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Note

Separation and purification of several anabolics present in bovine urine by isocratic high-performance liquid chromatography

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The techniques widely used to trace anabolics in the urine of cattle are radioimmunoassay (RIA)[1–3], thin-layer chromatography (TLC)[4–6] and/or gas chromatography–mass spectrometry (GC–MS)[7–9], Problems in the determination and identification of trace amounts of anabolics at the (sub)nanogram level are often caused by interferences from the urine matrix. Consequently, the specificity of RIA can be lowered by the possible presence of relatively large amounts of other known cross-reacting compounds or unknown compounds which interfere in the immunochemical reaction. Using TLC, the identification can be made considerably more difficult by the complexity of the two-dimensional chromatogram.

In this paper we describe the separation and isolation of various anabolics like stilbene derivatives, steroids and resorcylic acid lactones by means of isocratic reversed-phase high-performance liquid chromatography (HPLC), followed by quantification via RIA or identification via TLC or GC–MS.

MATERIALS AND METHODS*

All anabolics were identified by infra-red, ultra-violet (UV) and mass spectroscopy and checked for purity by HPLC, TLC and GC–MS.

The anabolics in 10 ml of urine were deconjugated by enzymatic hydrolysis with glucuronidase/sulphatase (Suc d'*Helix pomatia*; IBF, France) during at least 1 h at 37°C after addition of, for instance, tritiated diethylstilbestrol (DES) as a recovery tracer in case of RIA for DES. Then the free compounds were extracted twice with 5 ml of diethyl ether. After addition of 225 μl distilled water, the ether was evaporated under nitrogen and 400 μl of methanol were added. From this methanol–water mixture, 250 μl were applied to the HPLC column. The RIA procedure following HPLC purification will be described in detail elsewhere[10]. A detailed description in Dutch of the RIA procedure, including the HPLC purification, is available in ISO layout[11] upon request.

* Reference to a company and/or product is for purpose of information and identification only and does not imply approval or recommendation of the company and/or the product by the National Institute of Public Health and Environmental Hygiene, to the exclusion of others which may also be suitable.

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All HPLC separations described were performed on a column (150 × 4.6 mm I.D.) with Valco fittings (Chrompack) packed with 5-μm Hypersil ODS (Shandon) protected by a micro guard column (75 × 2.1 mm I.D.) packed with Corasil C_{18} (Waters Associates). The main column was packed with a Column Packing Instrument (Shandon). The column was eluted in an isocratic mode with methanol–water (see Results) at 2.0 ml/min. All HPLC instrumentation was obtained from Waters Associates and consisted of an automatic injector (Model WISP), two solvent-delivery systems (Model 6000 and M 45) operated by a gradient controller (Model 660), UV detector (Model 450) and printer-plotter-integrator (Data module). The separated fractions were collected in a fraction collector (Redirac, LKB) modified with an electric three-way valve (Pharmacia)[3]. Both the fraction collector and valve were operated automatically by timed events from the integrator mediated by a home-built interface (Model SE 459). The electronic scheme of this interface is available upon request. After each run the tube between the three-way valve and the fraction collector was washed automatically with 1 ml eluent to avoid cross-contamination.

RESULTS

Separation of stilbene derivatives for immunochemical quantification

trans-Diethylstilbestrol (DES), α- and β-dienestrol (DE) and *meso*-hexestrol (HEX) are chemically closely related and can hardly be separated by conventional liquid chromatographic purification techniques. By means of reversed-phase HPLC these stilbene derivatives can be separated completely (Fig. 1) with methanol–water (60:40, v/v) and subsequently collected and determined individually via RIA. In this system *cis*-DES elutes at between 16 and 18 min[12].

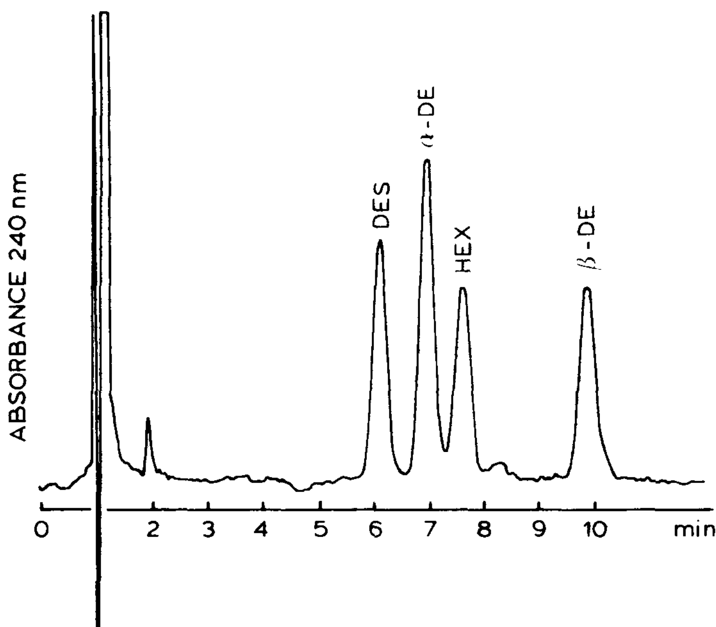

Fig. 1. Isocratic HPLC separation of a standard solution of *trans*-diethylstilbestrol (DES), α- and β-dienestrol (DE) and *meso*-hexestrol (HEX) (100–500 ng). For experimental conditions, see text.

Separation of Zeranol and derivatives for RIA

Of the anabolic estrogen zearalanol [Zeranol® (Z)], a member of the group of resorcylic acid lactones, a number of chemically closely related derivatives are known, *e.g.*, its metabolite zearalanone (Za) and its parent compound the mycotoxine zearalenone (Ze). Since these compounds may possess a significant cross-reactivity towards an antiserum against Z[13,14], it is necessary to separate them prior to the immunochemical detection and quantification. Fig. 2 shows such a separation with methanol–water (58:42, v/v).

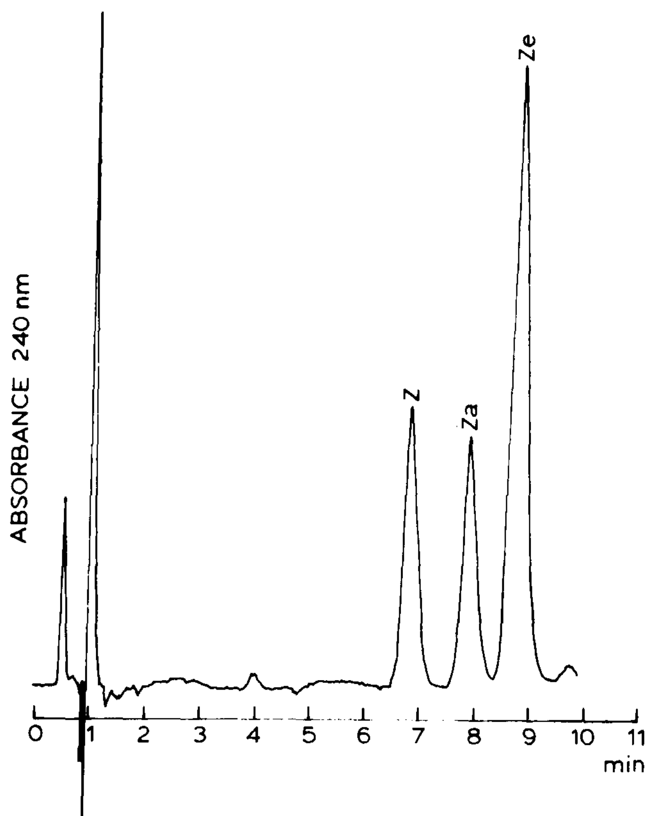

Fig. 2. Isocratic HPLC separation of a standard solution of Zeranol (Z), zearalanone (Za) and zearalenone (Ze) (100 ng). For experimental conditions, see text.

Separation of anabolics for TLC and GC–MS

Besides Z, its derivatives and the stilbene derivatives DES, DE and HEX, also residues of xenobiotic anabolic steroids can be found in bovine urine like 17 β-Trenbolone® (TB), 19-nortestosterone (NT), 17α-methyltestosterone (MT) and/or medroxyprogesterone (MP). These residues can be separated completely (Fig. 3) with methanol–water (60:40, v/v) and subsequently collected and detected individually or in suitable combinations by TLC or GC–MS. Only 10 ml of bovine urine are needed for a multi residue HPLC–TLC or HPLC–GC–MS analysis[15,16].

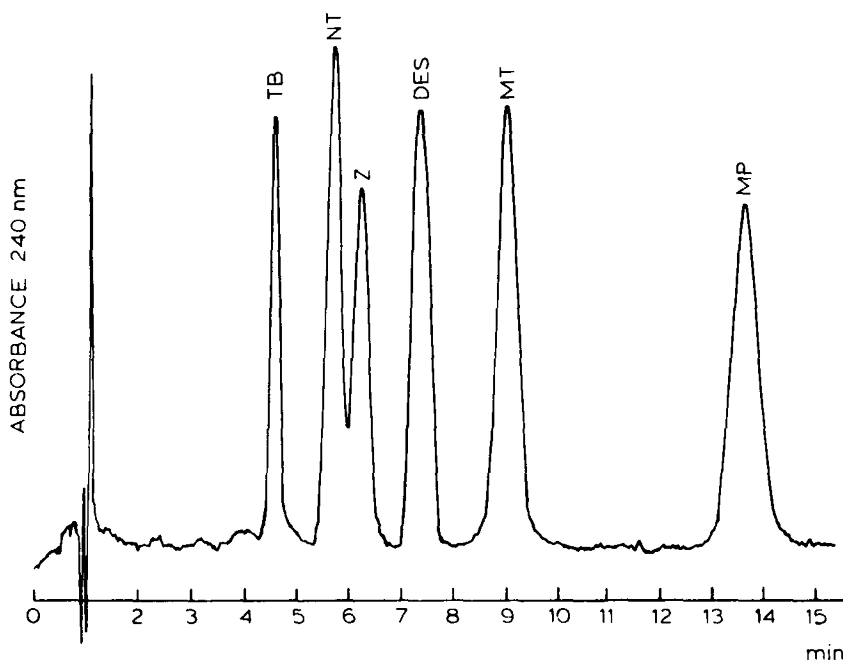

Fig. 3. Isocratic HPLC separation of a standard solution of 17β-Trenbolone (TB), 19-nortestosterone (NT), Zeranol (Z)), *trans*-diethylstilbestrol (DES), 17α-methyltestosterone (MT) and medroxyprogesterone (MP) (100 ng). For experimental conditions, se text.

DISCUSSION

A number of chemically closely related anabolic compounds or their metabolites can be separated from each other and from interfering matrix components by isocratic reversed-phase HPLC. The application of HPLC purification prior to a radioimmunochemical detection procedure results in a large increase in specificity. Consequently, the possibility of a false-positive classification is decreased substantially, as confirmed during a control program for the presence of DES in bovine urine[17,18]. Early in 1981 in the Netherlands 3746 samples of bovine urine were monitored for the presence of DES with RIA without chromatographic purification (direct RIA). In this study, the following cumulative frequency distribution for the immunochemical responses, expressed as μg/l DES, was observed: 19% = 0; 30% ⩽ 0.1; 42% ⩽ 0.2; 68% ⩽ 0.5 and 86% ⩽ 0.9. Of the samples, 14.3% were classified as "positive" according to a limit of 1 μg/l DES equivalent. After reinvestigation of these "positive" samples by HPLC–RIA, 25–30% proved to be false positive for DES, DE or HEX. This finding was confirmed by HPLC–GC–MS.

In a subsequent national control program with the same RIA after column chromatography on Celite (Celite RIA) the following cumulative frequency distribution for the immunochemical responses was observed, expressed as μl/l DES: 73% = 0; 91% ⩽ 0.1; 95% ⩽ 0.2; 97% ⩽ 0.5 and 98% ⩽ 0.9. In this investigation, 1.8% of 12,576 samples of bovine urine were found to be "positive" according to

the above limit. Of these samples about 6% were false positive for DES, DE or HEX as shown by HPLC–RIA and confirmed with HPLC–GC–MS.

In both control programs the HPLC–RIA gave no false positive results, as judged from the comparative investigation by HPLC–GC–MS. There were no indications for any false negative results. For this purpose, 10% of the negative samples from the screening were reinvestigated with HPLC–RIA.

In nearly 3 years, no discrepancies have been observed between the qualitative results of HPLC–RIA and HPLC–GC–MS. In addition, within the HPLC procedure it is possible to isolate or identify other cross-reacting compounds, which can be determined separately using RIA or an immunogram procedure. In practice, this procedure is operational for the immunochemical identification of DES, DE and HEX[19].

For use with chemiluminescence immunoassay (CLIA) the reported HPLC procedure also looks very promising[20].

The HPLC purification of urine hydrolysates for TLC detection results in very "clean" two-dimensional chromatograms. As a result the specificity is increased, the minimum detectable amount is decreased and the interpretability is strongly enhanced. Furthermore, compounds which cannot be separated by two-dimensional development on the TLC plate, like the dansyl derivatives of DES, DE and HEX, can be collected separately by HPLC and subsequently determined individually by TLC. In a comparative study with about 400 samples of urine, for the stilbenes and other anabolics no qualitative discrepancies have been observed between HPLC–TLC and HPLC–GC–MS.

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