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Note

Liquid chromatographic purification of tissue samples prior to chemiluminescence immunoassay of 19-nortestosterone

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Several anabolics are reported to be used mostly illegally in livestock breeding. Almost no reference was made to the androgen 19-nortestosterone (nandrolone) (19-NT) until its intensive use in The Netherlands was reported[1,2]. It seems that nowadays 19-NT is the most frequently used anabolic steroid in cattle fattening in The Netherlands and probably in Belgium as well.

Almost no relevant methods for detection and quantitative assay are available. The multi-residue method of Verbeke[3] for detection of anabolics in urine and in tissues includes 19-NT. Jansen *et al.* have developed an immunoassay for urine, employing either radioactive[4] or chemiluminescent labels[2]. Its combination with high-performance liquid chromatography (HPLC) was shown to be necessary in order to avoid false positive results[5,6].

As far as I know, no methods for detection and quantitative assay in muscle tissue have yet been described. From a practical point of view, screening of urine samples is much easier to perform, however meat is virtually the only sample type which is available from slaughtered animals or the retail trade.

The present paper describes the extraction and clean-up of tissue samples to such a degree of purity that the chemiluminescent immunoassay for 19-NT described by Jansen *et al.*[2] can be successfully applied.

EXPERIMENTAL

Reagents and instrumentation

All solvents were reagent grade and used as such, except diethyl ether which was shaken with 25% ferrous sulphate solution and washed three times with distilled water and subsequently distilled. 19-Nortestosterone was from Serva (F.R.G.). Subtilisin A (dialyzed and lyophilized, 29.3 Anson units/g) was from Novo Industri (Denmark). Lipidex-5000 was obtained from Pharmacia (Sweden).

The chemiluminescent label, *i.e.*, the N-(4-aminobutyl) N-ethylisoluminol conjugate of 19-NT, and the antiserum against 19-NT were kindly supplied by Drs. E. H. J. M. Jansen and R. W. Stephany, Laboratory for Endocrinology, National Institute for Public Health and Environmental Hygiene, Bilthoven, The Netherlands.

The HPLC equipment (Fig. 1) included a Waters pump and an automatic injector Wisp 710 B (Waters). UV absorption was monitored at 240 nm with a vari-
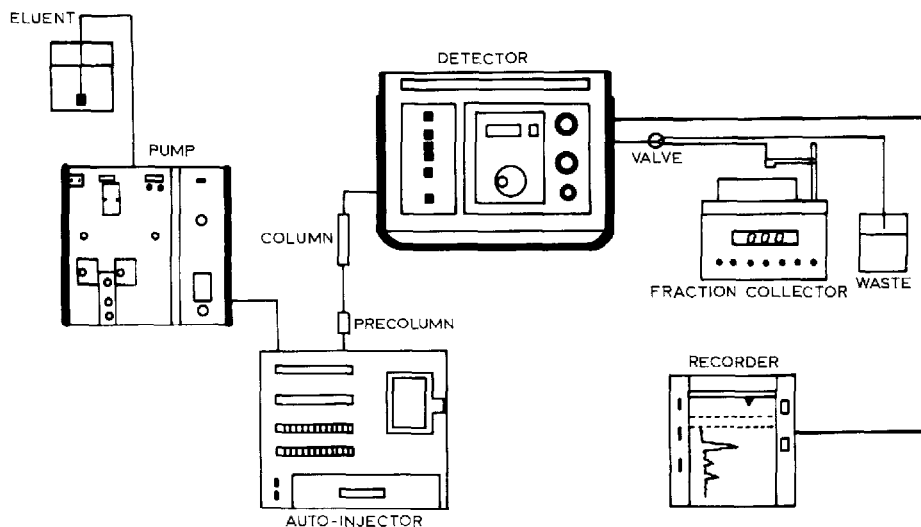
Fig. 1. Diagram of the instrumentation used in the HPLC purification step.

able wavelength detector (Pye Unicam, Model 4020). The HPLC column (250 mm × 4.6 mm) was packed with LiChrosorb RP-18 (5 μm, Merck) and was protected by a guard column (75 mm × 2.1 mm; Chrompack, Cat. No. 28603); it was operated at ambient room temperature. The eluent was methanol–water (65:35, v/v) at a flow-rate of 1.5 ml/min. The fraction collecting system consisted of a LKB Model 2212 Helirac fraction collector.

Luminescence was measured in a Lumac 3M Biocounter M 2010 A. Quantitative results were calculated with a Tulip PC after logit/log transformation.

Isolation of 19-NT 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 digestate. The liquid suspension was chilled to room temperature and extracted twice with 5-ml portions of diethylether. The combined layers were evaporated to dryness in a stream of nitrogen at 35°C, yielding the crude extract.

Column chromatography on Lipidex-5000

The crude extract was taken up in 0.2 ml of hexane–dichloromethane (85:15, v/v) and applied on top of a small glass column (145 mm × 6 mm), 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 2.5 ml were discarded; the next 5 ml were collected and evaporated to dryness at 35°C in a stream of nitrogen.

HPLC

The residue obtained after evaporation of the Lipidex-5000 fraction was taken up in 100 μl of methanol and transferred to a vial suited for the Wisp. A diagram of

the instrumentation is given in Fig. 1. The eluate between 5.30 and 7.15 min is automatically collected. The total elution time per sample was 15 min. The 19-NT-containing fraction was evaporated to dryness at 45°C under nitrogen, yielding the final extract. The latter was taken up in 500 μl of buffer pH 7.0.

Chemiluminescence immunoassay (CLIA)

Duplicate 200-μl aliquots of the final extract were subjected to CLIA as described by Jansen et al.[2]. A standard curve covering the range 62.5–2000 pg was established by means of methanolic 19-NT solutions. Concentrations were calculated from the logit/log transformation of the experimental values.

RESULTS AND DISCUSSION

Enzymatic digestion or deproteination of tissue samples has been reported to be succesful in toxicological analyses for benzodiazepines, barbiturates, salicylic acid and other acidic compounds[7,8]. This approach yielded far higher recoveries than conventional extraction methods and the subsequent chromatographic analysis allowed rapid separation and detection of nanogram quantities of drugs without elaborate preliminary purification. It was not possible in the case of 19-NT to compare recoveries with those obtained by other methods, due to a lack of such methods. No radioactive tracer was available to measure them by radioactivity counting. Yet the enzymatic deproteination and extraction offers the considerable advantage that a large number of samples can be left to digest overnight.

Although the cornerstone of the purification step is the HPLC fractionation, the preliminary Lipidex column remains indispensable in view of the lifetime of the guard column and even of the analytical column. When fatty meat or sausage meat is extracted, a considerable amount of lipids is present in the crude extract and as much as possible should be removed. Six crude extracts can easily be handled simultaneously by one analyst in about half an hour, excluding column preparation time.

The HPLC phase allows automation of injection and of fraction collection, so that this step can be carried out overnight after the first analysis day. One cycle, including the preparation of the injection, the injection itself and the chromatographic analysis, is completed in about 16 min.

Fig. 2 shows a typical chromatogram of a certified blank meat sample (left) and of a meat sample of an experimentally treated animal (right). The concentration of 19-NT in the latter, as assayed by CLIA, was 54 ppb*. Highly positive samples show a peak with a retention time of *ca.* 6.15 min, corresponding to the retention time of 19-NT. The collection window, 1.45 min broad, indicated by the shaded zone on the chromatogram, is determined from the absorption at 240 nm of a 19-NT standard (100 ng). Special attention should be paid to possible contamination of the injection system by this relatively massive dosage.

According to information from the supplier, the antiserum against 19-NT shows a cross-reactivity of 5.1% for 17β-trenbolone and of 3.6% for 17β-testoster-

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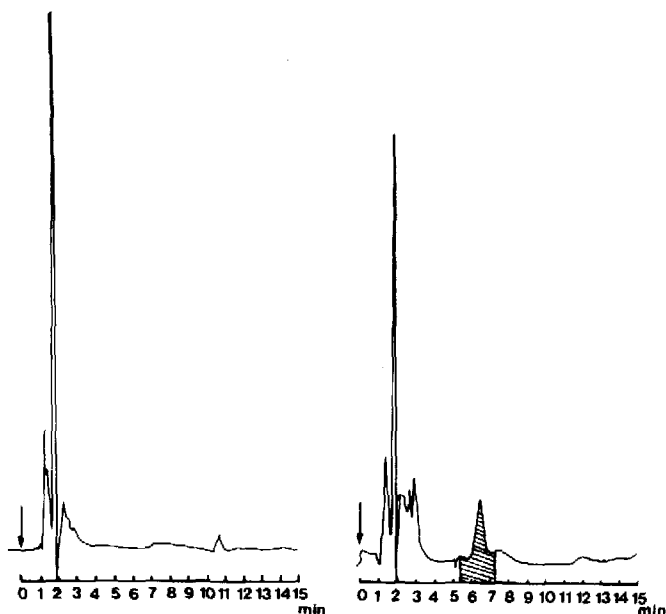* Throughout the article the American billion (10^9) is meant.

Fig. 2. Typical chromatogram of a blank meat sample (left) and of a sample from an animal experimentally treated with 19-NT (right).

one. High concentrations of the latter naturally occurring but also exogenously applied steroid could cause positive responses. It is obvious that the HPLC separation largely contributes to an increased specificity of the method which cannot be guaranteed completely by use of the antiserum alone.

It was not possible, as already mentioned, to standardize the developed method against existing methods or against a radioimmunoassay (RIA) method in particular. The main objective however of the method is to detect unwanted and illegal 19-NT residues in meat for human consumption. This means that, for forensic investigations, priority should be given to reliability rather than to accuracy. If the method is used for screening of 19-NT residues, it should be sensitive enough to detect all positive samples and it should be specific enough to avoid false positives. In other words, if the method is correctly applied, all negative samples should show negative signals and all positive samples should give a positive signal. In practice it is not always easy to distinguish between positive and negative responses. When certified blank meat samples, obtained from a bona fide breeder, are analysed, luminescence signals are obtained which clearly differ from the zero standard, showing the effect of quenching. Chemiluminescence suffers from the same disadvantages as β-scintillation counting[9]. The luminescence of a series of blank samples was significantly different ($p \leqslant 0.001$) from that of the 125-pg standard but not from that of the 62.5-pg standard. It was concluded that blank meat samples contain a certain amount of "background 19-NT" which is indistinguishable from 62.5 pg of 19-NT. This means that the limit of quantification is 125 pg per reagent tube or, expressed in terms of the concentration in muscle tissue, ca. 0.6 ppb. Therefore samples showing an apparent 19-NT content of less than 125 pg per reagent tube or 0.6 ppb in the tissue are considered negative.

The limit of quantification should not be mistaken for the limit of detection. The latter is the minimum quantity of analyte which can be detected on the standard curve. It depends on the slope of that curve but equally on the variance of the 0-pg standard. The limit of detection, which for the present method is 12 ± 8 pg ($n=8$), is determined as the quantity of analyte which yields a luminescence signal three standard deviations above (under) that of the 0-pg standard.

In each analysis series a certified negative sample and a spiked (1-ppb) sample are used as controls. Results are rejected when the negative control has an apparent 19-NT concentration higher than 0.6 ppb or when the positive control shows unexpected results, *i.e.*, an abnormally high response or a response lower than 0.6 ppb.

Despite the inherent but limited specificity of the antiserum and the additional specificity from the HPLC separation by which a number of possible interferences are excluded, immunoassays remain screening methods. This means that if the method is correctly applied, negatives may be considered as real negatives but that positives may be caused by nonspecific interferences. In other words, in forensic investigations, a confirmatory test by an independent technique is required. The sensitivity of the immunochemical methods can only be matched by chromatographic methods with specific or selective detection systems. Work is in progress in our laboratory to confirm positive CLIA results by gas chromatography–mass spectrometry. Several derivatives (heptafluorobutyrates, *tert.*-butyldimethylsilyl ethers) show excellent chromatographic and mass spectrometric properties which should enable unambiguous identification of the parent compound in the extract.

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