AMPLIFIED ENZYMEIMMUNOASSAY OF CLENBUTEROL

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INTRODUCTION

The ß-agonist drug clenbuterol is frequently used in veterinary fields for animal husbandry because it favors the development of muscle mass and decreases fat deposition. Residues of clenbuterol (CL) in meat pose health risk if ingested and Veterinary Laboratories recommend that clenbuterol residues are checked. Improvement of detection levels of residues is necessary to fulfill higher quality standards in meat production. We have developed two sensitive enzyme-immunoassays for the quantification of clenbuterol (CL). In both methods the working concentration range for CL was 10 to 0.01 ng/ml . The detection levels were ten times lower than commercial EIA and RIA kits.

MATERIALS AND METHODS.

Clenbuterol conjugated to bovine serum albumin (CL-BSA) and clenbuterol-alkaline phosphatase (CL-AP) were synthesized as described by Yamamoto e Iwata (1). A commercial antiserum anticlenbuterol (AbCL), raised in rabbits (C.E.R. Marloie, Belgium) was used.

Design A. Antibody tritration.
Wells (Polisorp Nunc, Denmark) were coated with 16 ng of CL-BSA in 100mM carbonate buffer pH=9, by overnight incubation, followed by blocking with Tris buffer saline solution with 0.02% Tween-20 (TBS-T) containing 20mg/ml of low fat powdered milk. The AbCL was incubated separately with samples or standard solutions of CL for 1 hour and transferred to wells for another 30 minutes. The AbCL bound to the solid phase was allowed to react sequentially with biotinylated antirabbit IgG antiserum (Biot-AB) and Extravidin-Alkaline Phosphatase (E.AP), for one hour each. The enzymatic activity in the resulting complex was measured by hydrolysis of p-nitrophenylphosphate 1mg/ml in 1M diethanolamine buffer, pH= 9,8 $MgCl_2$ 5mM. The reaction was stopped with one volume of 1M NaOH, and the absorbances were measured at 405nm. All incubations were carried out at room temperature in TBS-T with 0,1% BSA.

Design B. Competitive assay.
The immunoglobulin fraction of an antiserum against rabbit IgG was partially denatured by incubation in HCl pH= 2,5 100 mM NaCl for 10 minutes, diluted in TBS and used for coating of wells overnight. Residual active sites were blocked with 5% BSA in TBS. Samples or standards and tracer CL-AP, were incubated with the antibody AbCL separately in TBS-T 0,1% BSA, at 4°C overnight. The mixture was incubated in the anti-IgG-wells for 2 hours, and the immobilized enzyme from the immune complex was measured by the ELISA amplification system (Gibco BRL) (2).

Sample preparation.
Bovine eyes were obtained from a local slaughterhouse. Choroid tissue was isolated and supplemented with known amounts of CL according to Sauer & Anderson (3), and then extracted using C_{18}-separation columns (LiChrolut RP-18. Merck). The recovery was calculated using [^{3}H]-clenbuterol (C.E.R. Marloie, Belgium).

RESULTS AND DISCUSSION

The calibrations graphs obtained in both designs, are shown in figures 1 and 2. For the antibody titration method the graph was linear over the range of 1000 to 10 pg, when the absorbance was plotted against the log of CL concentration. Samples of bovine choroid tissue containing different amounts of CL were quantitated by this method. From the results depicted in figure 1 and table 1, we can deduct that the matrix effect is negligible, since the concentrations obtained in the assay were proportional to the amount of CL added.

In design A, the improvement in the color signal is obtained by increasing the number of enzyme molecules per immune complex (Biotin-Avidin amplification). In design B, AP activity in immune complex can be amplified using substrate recycling system.

The methods described have better detectability than commercial kits, with comparable sensitivity. These characteristics would make these methods suitable for the measurement of very low levels of CL.

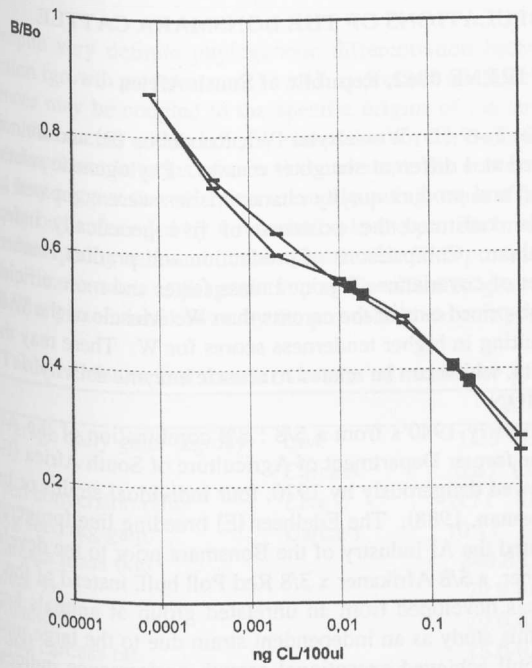

Figure 1. Calibration curve for Design A.
(■) Samples in duplicate.

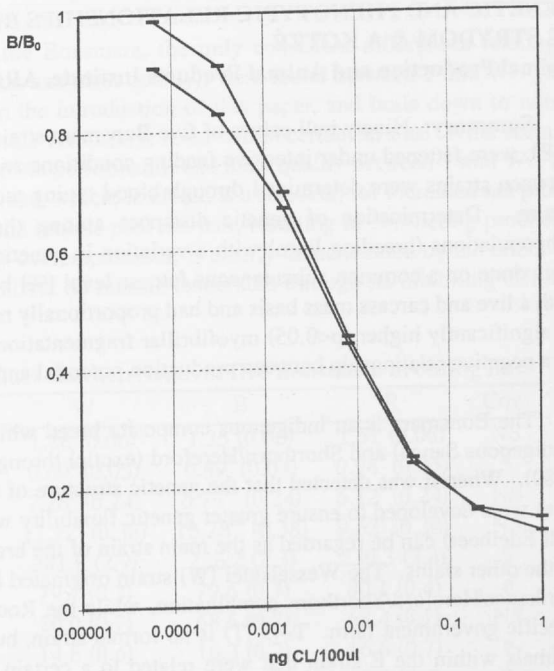

Figure 2. Calibration curve for Design B.

Table 1. Recovery and measurement of clenbuterol in bovine choroid tissue.

Added (ng/g of tissue)	Recovery (%)	Measured (ng/g of tissue)	Expected (ng/g of tissue)
10 ng/g	63.5	(7.6 , 5.1)	0.63
1 ng/g	47.5	(0.40 , 0..56)	0.47

Bibliography.

1. Yamamoto, I. and K. Iwata. 1982. Enzyme immunoassay for clenbuterol, and β2-adrenergic stimulants. *J. Immunoassay* 3:155-171.

2. Lövgren, U., K. Kronkvist, G. Johansson, and L.-E. Edholm. 1994. Enzyme amplified immunoassay for steroids in biosamples at low picomolar concentrations. *Anal. Chim. Acta* 288:227-235.

3. Sauer, M. J. and S. P. L. Anderson. 1994. *In vitro* and *in vivo* studies of drug residue accumulation in pigmented tissues. *Analyst* 119:2553-2556.

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