Journal of Chromatography, 564 (1991) 469–475
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5708

Detection of the illegal use of ethinylestradiol in cattle urine by gas chromatography–mass spectrometry

E. DAESELEIRE*, A. DE GUESQUIÈRE and C. VAN PETEGHEM

Laboratory of Food Analysis, Faculty of Pharmaceutical Sciences, State University of Ghent, Harelbekestraat 72, B-9000 Ghent (Belgium)

ABSTRACT

A method for the detection of ethinylestradiol in cattle urine is described, based on enzymic hydrolysis of the sample, clean-up by means of disposable octadecyl and amino solid-phase extraction columns, fractionation by reversed-phase high-performance liquid chromatography, and detection by gas chromatography–mass spectrometry (selected-ion monitoring). Identification is based on both gas chromatographic and mass spectrometric data. The method has been tested on urine samples for a collaborative study and all the results found were correct.

INTRODUCTION

Hormones are extensively used in the animal breeding sector in most countries of the world, including Belgium. One of them is ethinylestradiol, which is frequently found in injection sites taken from animals in slaughter-houses. The Belgian Public Health Authorities have taken measures, and the urine of the cattle is regularly screened for ethinylestradiol by radioimmunoassay. Positive results need to be confirmed by a second analysis, based on a different analytical technique.

Ethinylestradiol, a frequently used contraceptive in human medicine, has been measured in plasma by gas chromatography–mass spectrometry (GC–MS) [1–3]. The same technique has been applied for its detection in human urine [3], in bovine biological fluids [4], and in muscle tissue [5,6].

This paper describes a GC–MS method for the confirmation of the presence of ethinylestradiol in urine samples found positive by immunochemical methods. Emphasis is laid on sample pretreatment in order to increase the selectivity and thus the reliability of the method.

0378-4347/91/$03.50

EXPERIMENTAL

Chemicals, glassware and solvents

The disposable derivatization vials were silanized with a solution of dimethyldichlorosilane (Merck, Darmstadt, Germany) in toluene (Merck) before use. Methanol and water were HPLC grade from Alltech Assoc. (Deerfield, IL, U.S.A.). Ethyl acetate was obtained from Janssen Chimica (Geel, Belgium). Octadecyl (6 ml) and amino (3 ml) disposable extraction columns were from J. T. Baker (Phillipsburg, NJ, U.S.A.).

N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) from Aldrich (Milwaukee, WI, U.S.A.), trimethylchlorosilane (TMCS) from Fluka (Buchs, Switzerland) and pyridine from Pierce (Rockford, IL, U.S.A.) were used as supplied.

Helix pomatia (β-glucuronidase plus arylsulphatase) was obtained from Boehringer (Mannheim, Germany).

Enzymic hydrolysis and clean-up

Urine samples were purified according to the method of Schmidt *et al.* [7]. The pH was adjusted to 4.6 with a 3 *M* acetate buffer (40.83 g sodium acetate trihydrate + 18.015 g acetic acid in 100 ml of water). The urine (10 ml) was then concentrated on a octadecyl (C_{18}) (6 ml) column, previously conditioned with two 5-ml volumes of methanol and two 5-ml volumes of water. The column was washed with two 5-ml volumes of water, and the analyte was eluted with 2 ml of methanol.

After evaporation of the methanol under a stream of nitrogen at 40°C, the residue was taken up in 100 μl of methanol, and 5 ml of a 0.2 *M* acetate buffer (pH 4.6) and 50 μl *Helix pomatia* digestive juice were added.

The sample was incubated for 16 h at 37°C or for 3 h at 55°C. After cooling to room temperature, the sample was applied to the previously used and reconditioned C_{18} column. An amino (NH_2) (3 ml) column was conditioned with two 3-ml volumes of ethyl acetate, and the C_{18} column was placed above the NH_2 column. Ethinylestradiol was eluted from both cartridges with two 1-ml volumes of ethyl acetate.

After evaporation to dryness, the residue was redissolved in 100 μl of methanol, 50 μl of which were injected into the HPLC system. The system consisted of a pump (Model 6000A), and automatic injector (Wisp 710 B) both from Waters (Milford, MA, U.S.A.), a fraction collector (2212 Helirac, Pharmacia-LKB, Uppsala, Sweden) and a UV detector (Pye Unicam, Model PU 4020), set at 280 nm.

The analytical column was a LiChrospher RP 18 (125 mm × 4 mm I.D., 5 μm) (Merck) and was protected by a guard column (pellicular reversed phase 30–50 μm, 75 mm × 2.1 mm I.D.) (Chrompack Cat. No. 28603, Middelburg, The Netherlands). The mobile phase was methanol–water (65:35, v/v) and was pumped at a flow-rate of 1 ml/min.

The methanol–water fraction (3 ml, 4–7 min) was evaporated to dryness under a stream of nitrogen.

Derivatization

For the formation of trimethylsilyl (TMS) ether derivatives, the residue obtained after HPLC fractionation was dissolved in 100 μl of MSTFA–TMCS–pyridine (3:1:10, v/v) and heated at 60°C for 1 h. After evaporation of the excess of reagent, the final residue was dissolved in 50 μl of *n*-hexane, 2 μl of which were injected into the GC–MS instrument.

Samples

Eight control samples of unknown ethinylestradiol concentration (blanks and positives), provided by the Laboratoire d'Hormonologie (Marloie, Belgium) and intended for a collaborative study, were analysed as described above.

Gas chromatography–mass spectrometry

The analyses were carried out on an HP 5970 mass-selective detector (Hewlett Packard, Palo Alto, CA, U.S.A.) linked to a HP 5890 gas chromatograph equipped with a HP Ultra-2 (5% phenylmethylsilicone) fused-silica capillary column (25 m × 0.2 mm I.D., 0.33 μm film thickness) and an all-glass moving-needle injection system.

The carrier gas was high-purity helium (L'Air liquide, Liège, Belgium) at a flow-rate of 0.47 ml/min. The injector and the interface temperatures were maintained at 290°C. The oven temperature was programmed from 200 to 280°C at 5°C/min. The final oven temperature was held for 10 min. The analyses were performed in the selected-ion monitoring (SIM) mode, using dwell times of 100 ms.

RESULTS AND DISCUSSION

The digestive juice of *Helix pomatia* is the most versatile enzymatic preparation for steroid glucuronic and sulphate conjugates hydrolysis. Under normal conditions, the buffered urinary extract is usually incubated at 37°C for 16 h. However, when the analysis has to be done fast, hydrolysis can be conducted at 55°C for 3 h with no apparent losses of the steroid of interest [8].

The extraction on C_{18} disposable extraction columns was preferred to that on Amberlite XAD-2 polystyrene resin [9,10]. Both conjugated and unconjugated metabolites of anabolic steroids are adsorbed from the urine. Recovery experiments were carried out by means of a radioactive tracer of ethinylestradiol [tritium-labelled, specific activity 59 Ci/mmol, Amersham (Little Chalfont, U.K.)] and liquid scintillation counting. The results were 99.3 ± 1% (n = 5) for the C_{18} column, 73.2 ± 3.3% (n = 5) for the combination of the C_{18} and the NH_2 columns and 95% (n = 1) for the HPLC column.

The HPLC fractionation can be fully automated by the use of a programmable injection system and fraction collection unit. This means that this time-consuming step can be performed overnight.

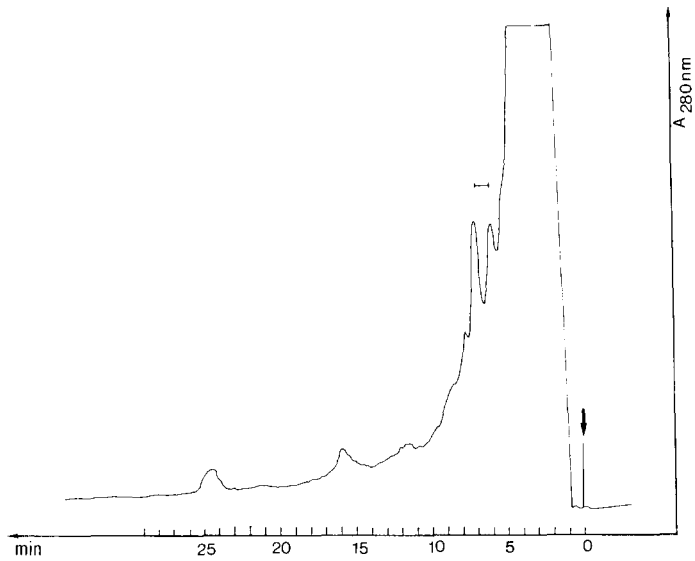

Fig. 1. Chromatogram of a urine sample: the brackets indicate the collected fraction.

The collection region was determined by injecting 25 ng of ethinylestradiol standard. The shift of this region as a function of time or ambient temperature is minimal and should be controlled only once a month.

Fig. 1 shows a typical chromatogram of a urine sample. The brackets indicate the collected fraction.

The most suitable derivative of ethinylestradiol in view of the GC–MS detection was obtained with MSTFA–TMCS–pyridine (3:1:10, v/v). The heptafluorobutyric acid derivative is less suitable because the abundance of the ion *m/z* 492, which is the molecular ion, is too weak to give sufficient signal when analysing the urine samples. The spectrum of the TMS derivative is shown in Fig. 2.

Urine samples were analysed in the SIM mode. The selected ions were *m/z* 440, 425, 300 and 285. A sample was declared positive when all ions simultaneously appeared at the right retention times, expressed as methylene unit value (M.U. = 28.43), and checked with the retention behaviour of an authentic standard.

The results of the analyses of the test samples and the results that had to be found are shown in Table I. They show that, even without the use of an internal standard, it was possible to estimate the ethinylestradiol concentration on a semi-quantitative base and that no false-positive or false-negative results were obtained. Typical fragmentograms of a positive and a negative sample are shown in Figs. 3 and 4.

Specificity is guaranteed by the combination of GC retention data and MS data (selected ions must appear simultaneously at the correct retention times). It has been demonstrated with certified nortestosterone-containing urine samples

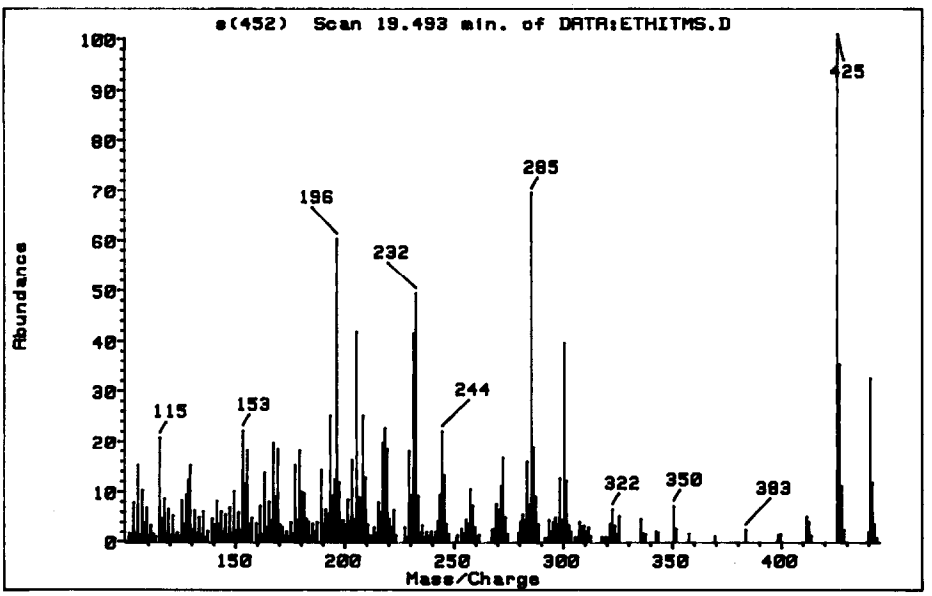

Fig. 2. Electron-impact (70 eV) mass spectrum of the di-TMS derivative of ethinylestradiol.

that intensity ratios of the selected ions are not a suitable criterion, because a number of samples would have had to be declared negative because they did not entirely obey the rules prescribed within the EEC [11].

The detection limit was 0.7 ppb (all ions present, signal-to-noise ratio >3 for ion m/z 440). Although this is not as low as described by Tetsuo *et al.* [3] (5 pg/ml for human urine), it can be concluded that this method is very suitable for the

TABLE I

COMPARISON OF GC–MS RESULTS WITH THE ASSUMED CONCENTRATIONS, AS KNOWN TO THE ORGANIZER OF THE COLLABORATIVE STUDY

Sample No.	Semi-quantitative estimation by GC–MS[a]	Assumed concentration of ethinylestradiol (ng/ml)
33	Negative	0
34	Positive + + +	4
35	Positive +	2
36	Positive + + + +	8
37	Positive + + +	6
38	Negative	0
39	Positive + +	4
40	Positive + + +	6

[a] The number of plus signs indicates the relative concentration found by GC–MS.

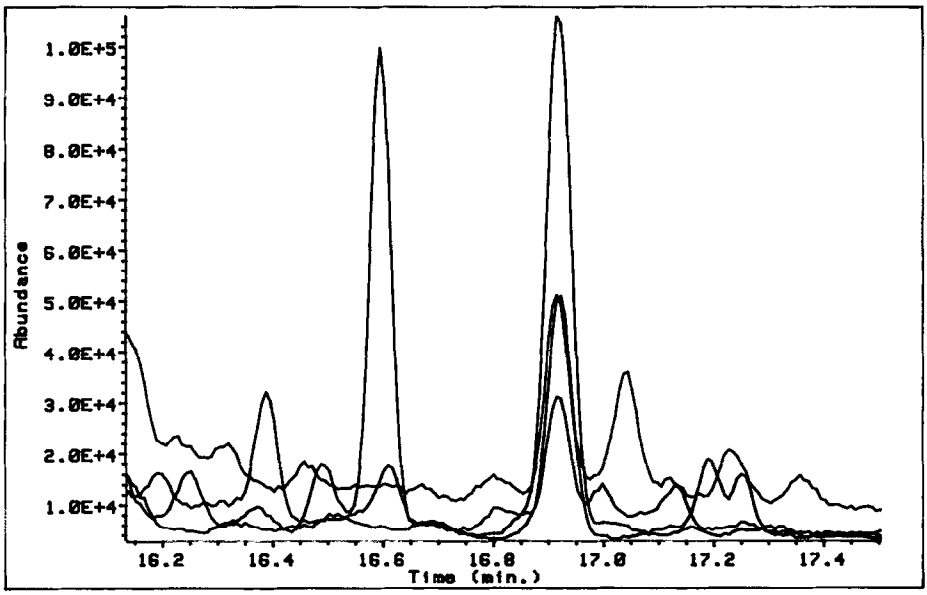

Fig. 3. Fragmentogram of a positive sample (ions *m/z* 440, 425, 300 and 285; retention time, 16.92 min).

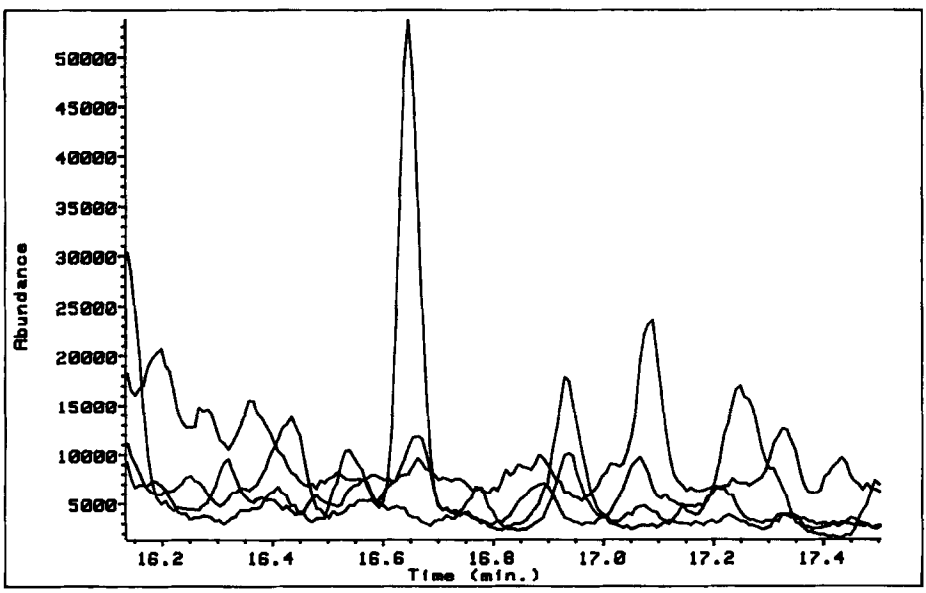

Fig. 4. Fragmentogram of a negative sample (four ions not present at 16.92 min).

confirmation of urine samples that were found positive for ethinylestradiol after screening control.

The clean-up procedure is less labour-intensive and less expensive than that used by Tetsuo *et al.*, [3] but is comparable with the method described by Marlier *et al.* [4], who have worked on cattle urine with a detection limit of *ca.* 1 ppb.

REFERENCES

1 K. Fotherby, J. O. Akpoviroro, L. Siekmann and H. Breuer, *J. Steroid Biochem.*, 14 (1981) 499.
2 L. Siekmann, A. Siekmann and H. Breuer, *Z. Anal. Chem.*, 290 (1978) 159.
3 M. Tetsuo, M. Axelson and J. Sjövall, *J. Steroid Biochem.*, 13 (1980) 847.
4 M. Marlier, H. Lognay, Ph. Gaignage and M. Severin, *Abstracts of the Second International Symposium on Applied Mass Spectrometry in the Health Sciences, Barcelona, April 17–20, 1990*, pp. 199.
5 B. Bergner-Lang and M. Kächele, *Dtsch. Lebensm. Rundsch.*, 83 (1987) 349.
6 H. J. Stan and B. Abraham, *Lebensm. Chem. Gerichtl. Chem.*, 34 (1980) 73.
7 N. A. Schmidt, H. J. Dorburgh, T. J. Penders and C. W. Weykamp, *Clin. Chem.*, 31 (1985) 637.
8 R. Massé, C. Ayotte and R. Dugal, *J. Chromatogr.*, 489 (1989) 23.
9 H. L. Bradlow, *Steroids*, 11 (1968) 265.
10 H. L. Bradlow, *Steroids*, 30 (1977) 581.
11 W. G. De Ruig, R. W. Stephany and G. Dijkstra, *J. Chromatogr.*, 489 (1989) 89.