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Note

Identification of anabolic stilbene derivatives in bovine urine by high-performance liquid chromatography and off-line chemiluminescent immunochemical detection

R. H. VAN DEN BERG, E. H. J. M. JANSEN, G. ZOMER, C. ENKELAAR-WILLEMSEN, R. BOTH-MIEDEMA and R. W. STEPHANY*

National Institute of Public Health and Environmental Hygiene, P.O. Box 1, 3720 BA Bilthoven (The Netherlands)

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Forensic control of the illegal use of anabolics in fattening of cattle and veal calves has been focused in the Netherlands on the group of so-called "stilbenes": diethylstilbestrol (DES), dienestrol (DE) and hexestrol (HEX). Detection and identification methods have been developed, such as radioimmunoassay (RIA) following chromatographic purification of urine extracts on celite[1], on paper[2] and by high-performance liquid chromatography (HPLC)[3]. Reversed-phase HPLC purification and fractionation[4] turned out to be very useful and selective. In addition a specific HPLC immunogram procedure was developed[5], which consisted of fractionation of urine extracts by isocratic reversed-phase HPLC followed by off-line radioimmunochemical detection. As judged against final confirmation by combined HPLC–gas chromatography (GC)–mass spectrometry (MS), the combination of HPLC retention time, peak profile in the immunogram and specificity of the antiserum turned out to be also very specific for the identification of DES, DE and HEX in a 3-year control programme[6]. Here the HPLC immunogram procedure is presented with chemical detection by chemiluminescence rather than radiochemical detection.

MATERIALS AND METHODS*

The preparation of the urine extracts, the HPLC immunogram procedure and the HPLC equipment used have been described in detail elsewhere[5].

The chemiluminescent immunoassay (CLIA) for HEX was similar to that described for 19-nortestosterone[7] or 17α-methyltestosterone[8]. The antiserum (code H 152456) used in the assay in a final dilution of 1:15 000, was raised in rabbits by immunization against an immunogene containing as different haptens the carboxypropyl ethers of DES, DE and HEX simultaneously conjugated to bovine serum

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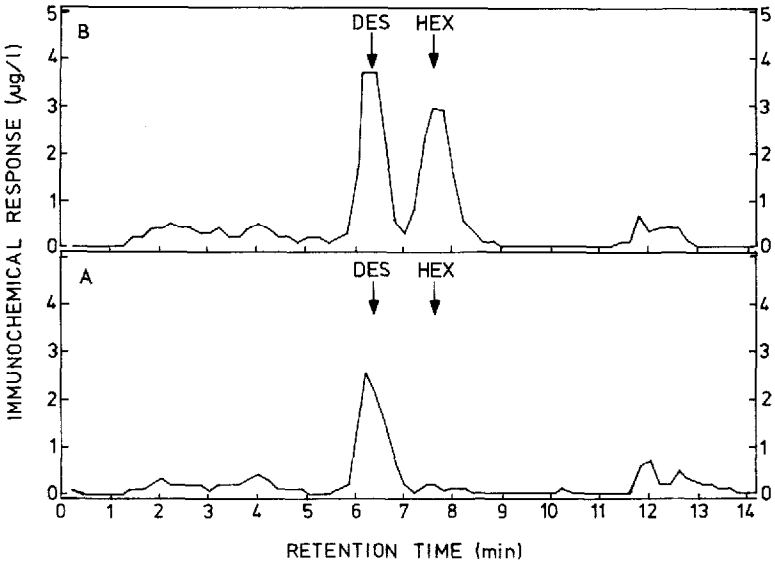

Fig. 1. HPLC immunograms of extracts of bovine urine H 155018 before (A) and after (B) enrichment with 10 ng HEX/ml urine. The retention times of standard *trans*-DES and standard HEX are indicated by arrows. HPLC conditions: stainless-steel column (150 mm × 4.6 mm I.D.), packed with Hypersil ODS (5 µm), eluted isocratically with methanol–water (6:4) for 8 min followed by pure methanol for 2 min both at a flow-rate of 2 ml/min.

albumin (BSA). Chemiluminescent measurements were performed with an adapted Biocounter (Lumac/3M). The synthesis of the chemiluminogenic DES-, DE- and HEX-label and details about the CLIA will be published elsewhere.

RESULTS AND DISCUSSION

Chemiluminescent N-(4-aminobutyl)-N-ethylisoluminal (ABEI) labels were prepared of DES, DE and HEX in order to replace radioactive labels in immunoassays for these anabolic compounds. The ABEI labels were tested in a "total stilbene" assay using antiserum raised against an immunogene consisting of bovine serum albumin (BSA) conjugated with equal amounts of DES-, DE- and HEX-carboxypropyl ether. Only the HEX-ABEI label was suitable for use in a CLIA. However, both DES and DE can also be detected in this HEX CLIA on account of the cross-reactivities of 100% and 16%, respectively. The standard line in the HEX CLIA covers the range from 10 pg (90% relative binding) to 600 pg (10% relative binding) per test tube. An equivalent of 0.67 ml of urine is fractionated by HPLC and subsequently introduced into the CLIA.

As a practical example, Fig. 1 shows two HPLC immunograms of extracts of the urine of a slaughtered bull. The urine sample (H 155018) contained *ca.* 9 µg/l DES-equivalents as determined with celite RIA. The immunochemically responding urinary compound was subsequently identified as DES via HPLC–thin-layer chromatography and final confirmation via HPLC–GC–MS. Fig. 1B shows the immunogram of the same urine after enrichment with 10 µg/l HEX. Since the peaks of the

CLIA responses correspond to the retention times of standard DES (6.3 min) and HEX (7.6 min), respectively, these peaks disclose the presence of DES (Fig. 1A) or DES and HEX (Fig. 1B). CLIA responses, calculated as μg/l DES, should be considered as semiquantitative only because no recovery corrections are applied.

Unlike the radioactive [^{3}H]HEX label, the HEX-ABEI label is not suitable as a recovery tracer. However, replacement of radioactive labels by chemiluminescent labels in immunochemical detection methods has a number of advantages. Besides socio-economic reasons, such as radioactive waste disposal, special equipment and legal permission, a number of practical advantages are important, such as sensitivity and speed. In theory, and also in practice, the minimum detectable amount is generally lower in the CLIA than in the RIA. Another practical advantage of the CLIA is the speed of detection of the chemiluminescence. Routinely we use integration times of 2 or 6 s, which means that an assay of 160 test tubes can be counted within 30 min, whereas the tritium-counting in the corresponding DES-RIA takes 8–16 h. In practice the results of the CLIA will be available within one working day, whereas the results of the RIA are in general not available until the next day. Possible corrections or HPLC–CLIA confirmation of responding samples in the RIA-screening can be made on the same day that the screening results become available. This speed of analytical sample throughput and confirmation is especially important if non-negative responses in the screening will condemn the carcass of the corresponding slaughtered animal to be stored in the freezer to await final confirmation of the screening result.

Further work is in progress to develop an even quicker solid-phase CLIA for DES, DE and/or HEX in order to screen urine samples for these legally banned anabolic compounds.

ACKNOWLEDGEMENT

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