Journal of Chromatography, 347 (1985) 379–383
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 067

Note

Screening of parts-per-billion levels of diethylstilbestrol in bovine urine by high-performance liquid chromatography with ultraviolet detection

E. H. J. M. JANSEN*, P. W. ZOONTJES, R. BOTH-MIEDEMA, H. VAN BLITTERSWIJK and R. W. STEPHANY

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

(Received July 26th, 1985)

In the Netherlands the screening of bovine urine for the presence of the illegal anabolic estrogen diethylstilbestrol (DES) is performed by radioimmunoassay (RIA) after chromatographic purification steps[1,2]. Confirmation of the identity of DES is performed by gas chromatography–mass spectrometry (GC–MS)[3] after purification of the urine extract by high-performance liquid chromatography (HPLC)[4]. After introduction of this procedure in a National Control Program, the use of the carcinogen DES for fattening purposes stopped almost completely in the Netherlands[5]. For practical reasons we are interested in screening methods other than RIA. Detection procedures for DES in urine using HPLC with various detection principles, such as post-column photochemical detection[6,7], dansylation with fluorescence detection[8], electrochemical detection[9] and detection with GC–MS[10] have been reported recently. None of these detection methods proved to us to be suitable for large scale screening, owing to laborious procedures or insufficient practical evaluation or validation.

To control for residues of the synthetic androgen trenbolone (TB) in bovine urine a fast screening method has been developed using HPLC with on-line UV detection at 350 nm[11]. Here a rather simple detection method using HPLC with UV detection at 240 nm is presented and evaluated for large scale screening of bovine urine samples for the anabolic DES.

MATERIALS AND METHODS*

Apparatus

The HPLC equipment consisted of an automatic injector (WISP, Waters Assoc.), a solvent delivery system (Model 2150, LKB) equipped with a solvent switch (Model PSV-3, Pharmacia), a variable-wavelength detector operated at 240 nm

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0021-9673/85/$03.30

(Model 773, Kratos) and a printer–plotter–integrator (Data module, Waters Assoc.). The chromatographic columns (150 × 4.6 mm I.D.), obtained from Chrompack, were packed with LiChrosorb Diol 5 μm (Merck) or Hypersil ODS 5 μm (Shandon) using a Column Packing Instrument (Shandon). The DES fractions were collected with a modified fraction collector (Redirac, LKB), equipped with a electric three-way valve (Model PSV-3, Pharmacia). Both fraction collector and valves were operated by the timed events of the integrator mediated by a laboratory-made interface (Model SE 459). The columns were thermostated at 30°C by a laboratory-made metal holder. Elution conditions for the diol column were 8 min isooctane–ethanol (97:3, v/v) and 2 min isooctane–ethanol (60:40, v/v) to clean the column of tightly bound urine matrix components. Elution conditions for the reversed-phase column were 8 min methanol–water (60:40, v/v) and 2 min methanol to clean the column of tightly bound compounds. A constant flow-rate of 2.0 ml/min was used. DES was quantified using a 3390A integrator (Hewlett-Packard).

All solvents were of analytical grade (Merck). Anabolic standards were checked for purity by melting point, HPLC and infrared spectroscopy.

Sample clean-up and detection

Bovine urine (2 ml) was hydrolysed enzymatically[1] during 2 h at 37°C with glucuronidase/sulfatase (*Suc d'Helix pomatia,* IBF, France). After *n*-hexane extraction (10 ml) of the urine hydrolysate, the organic layer was evaporated under nitrogen and the dry residue was dissolved in 0.300 ml of the HPLC mobile phase of the diol column. After application of 0.250 ml of the extract to a column with LiChrosorb diol the *trans*-DES fraction was collected automatically for 1.5 min around the retention time of *trans*-DES. The eluting solvent of the DES fraction was evaporated and the dry residue was redissolved in 0.300 ml of the HPLC mobile phase of the reversed-phase column. During the HPLC separation on Hypersil ODS of 0.25 ml of the extract, *trans*-DES was detected by on-line UV absorption at 240 nm. With this procedure 40 urine samples can be processed and analysed by a single technician within 48 h. Both HPLC purification of urine extracts on LiChrosorb diol and HPLC detection on Hypersil ODS can be performed automatically during two sequential nights.

RESULTS AND DISCUSSION

The chromatographic behaviour of *trans*-DES on an isocratic reversed-phase HPLC system is shown in Fig. 1. From this figure it has been calculated that the absolute detection limit of standard *trans*-DES is 0.4 ng, with a signal-to-noise ratio of 2.

Early attempts to monitor nanogram amounts of DES in purified urine extracts with UV detection failed on account of the interfering absorption of matrix components. Also results obtained with combinations of two HPLC columns in series were not fully satisfactory. Only for trenbolone, which can be monitored at 350 nm, could UV detection be performed in urine extracts using one normal-phase HPLC column[11].

In these procedures the first purification step was an extraction with diethyl ether. On testing other organic solvents, it appeared that extraction of DES from

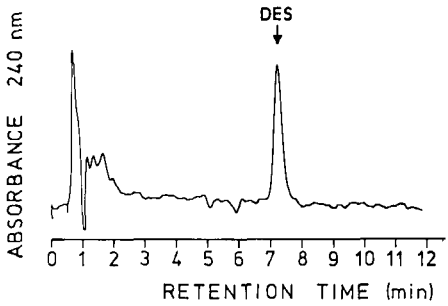

Fig. 1. HPLC chromatogram of 5 ng of *trans*-DES standard on Hypersil ODS (5 μm) in methanol–water (6:4, v/v) at a flow-rate of 2 ml/min.

urine hydrolysates with *n*-hexane was as efficient as with ether, but in addition the extracts appeared to be much cleaner. Application of *n*-hexane extracts in a double HPLC separation and clean-up procedure resulted in very "clean" chromatograms in which *trans*-DES can be detected at microgram per litre (ppb) concentrations. Detection was performed at 240 nm rather than 195 or 200 nm. In Figs. 2 and 3 two examples are shown of chromatograms of extracts of bovine urine samples from practice containing DES in concentrations of 1.2 and 8.5 μg/l, respectively, according to HPLC–RIA[1]. The identity of DES in these samples was confirmed by HPLC–GC–MS. It can be concluded from these Figures that DES can be identified and quantified at the ppb level based on its characteristic retention time. At present, after

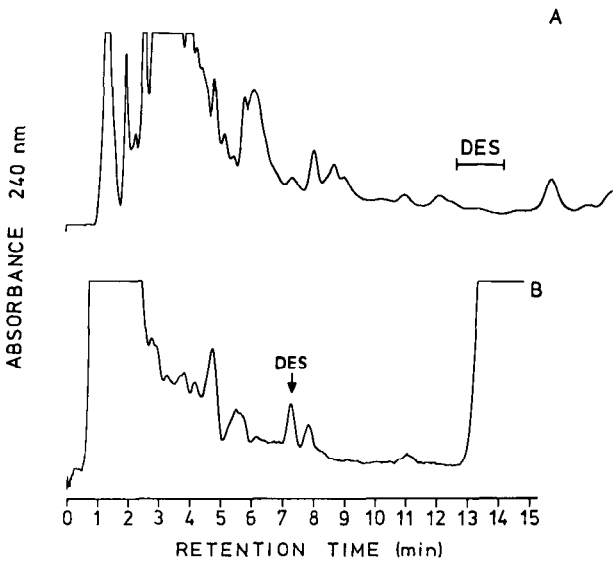

Fig. 2. HPLC chromatogram (A) of an extract of 1.67 ml of urinary DES containing bovine urine (H 146743) on LiChrosorb Diol (5 μm). The *trans*-DES fraction was collected automatically for a total of 1.5 min as indicated and applied (5/6 part, an aliquot of 1.39 ml of urine) to a Hypersil ODS (5 μm) column (B). From Figure B an amount of 2.6 ng of *trans*-DES (1.9 μg/l) has been calculated. The retention time of *trans*-DES is indicated by the arrow. In Figure B the absorbance scale was increased 20-fold.

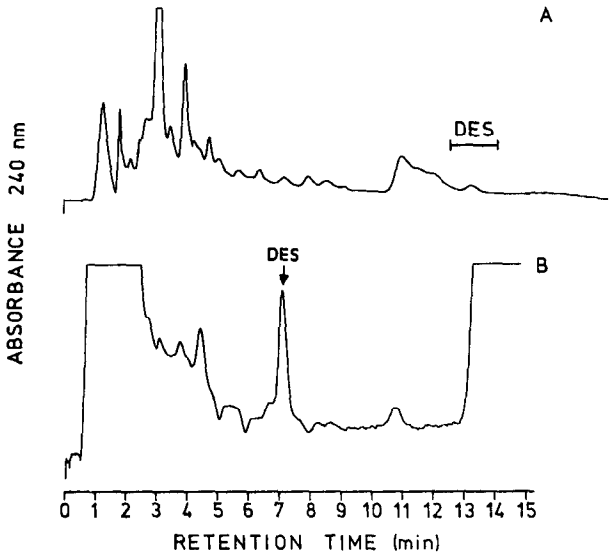

Fig. 3. HPLC chromatograms of an extract of 1.67 ml of urinary DES containing bovine urine (H 146841) on (A) LiChrosorb diol (5 μm) and (B) Hypersil ODS (5 μm). Further conditions as in Fig. 2. From Figure B an amount of 8.5 ng of *trans*-DES (6.1 μg/l) has been calculated.

preliminary analysis of 20 blank urine samples, no false positive responses were observed. Using this clean-up procedure the recovery of *trans*-DES was 73.3% ± 9.7% (S.D.) at the level of 5 μg/l ($N = 15$). In addition, 24 samples of bovine urine from the control programme of 1983 have been analysed and the quantitative results (X) compared with those obtained with the HPLC–RIA (Y) for DES. After orthogonal regression analysis a good correlation was found with a correlation coefficient of 0.987, a slope of 1.57 and an intercept of 0.10 ($Y = 1.57X + 0.10$).

The reported detection method is a fairly simple, relatively fast and partially automated screening method for the detection of DES in bovine urine in concentrations down to the 1 ppb level. Therefore this screening method can be considered as an alternative to RIA in certain applications. If necessary the reliability of the identification can be improved by co-chromatography of standard *trans*-DES in the reversed-phase run. In the case of urinary DES only an increase of peak height should be observed without peak broadening. At present similar methods are under development for monitoring other anabolics, such as xenobiotic androgens and other stilbene derivatives such as hexestrol and dienestrol.

ACKNOWLEDGEMENT

This investigation was performed as part of project 368301 of the Dutch Veterinary Inspectorate of Public Health.

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