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CHEMILUMINESCENCE IMMUNOASSAY FOR THE DETECTION AND QUANTIFICATION OF METHYLTESTOSTERONE RESIDUES IN MUSCLE TISSUE

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SUMMARY

Chemiluminescence as a detection method for immunoassays has succesfully been applied to the measurement of methyltestosterone (MT) residues in muscle tissue. The sample is digested enzymatically, extracted with diethyl ether and purified on a Lipidex-5000 column. An optional clean-up utilized disposable C_{18} columns. As the luminescent label the N-(4-aminobutyl)-N-ethylisoluminol conjugate of MT was used. The antiserum was raised in a rabbit against MT-3-carboxymethyloxime-bovine serum albumin. The detection limit of the assay was 14 $\pm$ 7 pg ($n = 13$), with a limit of quantification in muscle tissue of 0.125 ppb.

INTRODUCTION

Several anabolic steroids are reported to be used, mostly illegally, in livestock breeding. Diethylstilbestrol (DES) is probably the most intensively used substance. Because of its potential carcinogenic properties, many efforts have been made to detect it in samples of animal origin [1–9] and have made a large contribution to a dramatic decrease in the administration of this compound. As a direct consequence other products have gained importance, two of these being 19-nortestosterone (19-NT) and methyltestosterone (MT). Methods for the detection and/or quantification have been published for 19-NT [10,11]. The analytical procedures for sample preparation [10] and detection [11] have now successfully been extended to MT. The original method could even be shortened and simplified as high-performance liquid chromatographic (HPLC) purification was no longer necessary.

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EXPERIMENTAL

Reagents and instrumentation

All solvents were of analytical-reagent grade and were used as received, except diethyl ether, which was shaken with 25% iron(II) sulphate solution and washed three times with distilled water and subsequently distilled. 17-Methyltestosterone (17β-hydroxy-17-methylandrost-4-en-3-one) was obtained from Serva (Heidelberg, F.R.G.) and subtilisin A (dialysed and lyophilized, 29.3 Anson U/g) from Novo Industri (Copenhagen, Denmark). Lipidex-5000 was obtained from Packard Instruments (Brussels, Belgium) and Bond Elut C_{18} columns (1 ml) from Analytichem International (Harbor City, CA, U.S.A.).

The chemiluminescent label, i.e., the N-(4-aminobutyl)-N-ethylisoluminol conjugate of MT, was kindly supplied by Dr. G. Zomer and Dr. R.W. Stephany of the National Institute for Public Health and Environmental Hygiene (Bilthoven, The Netherlands). The antiserum raised in a rabbit against MT-3-carboxymethyloxime–bovine serum albumin was purchased from the Laboratoire d'Hormonologie (Marloie, Belgium).

Luminescence was measured in a 3M Biocounter M 2010 A (Lumac, Schaesberg, The Netherlands). Quantitative results were calculated with a Tulip personal computer (Compudata, Malines, Belgium) after logit/log transformation.

Isolation of methyltestosterone from tissue samples

A 1.0-g sample of minced meat was deproteinated enzymatically with 1 mg of subtilisin A in 4 ml of 0.1 *M* Tris solution (pH 9.5) for at least 2 h at 60°C in a water-bath. Overnight digestion does not affect the quality of the digested material. The liquid suspension was chilled to room temperature and extracted twice with 5-ml portions of diethyl ether. The combined layers were evaporated to dryness in a stream of nitrogen at 35°C, yielding the crude extract.

Column chromatography on Lipidex-5000 and Bond Elut C_{18}

The crude extract was taken up in 0.2 ml of hexane–dichloromethane (85:15, v/v) and applied on the top of a small glass column (145 mm × 6 mm I.D.), plugged at the bottom with glass-wool and filled with 6 cm of Lipidex-5000 swollen and conditioned with the same solvent. The column was then eluted with the same mixture. The first 1.5 ml were discarded; the next 5 ml were collected and evaporated to dryness at 35°C in a stream of nitrogen.

For the optional clean-up step, the Bond Elut C_{18} columns were conditioned with 2 ml of methanol and 2 ml of doubly distilled water. The dry extract from the Lipidex-5000 column was taken up in 0.2 ml of methanol, diluted with 4 ml of doubly distilled water and passed on to the column. Washing was carried out with 1 ml of doubly distilled water, 1 ml of methanol–water (55:45) and 0.5 ml of hexane. The analyte was eluted with 0.5 ml of ethyl acetate. After evaporation to dryness at 40°C, the final extract was dissolved in 0.5 ml of phosphate buffer (pH 7, 0.1 *M*), to which 0.05% gelatine was added.

Chemiluminescence immunoassay (CLIA)

Duplicate 200-μl aliquots of the final extract were subjected to CLIA as described for 19-NT by Jansen et al. [11]. A calibration graph covering the range 0–800 pg was established by means of methanolic MT solutions. Concentrations were calculated from the logit/log transformation of the experimental values.

RESULTS AND DISCUSSION

The usefulness of enzymatic digestion of tissue samples has been indicated in the analysis of 19-NT residues [12]. Because the tissue structures visually completely disappeared, a higher release and hence a higher recovery could be expected. This has been proved for benzodiazepines, barbiturates, salicylic acid and other acidic compounds in toxicological analysis [13,14].

Depending on the fat content of the sample, which for sausage meat could be high, a more or less perceptible crude extract was obtained after the diethyl ether extraction. Delipidation by passing the crude extract through a Lipidex-5000 column was partially effective in that, after evaporation of the eluent, a dry extract was obtained. However, for the detection and quantification of nortestosterone residues further purification was required. High blank values resulting in false-positive readings made additional clean-up necessary for which HPLC with automated injection and fraction collection was succesful. The main disadvantage was that after each series of chromatographic runs (six samples plus two controls) the column had to be rinsed in order to eliminate slowly eluting and interfering compounds. This means that a discontinuity of about half a day must be tolerated.

For the detection and quantification of MT residues the additional HPLC procedure was not necessary. This was assessed from readings for blank meat samples obtained from a bona fide breeder. When these samples were digested, extracted and purified according to the procedure described above, and subsequently analysed as such by CLIA signals were read which coincided with the 0-pg standard. This means that there are no substances in the extract which interfere with the light emission of the chemiluminescent phenomenon, in other words, that there is no quenching. Indeed, a decrease in the amount of light emitted is detected as a positive signal, as on the calibration graph the amount of light emitted is inversely proportional to the concentration of the analyte. An example of such a calibration graph is given in Fig. 1. In order to exclude the possibility that foreign substances interfere with the antigen–antibody binding, which is the basis of the immunochemical detection, samples of the same 'certified' blank meat were spiked with known amounts of MT. Recoveries of more than 80% were obtained, indicating that the method does not induce false-negative results.

The reliability, i.e., the possibility that false-positives are detected, is of course basically determined by the quality of the antiserum. Substances that show a limited cross-reactivity for the antibodies, but which sometimes occur at concentrations which may be several orders of magnitude higher than that of the analyte, constitute a real threat to the qualitative and quantitative results. According to the information from the supplier, the antiserum against MT shows a cross-

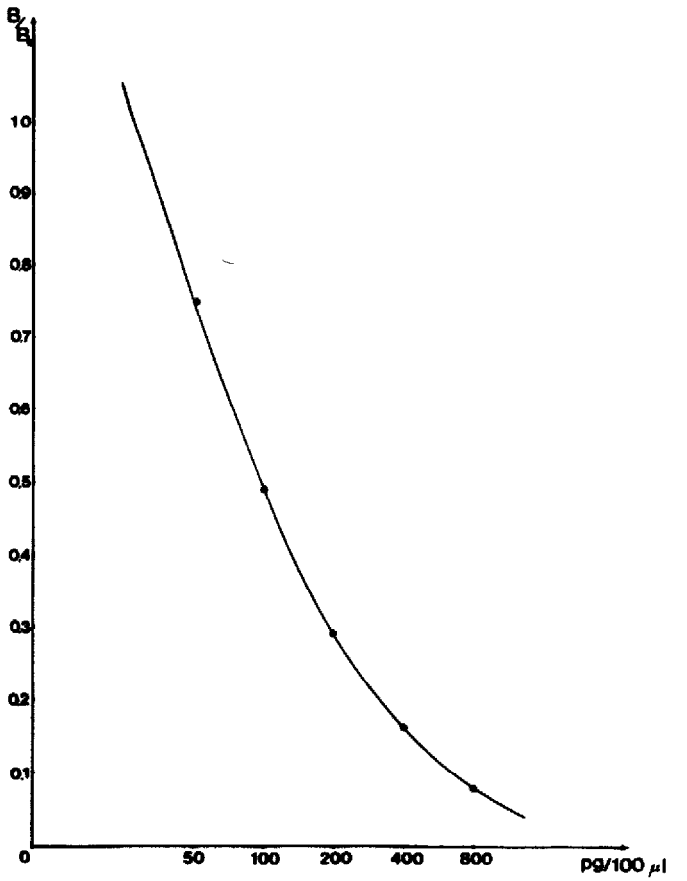

Fig. 1. Typical calibration graph for the MT assay. After logit/log transformation a graph corresponding to the equation $y=a_1x+a_0$ is obtained where $y=\log[(B/B_0)/(1-B/B_0)]$ and $x=\log$(concentration of analyte) (B/B_0=fraction of luminescent label bound in standard or sample tube relative to maximum binding). The average value of a_1 (the slope of the graph) is -0.950 ± 0.124 ($n=13$).

reactivity of 6.5% for testosterone, 2.8% for dihydrotestosterone and 0.8% for nortestosterone. Obviously the cross-reactivity against foreign substances of unknown structure is not reported.

The validation of the results of an immunochemical method can be achieved most efficiently by a technique based on a completely independent analytical principle, such as gas chromatography coupled with mass spectrometry (GC–MS). Work is in progress in this laboratory to develop a GC–MS method for the confirmation of the results of the screening method based on CLIA.

In order to improve the reliability of the CLIA results and whilst awaiting the development of a GC–MS method, an additional clean-up step can be incorporated for the confirmation of positive screening results, consisting of a solid-phase extraction on Bond Elut C_{18}. After this additional purification step, the certified blank samples always show readings which actually coincide with the 0-pg standards.

The limit of detection (LOD) of any immunochemical method is defined as

the minimum amount that can be detected on the calibration graph. It depends on the slope of the graph, which is a measure of sensitivity, but equally on the variance of the 0-pg standard. It can be specified as the amount of analyte that yields a luminescence signal three standard deviations above that of the 0-pg standard. Although the calibration graph is very steep, the limit of detection is affected by the variance of the duplicate readings on the luminometer, which is one of the disadvantages of CLIA compared with, e.g., radioimmunoassay. From the definition mentioned it was calculated that the limit of detection of the CLIA is 14 ± 7 pg ($n = 13$).

The limit of detection should not be mistaken for the limit of quantification (LOQ). As already indicated, blank meat samples, obtained from a bona fide breeder, yield luminescence signals which coincide or almost coincide with the 0-pg standard and which are statistically different from the signals of the lowest standard on the calibration graph, i.e., 50 pg per tube. This means that the apparent or background MT concentration never exceeds 50 pg per tube, which corresponds taking into account the dilution factors, to 125 pg/g of meat, i.e., 0.125 ppb. This value is considered to be the LOQ.

It should be made clear that the LOQ has nothing to do with the limit of decision, which in some countries is employed in the control of the illegal use of anabolics. It is then the fixed official value under which there is no transgression of the regulations and there will be no legal action.

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