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Combined high-performance liquid chromatography and radioimmunoassay for the screening of 19-nortestosterone and methyltestosterone residues in meat samples

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ABSTRACT

A procedure is described for the detection of 17β-19-nortestosterone (17β-19-NT) and 17α-methyltestosterone (17α-MT) in muscle tissue by a combination of high-performance liquid chromatography and radioimmunoassay. The steroids were released from the muscle tissue by enzymic digestion, and the extracts were purified by solid-phase extraction. A meat sample from the retail trade, which was proved to contain 17β-19-NT and 17α-MT residues by gas chromatography–mass spectrometry, and blank meat samples obtained from non-treated experimental animals were purified and the extracts injected into the liquid chromatograph. With an automatic fraction collector, fifteen fractions of 1 ml each were obtained, which after evaporation were subjected to a radioimmunoassay for the steroid concerned. It was demonstrated that none of the fractions from the blank meat samples contains any substance that interfered with the immunochemical detection system. In addition, a good qualitative agreement between the two sets of results was obtained. Although the sample preparation step is rather labour-intensive, the method can be successfully applied as a reliable confirmation method for positive radioimmunoassay screening results in circumstances where no gas chromatography–mass spectrometry facilities are available.

INTRODUCTION

Several anabolic steroids are reported to be used, mostly illegally, in livestock breeding. In particular, 17β-19-nortestosterone (17β-19-NT) and 17α-methyltestosterone (17α-MT) are frequently used in Belgium to fatten cattle. The screening of meat samples for the presence of those two anabolics can be performed by radioimmunoassay (RIA), which is a very sensitive method, but which has the disadvantage of possible cross-reactivities. In order to reduce that possibility, a combined high-performance liquid chromatographic (HPLC) and RIA method has been developed, in which the meat is purified and fractionated so that only the fractions containing 17β-19-NT and 17α-MT are subjected to the RIA procedure. The HPLC–RIA method has already been used to detect estrogens in urine samples [1–3].

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EXPERIMENTAL

Chemicals

Subtilisin A was obtained from Novo Industri (Copenhagen, Denmark). Diethyl ether was from Janssen Chimica (Geel, Belgium). Methanol and water were HPLC grade from Alltech (Deerfield, IL, U.S.A.). Octadeyl (C_{18}) disposable extraction columns were from Analytichem (Harbor City, CA, U.S.A.).

The 19-nortestosterone and methyltestosterone antisera were obtained from Laboratoire d'Hormonologie (Marloie, Belgium). The tracers [6,7-^{3}H]nortestosterone and [1,2-^{3}H]methyltestosterone were from Amersham (Little Chalfont, U.K.).

HPLC equipment

The HPLC equipment included a Pye Unicam pump (Model PU 4011) directed by a controller (Model PU 4030), an automatic injector (Model Gilson 231), an automatic fraction collector (Model Gilson 203) and an UV detector (Model PU 4020) fixed at 254 nm.

The analytical column was a LiChrospher RP 18 (125 mm × 4 mm I.D., 5 μm) from Merck (Darmstadt, Germany) and was protected by a guard column (pellicular reversed phase, 30–50 μm, 75 mm × 2.1 mm I.D., Chrompack Cat. No. 28603, Middelburg, The Netherlands). It was operated at room temperature. The mobile phase was methanol–water (65:35, v/v) and was pumped at a flow-rate of 1 ml/min.

Isolation from 17β-19-NT and 17α-MT from tissue samples

A 1.0-g meat sample was digested enzymically with 1 mg of subtilisin A in 4 ml of 0.1 *M* trishydroxymethylaminomethane solution (pH 9.5) for at least 2 h at 60°C in a water bath. The liquid suspension was cooled to room temperature and extracted twice with 5-ml portions of diethyl ether. The combined layers were evaporated to dryness under a stream of nitrogen at 35°C, yielding the crude extract.

Purification on C_{18} disposable extraction columns

The dry residue was dissolved in 0.1 ml of methanol and 4 ml of water. This solution was applied to a C_{18} disposable column, previously conditioned with 5 ml of methanol and 5 ml of water. The column was washed with 5 ml of water and the steroid residues were eluted with 2 ml of methanol, which was evaporated to dryness under a stream of nitrogen.

Fractionation by HPLC

The dry residue was dissolved in 200 μl of methanol, of which 100 μl were injected into the HPLC instrument. Fractions of 1 ml were collected automatically during 15 min. The fifteen fractions of each sample were evaporated to dryness under a stream of nitrogen.

Radioimmunoassay

The antisera used were raised in rabbits against 19-nortestosterone-17-hemisuccinate–bovine serum albumin (BSA) and methyltestosterone-3-carboxymethyloxime–BSA. 19-[6,7-^{3}H]Nortestosterone and [1,2-^{3}H]methyltestosterone were used as the radioactive labels. Their specific activities were 19 and 60 Ci/mmol, respectively.

The final fractions from HPLC after evaporation were taken up in 1.0 ml of a 0.5 ng/ml gelatine phosphate buffer (pH 7.4, 0.1 *M*). A 300-μl aliquot was subjected to the RIA procedure as described by Evrard *et al.* [4]. Calibration curves covering the range 50–800 pg were established by means of methanolic 17β-19-NT and 17α-MT solutions.

Samples

Five blank meat samples and one meat sample that was found to be positive for 17β-19-NT and 17α-MT by GC–MS were subjected to the procedure.

RESULTS AND DISCUSSION

The usefulness of enzymic digestion of tissue samples has been indicated by a study in which the efficiencies of different enzymes were compared [5], and in the analysis of 17β-19-NT residues [6]. This method yields far higher recovieres than conventional extraction methods, as already proven for benzodiazepines, barbiturates, salicylic acid and other acidic compounds in toxicological analysis [7,8].

After the diethyl ether extraction, the crude extract was passed through a C_{18} disposable extraction column. However, for the detection of 17β-19-NT and 17α-MT a further clean-up was required. Therefore HPLC fractionation was carried out in order to eliminate possible interfering substances and thus false-positive results. Recoveries from both purification on C_{18} and fractionation by HPLC were calculated by means of radioactive tracers and liquid scintillation counting. The results were 97 $\pm$ 4% ($n=5$) and 99 $\pm$ 1% ($n=5$) after C_{18} extraction and 92% ($n=1$) and 99% ($n=1$) after HPLC for 17β-19-NT and 17α-MT, respectively.

A typical chromatogram of a meat sample extract is shown in Fig. 1. The brackets indicate the regions containing 17β-19-NT and 17α-MT. The concentrations normally encountered do not permit visual detection of the 17β-19-NT and 17α-MT peaks. The elution time of the two compounds is determined by injecting 25 ng of the corresponding standards.

Figs. 2 and 3 show the immunochemical responses for 17β-19-NT and 17α-MT, respectively, of the fifteen fractions collected from the positive meat sample. The radioactivity of the bound label in the presence or absence of 17β-19-NT and 17α-MT is expressed as B and B_0 respectively. There was a positive signal for 17β-19-NT in fractions 5, 6 and 7, and for 17α-MT in fractions 5, 6, 7, 8, 9, 10 and 11.

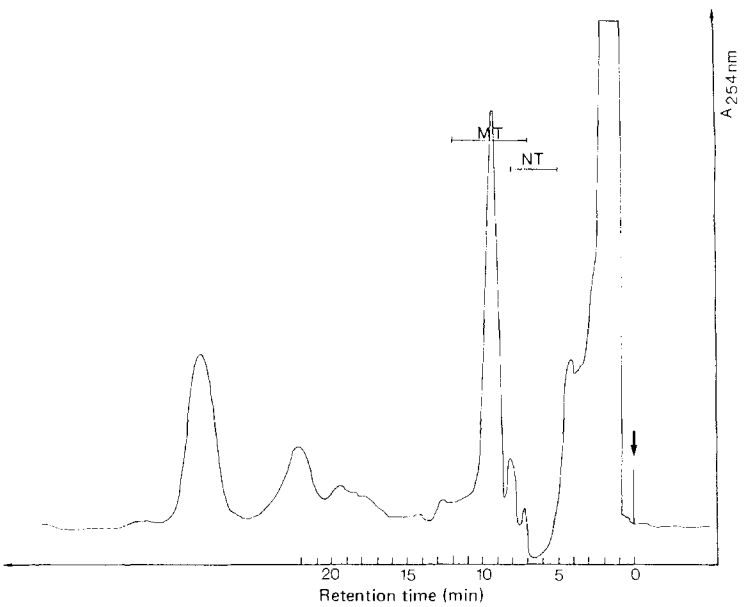

Fig. 1. Chromatogram of a meat sample extract; the brackets indicate the regions containing 17β-19-NT and 17α-MT.

None of the blank meat samples showed a positive signal in any collected fraction. The mean values of the ratio B_0/B were 0.98 ± 0.03 [$n = 15$, coefficient of variation (C.V.) = 3%] for 17β-19-NT and 0.97 ± 0.04 ($n = 15$, C.V. = 4%) for 17α-MT. This proves that almost no cross-reactivity with endogenous compounds occurs.

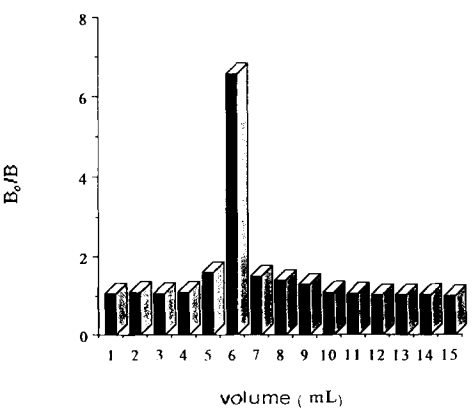

Fig. 2. Immunochemical response for 17β-19-NT of the fifteen collected fractions from the positive meat sample.

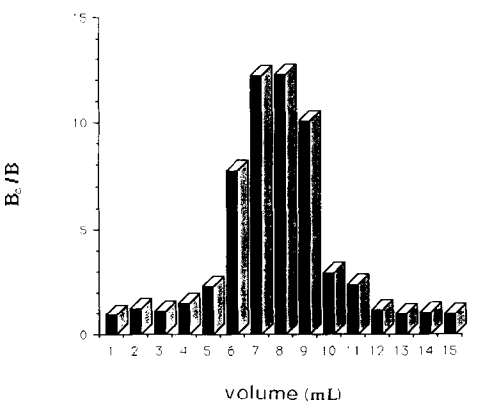

Fig. 3. Immunochemical response for 17α-MT of the fifteen collected fractions from the positive meat sample.

TABLE I

CROSS-REACTIVITIES OF HPLC INTERFERING COMPOUNDS

Compound	*anti*-Nortestosterone antiserum (%)	*anti*-Methyltestosterone antiserum (%)
17α-Testosterone	0.01	0.04
17β-Testosterone	0.03	6.5
17α-Estradiol	0.1	0.1
17β-Estradiol	0.01	0.1

The elution regions of possibly interfering substances such as 17α- and 17β-testosterone and 17α- and 17β-estradiol were 6.5–8 and 5.5–6.5 min, respectively. As they appear in the same fraction that contains 17β-19-NT and 17α-MT, and given their structural relationship with these analytes, possible interferences need to be excluded. Therefore, the cross-reactivities for the two antibodies were determined. The results are shown in Table I. All steroids except 17β-testosterone, showed cross-reactivities of 0.1% or less. The possibility that false-positive results would be obtained becomes very small. Under the HPLC conditions described, the selectivity for 17α-MT is rather limited as the radioimmunochemical response is spread over 7 min (or ml). Gradient elution would probably improve the resolution of the chromatographic system.

It can be concluded that this method can be used as a reliable confirmation method for positive immunoassay screening results in those circumstances when no GC–MS facilities are available. Further research is necessary to improve the method by using gradient elution instead of isocratic elution.

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