating the plate wells with secondary antibody using goat or sheep anti rabbit IgG (12). The amount of required hormone specific antibody for EIA has been found to be 10 times less than for RIA and 1000 times less than for sandwich ELISA (12).

In this study, 1:500,000 optimum dilutions were used for double side titration analyses after coating the plate wells with secondary antibody. This dilution was much lower than that used by Pell and Asthon (13), 1: 500 dilutions for oGH analyses with RIA. This shows that the same amount of antiserum can be used for more EIA analyses.

The incubation period of oGH assays for RIA is 3 to 5 days (2,14) while the incubation period for EIA is 48 h (2 days). This indicates that EIA analysis methods are faster than RIA.

The required quantity of the plasma samples (40 μl) in EIA does not affect the sensitivity of the analyses (Figure 2). It has been reported that 0.5 ng ml^{-1} is the minimal amount of RIA detection (14,15). In this study, it has been shown that the GH detection level of EIA sensitivity is not less than that of RIA methods. The minimum detection limit for the EIA method was found to be 0.38 ± 0.02 ng ml^{-1} and the relative binding value was 3.8 ± 0.18 ng ml^{-1}.

Interassay and intraassay variation tests have been developed for different animal species in order to assess the repeatability of GH – RIA methods (15-18). The difference between intraassay variations have been reported as 5% to 13% and interassay variations as 11% to 20% (15,16,18). In this project, intraassay variations were found to be 10.44% and 9.84%, using two different plasma samples containing different levels of GH (3.39 ± 0.12 and 13.45 ± 0.42 ng ml^{-1}). The difference between interassay variations were 10.26 % and 5.03% in two different plasma samples (6.18 ± 0.11 and 12.48 ± 0.11). These findings are in agreement with RIA variations.

The reliability of the developed EIA was checked using a recovery test. 3.13, 6.25 and 12.50 ng ml^{-1} of oGH were added to samples and recorvery rates of 97.6 %, 92.18 % and 100.03 % respectively were obtained. The variations were found to be 5.8 %, 7.97 % and 9.84 % respectively for the recovery tests (Table 1).

The utility of the developed EIA was checked using an Akkaraman lamb and a Merino lamb by injecting

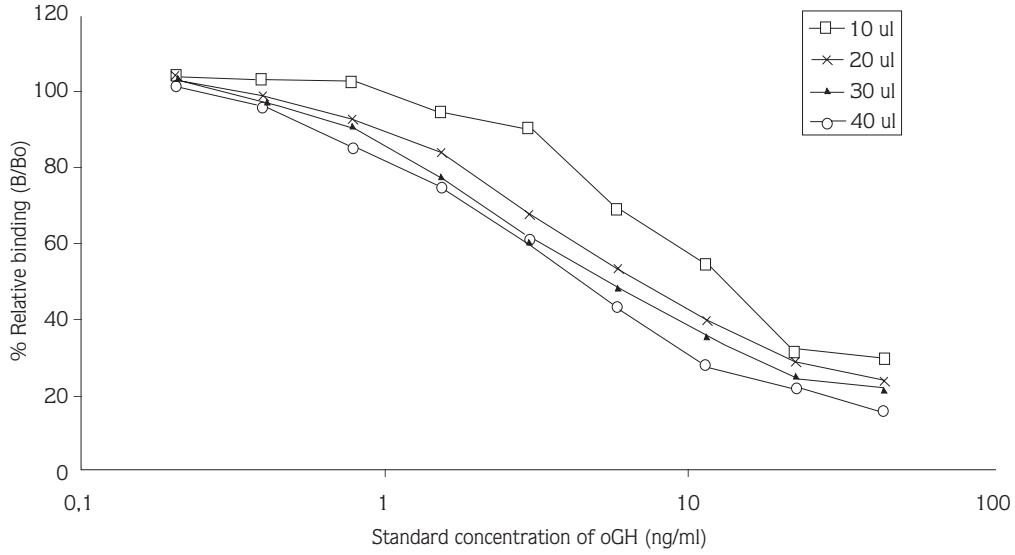

Figure 2. Influence of volume of standard on oGH binding.

intravenously 8 μg kg^{-1} body weight hGRF 1-29 after determining the basal level of oGH. It was found that EIA can detect the response of GRF stimulations at basal levels (Figure 3). This study shows that EIA is a reliable and sensitive detection method for plasma oGH level.

Acknowledgement

We would like to thank the Alexander von Humbauld Trust for its support. We are grateful to TUBITAK for financial support in the purchase of oGH and oGH-2 the from National Hormone and Pituitary Program (NIDDK, Baltimore, Maryland, USA).

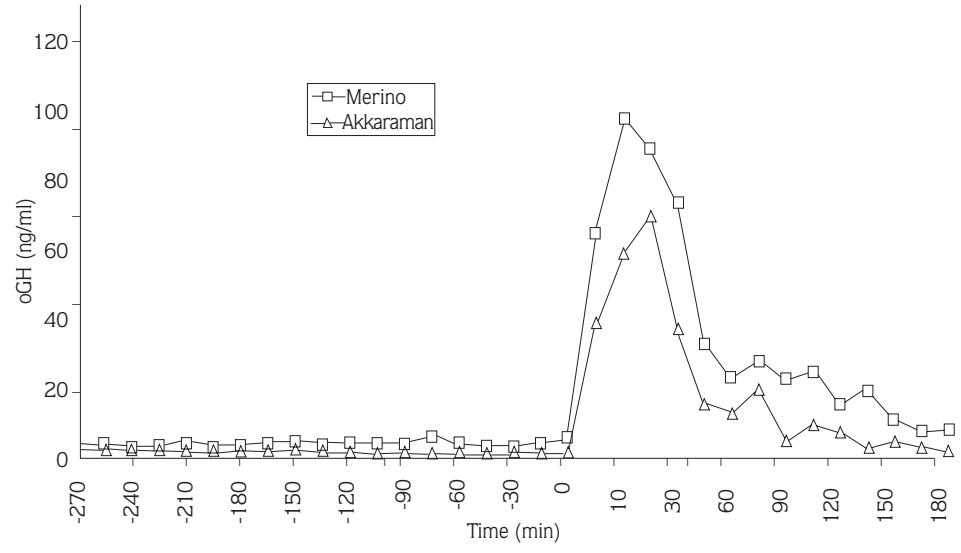

Figure 3. Plasma oGH levels in Merino and Akkaraman lamb before and after stimulation with Grf (8μg kg^{-1} body weight).

References

1. Hoffmann, B.: Biologische Grundlagen des Wachstums, in BST Symposium, 3.-4.- November, Landbauforschung Völkenrode, Sonderheft 88, Braunschweig, Germany, 1987; 159-180.
2. Richter, T.: Analytische Untersuchungen zur Wachstums-hormon-Somatomedin-Achse beim Wachsenden Rind, Inaugural Dissertation, die Tieraerztlichen Fakultaet der Ludwig-Maximilians-Universitaet München, Inaugural Dissertation, 1991.
3. Bauman, G: Growth hormone heterogeneity: genes, isohormones, variants and binding proteins, Endocrine Reviews, 1991; 12, (4): 424-449.
4. Raben, M.S.: Treatment of a pituitary dwarf with human hormone, J. Clin. Endocrinol., 1958; 18, 901.
5. Butenant, O.: Humanes Wachstumshormon: Wachstumsförderung beim Kind,BST-Symposium, 3.-4.-November, Lamdbauforschung Völkenrode, Sonderheft 88, Braunschweig, Germany, 1987; 72-77.
6. Cheek, D.B., Brasel, J.A., Elliot, D. and Scott, R.: Muscle cell size and number in normal children and dwarfs before and after treatment, Bull. Johns Hopk. Hosp., 1966; 119, 46.
7. Beeby, J.M. Haresighn, W. and. Swan, H: Endogenous hormone and metabolite concentrations in different breeds of beef on two systems of production, Anim. Prod., 1988; 47, 231-244.
8. Keller, D.G. Smith, V.G., Coiter, G.H. and King, G.J.: Serum growth hormone concentration in Hereford and Angus calves: effect of breed, sire, age of dam and diet, Canad. J. Sci., 1979; 59, 367-373.
9. Mutoyaba, B.U., Meyer, H.H.D., Schams, D. and Schallenberger, E.: Development of a sensitive enzyme immunoassay for LH determination in bovine plasma using the streptavidin-biotin technique, Acta Endocrinol. (Copenhagen) 1990; 122, 227-232.
10. Serpek, B., Elsaesser, F. and Meyer, H.H.D.: Development of an enzyme immunoassay for the determination of porcine growth hormone in plasma, Anal. Chim. Acta, 1993; 275, 183-187.
11. Brambilla, G.F. Berrini, A., Borromeo, V. and Secchi, C.: Determination of growth hormones in the plasma of cows treated with recombinant bovine growth hormone, Anal. Chim. Acta, 1993; 275, 173-176.
12. Meyer, H.H.D.: In Proceedings of International Symposium on the Use of Nuclear Techniques in Studies of Animal Production and Health in Different Environments, IAEA, 293, 3, FAO/Int. Atomic Energy Agency, Vienna, 1986, 255.
13. Pell, J.M. and Aston, R.: Active immunisation with a synthetic peptide region of growth hormone: increased lean tissue growth, J. Endocr., 1991;131, (1): 1- 4.
14. Bauer, M.: Wachstumshormon- und IGF-I Sekretion beim chronisch katheterisierten Schweinefetus, Inaugural. Dissertation, Institut für Tierzucht und Haustiergenetik der Georg-August-Universitat Göttingen, 1992.
15. Suttie, J.M. Lord, E.A. Gluckmann, P.D. Fennesy, P.F. and Littlejohn, R.P.: Genetically lean and fat sheep differ in their growth hormone response to growth hormone-releasing factor, Dom. Anim. Endocr., 1991; 8, (2): 323-329.
16. Beermann, D.H., Hogue, D.E., Fishell, V.K., Aronica, S., Dickson H.N. and Schricker, B.R.: Exogenous human growth hormone releasing factor and ovine somatotropin improve growth performance and composition of gain in lambs, J. Anim. Sci., 1990; 68, (12); 4122-4133.
17. Onischuk, L.A. and Kennedy, A.D.: Growth hormone, insulin, prolactin and glucose levels in ewe and ram lambs during normal and compensatory growth, Dom. Anim. Endocr., 1990; 7, (3): 365-381.
18. Van Kessel, A.G., Korchinski, R.S., Hampton, C.H. and Laarveld, B.: Effects of immunisation against somatostatin in the pregnant ewe on growth and endocrine status of the neonatal lambs, Domestic Animal Endocr., 1990; 7, (2): 217-228.