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Note

Assay of ethylestrenol in urine by isotope dilution—mass spectrometry

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Ethylestrenol is an anabolic steroid which may be illegally used with the aim of increasing performance in different power sports. To our knowledge, this compound or its metabolites have as yet not been detected in urine samples from competing athletes. In spite of that, the battery of tests used for the detection of illegal use of anabolic steroids should include analysis for ethylestrenol.

There is little detailed knowledge concerning the metabolism of ethylestrenol. It was recently shown in the rat that only about 17% of an intragastric dose was excreted in the urine, mainly as unmetabolized ethylestrenol [1]. In vitro studies with rat liver homogenates [2] as well as in vivo studies in man [3, 4] and monkeys [5] have shown that ethylestrenol may be hydroxylated in the 3-position. It has been reported that the major urinary metabolite of ethylestrenol in monkey and man is a 19-norpregnanetriol of unknown stereochemistry [3—5]. In the latter studies only small amounts of unmetabolized ethylestrenol could be detected in the urine.

In view of the severe legal consequences of the presence of anabolic steroids in the urine of competing athletes, the analytical method used must be highly accurate and specific. In previous works [6, 7] we demonstrated the usefulness of isotope dilution—mass spectrometry for such analyses. In the present work this technique was used also for the assay of ethylestrenol.

EXPERIMENTAL PROCEDURE

Preparation of [$^2H_5$]ethylestrenol

A solution of 4-estren-17-one (obtained from Steraloids Inc., Wilton, NH, U.S.A.), 50 mg in 2 ml of dried benzene, was added to a Grignard solution prepared from 37 mg of magnesium and 1.7 mmol of $C_2{}^2H_5I$ (obtained from

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CEA, Gif-sur-Yvette, France; 99% pure with respect to ^2H). The mixture was refluxed for 3 h. After cooling to 5°C, the complex was decomposed by the slow addition of 1 ml of ice water and 2 ml of 50% aqueous acetic acid solution. The mixture was then diluted with water and extracted with ethyl acetate. The organic phase was washed with water until neutral and dried over anhydrous sodium sulfate. The product was isolated by preparative thin-layer chromatography, using chloroform—ethyl acetate (1:1, v/v) as solvent. The product (27 mg) was pure as judged by thin-layer chromatography and gas—liquid chromatography—mass spectrometry (see Results).

Unlabelled ethylestrenol was obtained from Organon, Oss, The Netherlands, and was pure as judged by thin-layer chromatography with the same solvent as above.

Urine samples

Urine was collected from healthy males and females at the laboratory. The samples were kept frozen at −20°C until analyzed.

Radioimmunoassay

The kit for analysis of anabolic steroids was obtained from Prof. Brooks at St. Thomas's Hospital, London, Great Britain [8]. Instead of using the dextran-coated charcoal pellet for gamma counting, we used an aliquot of the supernatant.

The antibodies are directed towards 19-norsteroids, but react only to a small extent with ethylestrenol [8]. In spite of that, it has been reported that administration of ethylestrenol can be detected with these antibodies, probably due to the occurrence of metabolites with an oxygen function in the 3-position [8].

Preparation of samples for isotope dilution—mass spectrometry

The $^2H_5$-labelled ethylestrenol, 100 ng, was added to 5 ml of urine. The standard curve was prepared by adding 0—200 ng of unlabelled ethylestrenol to 100 ng of the internal standard and 5 ml of urine from an untreated subject. Sodium acetate buffer (0.15 *M*, pH 4.6) was added to the mixture and the conjugated steroids were hydrolyzed by adding 50 μl of *Helix pomatia* digestive juice. After mixing on a Vortex mixer, the solution was incubated at 37°C for 24 h. The free steroids were extracted with ethyl acetate, 10 ml, on a Rotary mixer for 30 min. The two phases were separated and the organic phase was washed twice with 0.1 *M* NaOH, 5 ml, and once with water, 5 ml. After treatment with anhydrous Na_2SO_4, the solvent was evaporated to dryness at 50°C under a stream of nitrogen. The residue was subjected to preparative thin-layer chromatography, using chloroform—ethyl acetate (1:1, v/v) as solvent. In order to locate the chromatographic zone containing ethylestrenol, [4-^{14}C]oestrone (15,000 dpm, obtained from the Radiochemical Centre, Amersham, Great Britain) was added to the mixture prior to the chromatography.

The radioactive zone (R_F = 0.88) was detected by radioscanning using a Berthold Dünnschicht Scanner II (Wildbad, G.F.R.). This zone was located just below the zone containing ethylestrenol (R_F = 0.93). The latter was scraped off and extracted with 5 ml of methanol. The solvent was evaporated under a stream of nitrogen, and the residue converted into a trimethylsilyl derivative

with trimethylsilylimidazole [9]. The reaction mixture was extracted with 50 μl of hexane and cooled in a freezer to separate the two phases.

Mass spectrometry

About 5 μl of the hexane phase were analysed by gas chromatography—mass spectrometry (GC—MS) using an LKB 9000 instrument equipped with a multiple ion detector and a 1.5% SE-30 column (Chromosorb W, 80—100 mesh, 1.4 m × 2 mm). The temperature of the column was 220°C whereas the flash heater and the ion source both had a temperature of 260°C. The carrier gas was helium and a flow-rate of 30 ml/min was used. The electron energy was 20 eV and the trap current 60 μA. The electron multiplier sensitivity was set to 240 and the amplification of both channels was 300 ×. The first channel was focused on the ion *m/e* 270 and the second channel at *m/e* 275.

RESULTS

The mass spectra of the trimethylsilyl (TMS) derivatives of unlabelled and deuterium-labelled ethylestrenol are shown in Fig. 1. The base peak in the mass spectrum of unlabelled ethylestrenol was at *m/e* 157 (cf. ref. 10). This ion is derived from the D-ring of the molecule, and corresponds to the ion at *m/e* 143 in the mass spectrum of the TMS derivative of methandienone [3]. In accordance with data given in ref. 10, prominent peaks were also seen at *m/e* 241 (corresponding to loss of the TMS group and the ethyl group from the molecular ion) and at *m/e* 270 (corresponding to loss of the TMS group from the molecular ion). In the mass spectrum of the TMS ether of deuterium-labelled ethylestrenol, the corresponding peaks were at *m/e* 162, *m/e* 241 and *m/e* 275. It may be concluded that five atoms of deuterium had been introduced and also that the common ion at *m/e* 241 is formed by loss of the ethyl group.

In the quantitative analysis of urine extracts, the ions at *m/e* 270 and *m/e* 275 were chosen. Contaminating compounds sometimes interfered when using the more intense ions at *m/e* 157 and *m/e* 162.

Fig. 2A shows the multiple ion detector recordings obtained in the analysis of a purified urine extract from an untreated subject. Deuterium-labelled ethylestrenol, 100 ng, had been added to the urine sample as an internal standard. A prominent peak was obtained in the recording of the ion at *m/e* 275, corresponding to the presence of internal standard. Only a very small peak was obtained in the recording at *m/e* 270, corresponding to unlabelled ethylestrenol. After ingestion of a small dose of Orgabolin®, 6 mg, by the same subjects as above, a corresponding analysis of a urine sample gave a prominent peak also in the recording of the ion at *m/e* 270, indicating the presence of unlabelled unmetabolized ethylestrenol (Fig. 2B). The concentration of ethylestrenol could be calculated from a standard curve, obtained by analysis of standard mixtures of different amounts of unlabelled ethylestrenol together with a fixed amount of deuterium-labelled ethylestrenol. It was shown that the ratio between the peaks of the tracings at *m/e* 270 and *m/e* 275 was linear with the concentration of ethylestrenol in the range 0—40 ng/ml of urine.

The possibility that endogenous compounds may interfere in the present

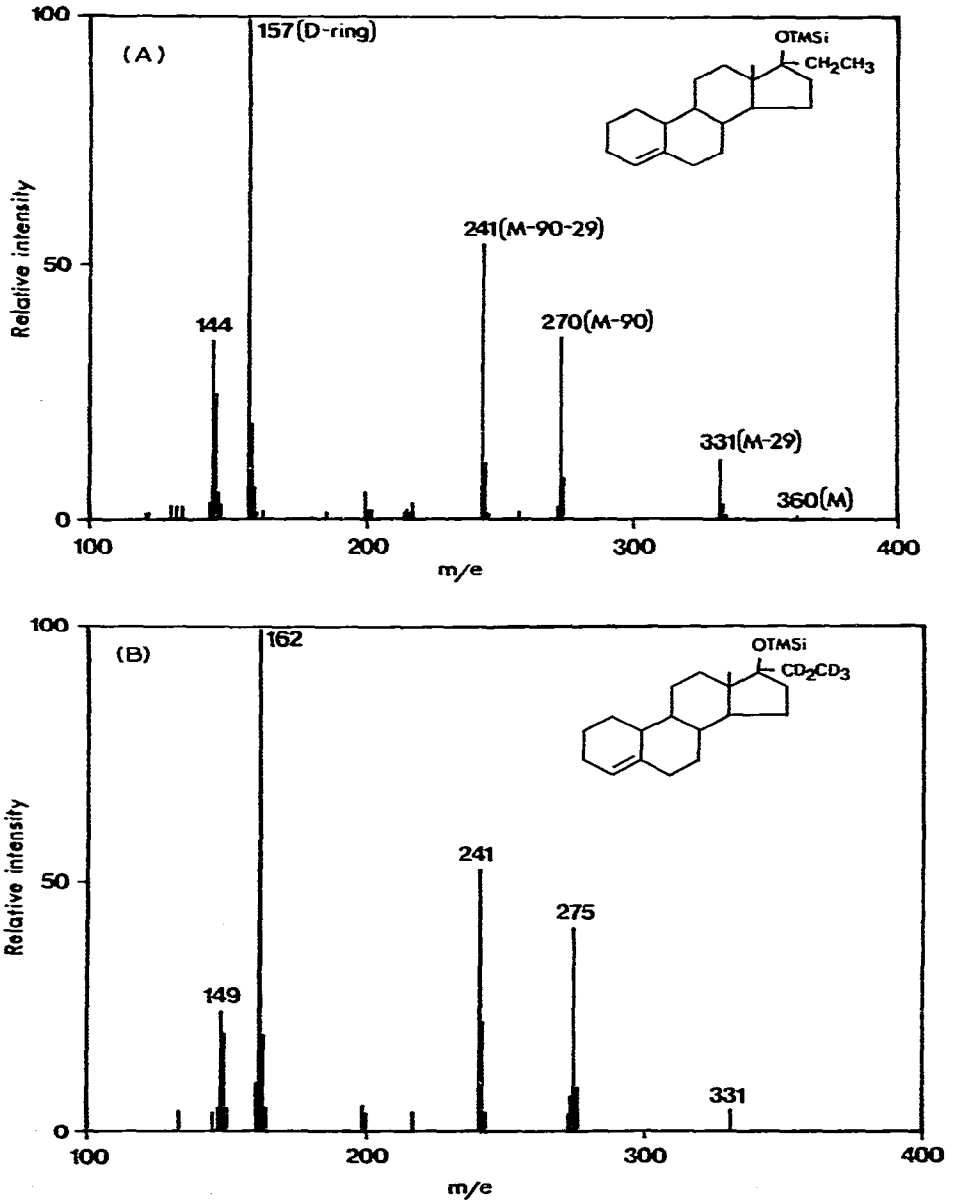

Fig. 1. Mass spectra of the TMS ethers of unlabelled (A) and deuterium-labelled (B) ethylestrenol.

assay was investigated by analysis of urine samples from ten untreated subjects. The apparent concentration of ethylestrenol in these urine samples never exceeded 1 ng/ml of urine. The concentration of ethylestrenol in the first 24-h portion of urine after ingestion of 6 mg of Orgabolin® was 16 ng/ml. It was not possible to detect significant amounts of ethylestrenol in the second 24-h portion of urine. Even after ingestion of 12 mg of Orgabolin, it was only pos-

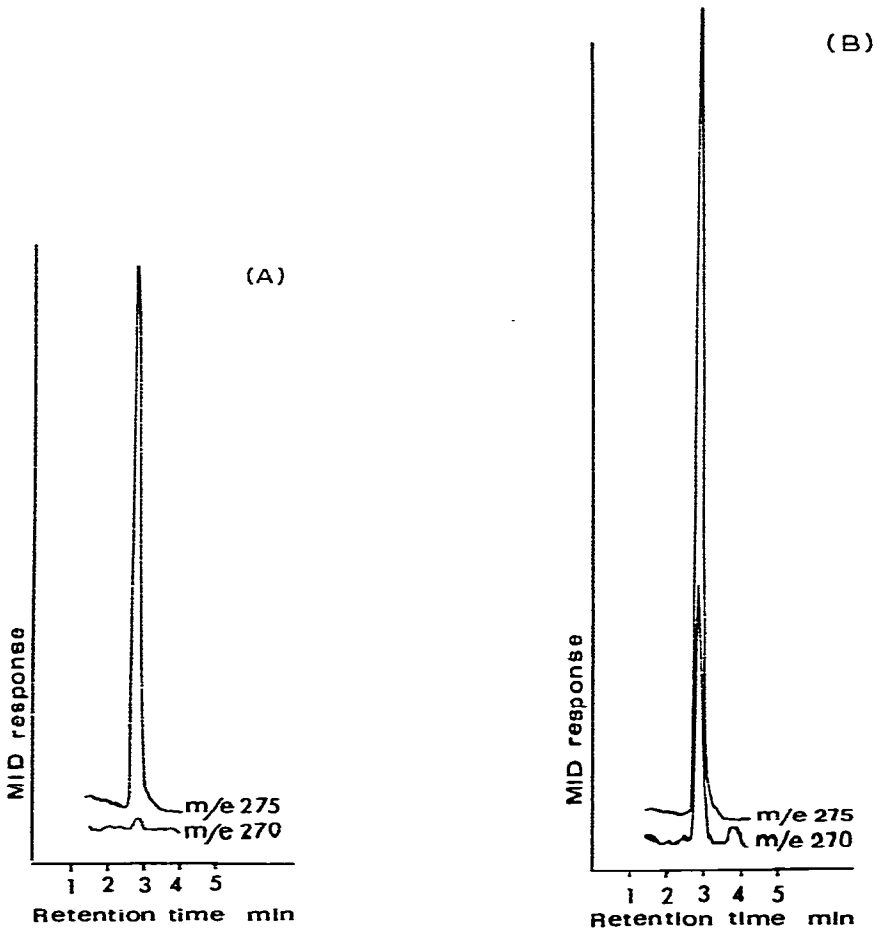

Fig. 2. Multiple ion detector recording of derivatives of a purified extract of urine from an untreated subject (A) and a subject treated with 6 mg of Orgabolin® (B). Deuterium-labelled ethylestrenol had been added as internal standard. For experimental details, see Experimental procedure.

sible to trace ethylestrenol in the first 24-h portion of urine. This was the case also when using radioimmunoassay with antibodies directed towards 19-norsteroids [1].

The coefficient of variation of the isotope dilution method, calculated from five replicate measurements of urine samples containing 10 and 50 ng/ml of ethylestrenol, was about 6% at each concentration.

DISCUSSION

In most doping laboratories, radioimmunoassay is used as a screening test for presence of anabolic steroids in urine. The presence of ethylestrenol or its metabolites in urine may be detected with use of antibodies directed towards 19-norsteroids [8]. In combination with a positive radioimmunoassay test, a positive identification with the present method should give sufficient evidence

that the athlete has taken ethylestrenol. Preferably, however, the identity should be confirmed by use of the other "diagnostic" ions at *m/e* 157 and *m/e* 144. In this low mass range there is always a risk of the presence of interfering compounds. In such cases it is advisable to analyse the urine sample also without the addition of internal standard. Under such conditions also the ions at *m/e* 241 and *m/e* 331 are "diagnostic" for the presence of ethylestrenol.

It may be added that Brooks et al. [10] have used the ion at *m/e* 157 for quantitation of the TMS ether of analogous 17α-ethylated steroids by single ion monitoring. Brooks and Middleditch [11] have also described the principles of an assay of the chloromethyl(dimethyl)silyl ether of ethylestrenol based on single ion monitoring of the ion at *m/e* 270.

In view of the extensive metabolism of ethylestrenol, a sensitive assay for the detection of its illegal use should preferably be directed towards the metabolites rather than towards unmetabolized ethylestrenol. Development of an isotope dilution assay for the major metabolite of ethylestrenol is now in progress.

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