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Development of a diphasic dialysis method for the extraction/purification of residues of ethinylestradiol in hair of cattle, and determination by gas chromatography–tandem mass spectrometry

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Abstract

A confirmatory method for the analysis of ethinylestradiol extracted from cattle hair was developed. After the extraction of the xenobiotic from the hair, by using alkaline digestion, the purification of the extract was carried out by employing diphasic dialysis. For the optimization of the technique several parameters was evaluated such as pH, extraction solvents, temperatures, times and agitation speeds. The detection and confirmation of the steroid was accomplished by using a GC–MS^2 ion trap system after trimethylsilylation. The calibration curve was linear over the range of 4–20 ng/g. The detection and quantification limit were 0.52 and 0.80 ng/g respectively; with recoveries up to 94%. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Diphasic dialysis; Ethinylestradiol

1. Introduction

The use of anabolic steroids in meat-producing animals are forbidden within the European Union [1] because of their carcinogenic potentiality [2,3]. Despite the ban, these substances have been still used as growth promoters for meat production [4,5]. Ethinylestradiol is an xenobiotic anabolic sex hormone which has a high oral bioavailability, and that have been use by farmers for cattle fattening in spite of the ban [6]. Urine and blood had been used as analytical matrixes for the detection of ethinylestradiol by using screening methods, such as ELISA, and confirmatory methods also (GC–MS) [7].

In the 90s, studies were initiated to determine the possibilities of using hair as a new matrix for the detection of clenbuterol misused in cattle fattening. These surveys demostrated that this β-agonist bound to the matrix, preferably to coloured hair, because of the presence of melanin [8–10]. Afterwards, Gleixner and Meyer [11] reported that oestradiol and testosterone could be detected in the hair of cattle using immunoassay for detection. Hair has the advantage over others matrixes, such as blood and urine, that once the drug is incorporated into the keratinized fibre it stays trapped there for months, given a larger detection window [6,12].

Liquid–liquid extraction methods has been used

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for the extraction and purification of steroids present in different animal tissues [13,14]. Recently a liquid–liquid extraction method named diphasic dialysis has been used for the extraction and purification of clenbuterol present in cattle urine, liver and hair [15–17]. This technique enables the direct extraction of analytes in organic extracts without the need of complementary purification steps [18,19].

Gas chromatography–mass spectrometry has been one of the confirmatory techniques of preference for the detection of steroids residues present in different animal tissues [20,21]. However, a large sensitivity can be achieved by using GC–MS^n [22].

In this study, we developed a method for the determination of ethinylestradiol present in the hair of cattle by using diphasic dialysis for the extraction and purification step, and GC–MS^2 for the confirmatory analysis.

2. Experimental

2.1. Reagents

Acetic acid, dichloromethane, ethyl acetate, *tert.*-butylmethyl ether, *n*-hexane, sodium hydroxide, sodium acetate and Tween 80 were purchased from Merck (Darmstadt, Germany). *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA), Iodotrimethylsilane (TMIS), DL-dithiothreitol (DTT), methyltestosterone, nandrolone, ethinylestradiol and testosterone d_3 were supplied by Sigma (St. Louis, MO, USA). Dialysis tubing was of the type 20/32, of regenerated cellulose with a molecular exclusion size of 10 000 Da (Visking, Serva, Feinbiochemical, Heidelberg, Germany). All the reagents and solvents were of analytical grade.

Stock solutions of the anabolic steroid was prepared in methanol at 100, 1 and 0.1 μg/ml and stored at −20°C. The internal standard (testosterone d_3) was also prepared in methanol at a concentration of 100 and 1 μg/ml. These solutions could be stored under cooling for no longer than 2 months.

The derivatizing agent was a mixture of MSTFA–TMIS–DTT (1000:5:5, v/v/w) and stored at -20 °C for 1 week.

2.2. Sample pretreatment

To remove potential external contamination with steroids, hair sample (400 mg) was washed three times with an aqueous solution of Tween 80 (10%), followed by three rinses with distilled water. This method was proposed by Fente and co-workers for the analysis of clenbuterol present in the hair of cattle [14]. The last wash was evaporated, derivatized and then analyzed to verify the absence of the steroid. The hair was dried at 40°C and cut finely with a scissors.

For the extraction of the steroid from hair, 200 mg was introduced into a glass tube; 50 μl of the internal standard solution (1 μg/ml) were added together with 1 ml of NaOH (1 *M*). The digestion was accomplished in a water bath at 100°C for 10 min.

2.3. Purification of the steroid

The digested hair was placed in a 500-ml beaker and the pH was adjusted to 8.4 with acetate buffer 0.2 *M*. Then, a previously wetted 25-cm long dialysis tubing, with an exchange surface of ca. 196 cm^2, and containing 25 ml of dichloromethane (extraction solvent), was introduced in the beaker. The extraction was performed by stirring at 150 rpm and 37°C for 4 h.

The content of the dialysis tube was then poured into a glass tube and evaporated to dryness under a nitrogen stream at 60°C.

2.4. Derivatization

The trimethylsilyl (TMS) derivatives of the steroids was produced by adding 50 μl of the derivatizing mixture. After vortex mixing, it was heated for 30 min at 60°C.

2.5. Apparatus

Thermostated incubator shaker, Model G25 & R25 (New Brunswick Scientific, Edison, NJ, USA).

Nitrogen evaporation system, with thermostated heating plate (New Brunswick Scientific, Edison, NJ, USA).

Table 1
Main MS^2 parameters for ethinylestradiol and the internal standard

Substance	Parent ion *m/z*	Isolation time (ms)	Excitation voltage (V)	Excitation time (ms)	Product ions *m/z*
Ethinylestradiol	425	12	0.90	30	397, 407
Testosterone d_3 (I.S.)	435	12	0.90	21	330, 420

The gas chromatograph was a TRACE GC 2000 series (Thermo Quest, Italy), equipped with an AS 2000 autosampler from Thermo Quest (Italy). The GC system was interfaced to a Finnigan GCQ_{plus} ion trap mass spectrometer (USA). Chromatographic separation was performed by using a capillary column (HP-5 MS; crosslinked 5% phenylmethylpolisiloxane; column length 30 m×0.25 mm with a 0.25-μm film thickness) from Agilent Technologies (USA). The GC temperature program was as follows: the initial temperature was 120°C for 2 min, then a temperature programme of 15°C/min to a temperature of 240°C which was held for 2 min, and increased to a final temperature of 300°C at 5°C/min. Samples (2 μl) were injected in the splitless mode. The injector temperature was set at 250°C. Helium was used as carrier gas at a flow-rate of 1 ml/min. The transfer line temperature was 250°C and the ion source temperature was 200°C. The MS instrument was operated in the electron impact ionization mode. For collision induced dissociation in MS^2, helium was used as collision gas. Other MS^2 instrumental conditions for the detection of trimethylsilyl derivatives of ethinylestradiol and the internal standard are indicated in Table 1.

3. Results and discussion

3.1. Diphasic dialysis extraction

For the extraction, some parameters of the technique were optimized such as pH, solvents, temperature, time, and agitation speed. All of the assays were performed three times with 200 mg of hair spiked with 50 μl of 1 μg/ml of ethinylestradiol. The results shown here are average values ($n=3$).

The first parameter studied was the pH. The pH values studied were between 3.8 and 10.8. The best results were achieved at pH 8.4. This was so because of the ionization of the steroids at lower or higher pH values, which diminish the recovery of the molecules.

Four extraction solvents were tested: dichloromethane, ethyl acetate, *tert*.-butylmethyl ether, *n*-hexane. Solvents were chosen according to their lack of miscibility with water. Dichloromethane was found to be the best solvent for the extraction of the steroid (with a 88% recovery). With this solvent, relatively clean extracts were obtained. With the use of the

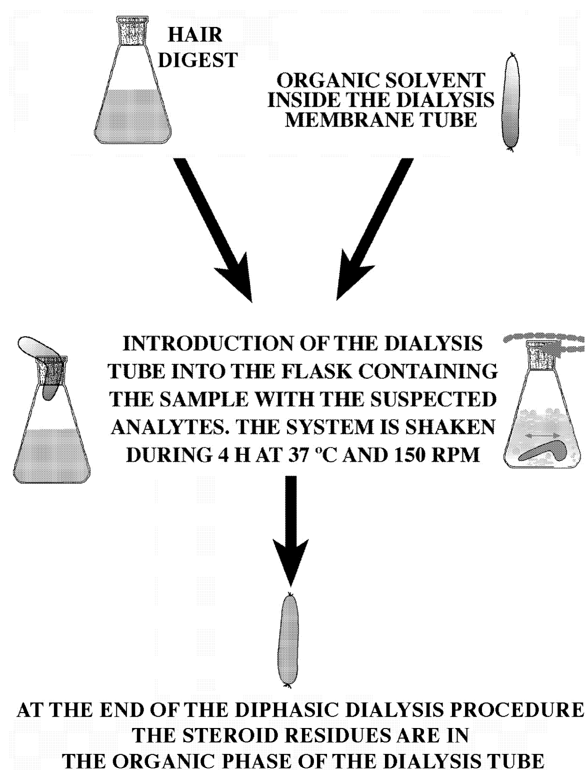
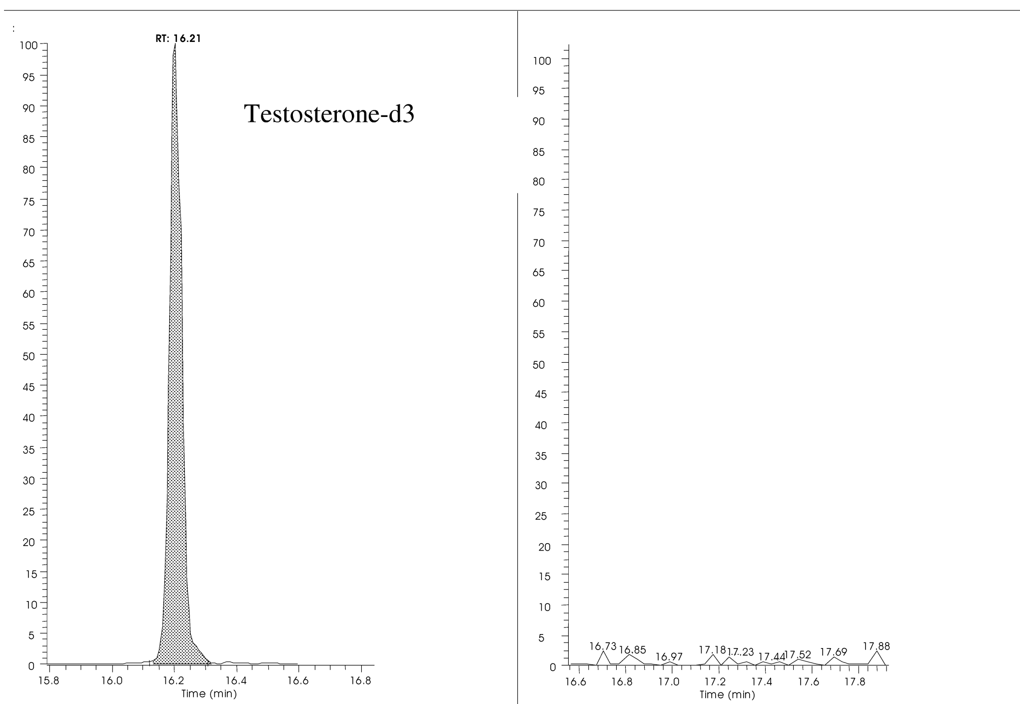


Fig. 1. Diphasic dialysis procedure for the extraction/purification of ethinylestradiol in cattle hair.



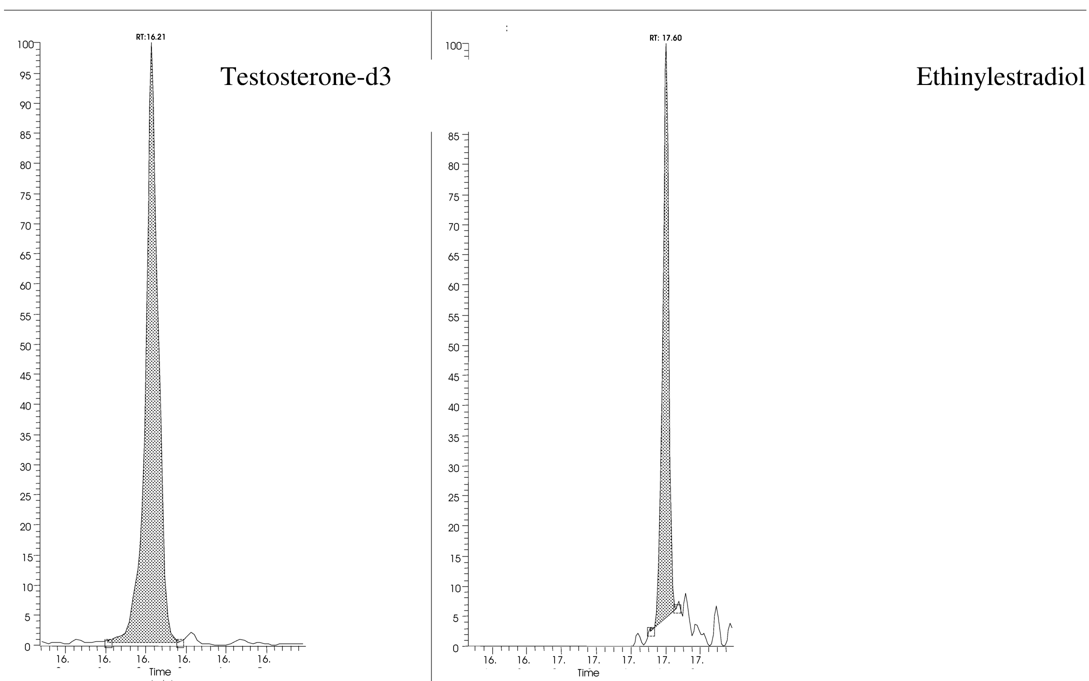


Fig. 2. (a) Total ion current (TIC) (sum of ions 397, 407 and 425) chromatogram corresponding to an animal hair before the treatment with 164 ng of ethinylestradiol. (b) Total ion current (TIC) of a positive sample obtained from the same animal, 35 days after the treatment.

other solvents indicated the recovery was always less than 70%.

The study for the diphasic dialysis optimum temperature was undertaken for values between 30° and 40°C. It was observed that extraction performance increased with temperature. The best recovery was obtained at 37°C. At higher temperatures an evaporation of the solvent was noticed.

Also, a better recovery can be obtained with an increase in extraction time, which reached a maximum when the extraction procedure was carried out at 4 h. It was noted that at 5 h no better recovery of ethinylestradiol was obtained.

Three agitation speeds were studied (100, 150 and 200 rpm). At 100 rpm the extraction was not effective (60%). At 200 rpm an evaporation of the extraction solvent was observed. The best recovery was obtained at 150 rpm. With the optimized conditions, a recovery percentage of 88% was achieved ($n=5$). Fig. 1 illustrates the diphasic dialysis procedure.

3.2. Gas chromatography–tandem mass spectrometry detection

The criterion followed for the election of the parent ion for the TMS derivative of ethinylestradiol were those of selectivity and intensity of the ion. After the formation of ions by electron impact ionization in the SCAN mode, the ion selected was m/z 425.

By using helium as a collision gas, the fragmentation of the parent ion was carried out to produce the daughter ions, which were selected by taking into account their selectivity and intensity. In this way, the m/z 397, 407 and 425 ions were selected for identification of ethinylestradiol in the SIR mode.

For the confirmatory analysis of residues of the drug present in the hair of cattle, the criteria specified for GC–MS^n and stated in the draft revised version of Commission Decision 93/256/EC, January 1999, was used [23]. Following this approach when GC–MS^n is used, at least two transition ions have to be monitored (m/z 407 and 397 for ethinylestradiol), and at least one ion ratio must correspond to that of the standard analyte at the same concentration between a permitted tolerance (397/407 and/or 425/407 for ethinylestradiol).

3.3. Validation

The standard calibration curve was obtained by adding to 500 mg of hair (ethinylestradiol free) 20, 40, 60, 80 and 100 μl of the ethinylestradiol (0.1 ng/μl) and 50 ng of testosterone-d3 ($n=3$). The linear regression equation was $y=0.141x-0.002$, with $r=0.998$.

Samples for intra- and inter-day assays and for recovery tests were prepared at three different concentrations, 4, 12 and 20 pg/mg. Each sample was analysed three times a day for 3 days. The relative standard deviations (RSD) of the intra-day precision ($n=3$) was 16.7%, and the inter-day precision ($n=9$) was 18.0%, both corresponding to a concentration of 4 pg/mg.

The overall recovery ($n=9$) was 74% at 4 pg/mg; 90% at 12 pg/mg and 94% at 20 pg/mg. It is likely that some hydrolysis took place, more so at low concentrations of the drug.

The detection limit (LOD), defined as the lowest value that differs from the blank with a 90% of confidence level (mean blank value + 3 standard deviations), was 0.52 pg/mg. The quantification limit (LQD), defined as mean blank value + 10 standard deviations, was 0.80 pg/mg.

The chromatograms in Fig. 2 correspond to a blank hair sample taken from a Rubia Gallega breed cattle (3 months old with a yellow/red hair colour), and to a positive hair sample took from the same animal 35 days after the intramuscular injection of 164 mg of ethinylestradiol (5.48 pg/mg ethinylestradiol).

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