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Gas Chromatography−Tandem Mass Spectrometry Determination of 17α-Ethinylestradiol Residue in the Hair of Cattle. Application to Treated Animals

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A gas chromatography−tandem mass spectrometry (GC−MS^2) method for the detection and quantification of 17α-ethinylestradiol in the hair of cattle has been developed, and uses an ion trap analyzer. After the digestion of 500 mg of hair by alkaline digestion using 1 M NaOH, extraction and purification of the steroid were performed in the same step by means of diphasic dialysis. This tecnique is a semipermeable-membrane technology developed for the direct extraction of relatively low-molecular-mass analytes. The process was performed by employing acetate buffer to homogenize the digested hair, dichloromethane as the extraction solvent at 37 °C, and stirring at 150 rpm for 4 h. The recovery was between 74 and 94%. The detection limit was 0.52 ng/g in hair. To evaluate the validity of the methodology, five animals, approximately 3 months old, received an intramuscular anabolic dose of the drug. The xenobiotic could be detected 7 or 14 days after the treatment (between 2.01 and 23.61 ng/g), and until the end of the study (day 98). No statistical difference between hair color and hair assay outcomes was found.

KEYWORDS: 17α-Ethinylestradiol; hair; diphasic dialysis; tandem mass spectrometry

INTRODUCTION

Several synthetic steroids, such as 17α-ethinylestradiol, have been used by farmers in meat production because they improve protein deposition, and consequently, increase the body weight of animals. Nevertheless, their use is forbidden within the European Union because of the carcinogenic potential of their residues (EEC Directive, 96/22/EEC, 1996, No. L 125/3, April 1996.). The presence of residues of this steroid in any animal tissue or fluid is proof of illegal administration. Urine (*1*, *2*), blood (*3*, *4*), muscle (*5*, *6*), and feces (*7*, *8*) have been used for the detection of steroids employed for cattle fattening. Different extraction and purification methodologies have been used, followed by screening and confirmatory techniques for the final analysis.

In the late 1990s, studies were started with the aim of detecting the presence of natural and exogenic steroids in the hair of cattle by using screening methods (*9*, *10*). Studies carried out in the field of forensic science in humans by using hair for the detection of many drugs have demonstrated that it has the advantage over other matrixes, such as urine and blood, that once analytes are incorporated into the queratinized fiber they can be retained there for months, whereas the detection period in urine or blood is only a few days (*11*). Also, many investigators have demonstrated, in their in vivo and in vitro studies, the affinity of natural and synthetic melanins to various drugs. It is generally accepted that melanin-containing tissue has the ability to accumulate and retain drugs (*12*)

In bovines, clenbuterol could be detected in the hair of calves treated with therapeutic and growth-promoting doses for months by using gas chromatography/mass spectrometry for detection (*13*, *14*). Also, research has been carried out with the aim of detecting the presence of anabolic steroids in the hair of livestock by using this same detection technique (*15*, *16*).

The development of confirmatory methods that lead to the determination of xenobiotic steroids used in cocktails at low concentrations in an illegal way for meat production are necessary. In this sense, the use of tandem mass spectrometry (MS^n), coupled to a gas or liquid chromatograph, has been gaining acceptance over GC−MS and LC−MS as an analytical method for the analysis of steroids present in complex biological matrixes because of its major sensitivity and selectivity (*4*, *17*, *18*). In comparison to GC−MS, GC−MS^n has several distinct advantages. First, complex mixtures can often be analyzed by MS^n with little or no sample cleanup. Second, the primary separation of sample components is analogous to that of GC−MS, but is rapid and is not restricted to a specific time. Last, MS^n strategies may have a wider range of applicability because compounds of a wider volatility range are more amenable to analysis by mass spectrometry than by GC (*19*).

The present study concerns the application of an analytical method developed for the detection and quantification of 17α-

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ethinylestradiol residue present in the hair of cattle treated by means of intramuscular injection with anabolic dosage of the drug. For the extraction/purification of ethinylestradiol, after alkaline digestion, diphasic dialysis was used and quantification was by GC−MS^2. Also, aspects such as the time in which the xenobiotic could be detected in hair, and the relationship between the outcomes of hair analysis and hair color are evaluated.

MATERIALS AND METHODS

Materials and Chemicals. Acetic acid, dichloromethane, methanol, sodium hydroxide, sodium acetate, and Tween 80 were purchased from Merck (Darmstadt, Germany). *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA), iodotrimethylsilane (TMIS), d,L-dithiothreitol (DTT), arachis oil, benzyl alcohol, 17α-ethinylestradiol, and testosterone d_3 were supplied by Sigma (St. Louis, MO). All the reagents and solvents were of analytical grade.

Stock solutions of the anabolic steroid were prepared in methanol at 100, 1, and 0.1 μg/mL; the internal standard (testosterone d_3) was also prepared in methanol at concentrations of 100 and 1 μg/mL. These solutions were stored at −20 °C.

The derivatizing agent was a mixture of MSTFA, TMIS, and DTT (1000:5:5, v/v/w).

Sample Pretreatment. To remove potential external contamination with steroids, hair samples (400 mg) were washed three times with an aqueous solution of Tween 80 (10%), followed by three rinses with distilled water. 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 scissors.

For the extraction of the steroid from hair, 500 mg was introduced into a glass tube; an amount of 50 μL of the internal standard solution (1 μg/mL) was added together with 2.5 mL of NaOH. The digestion was accomplished in a water bath at 100° C for 10 min.

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 tube of regenerated cellulose (with an exchange surface of ca. 196 cm^2 and a molecular exclusion size of 10 000 Da (Visking, Serva, Feinbiochemical, Heidelberg, Germany) and containing 25 mL of dichloromethane extraction solvent) was introduced into the beaker. The extraction was performed by stirring at 150 rpm at 37° C for 4 h in a thermostated incubator shaker (model G25 & R25, New Brunswick Scientific, Edison, NJ).

The dialysis contents were then poured into a glass tube and evaporated to dryness under a nitrogen stream at 60° C for derivatization.

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

Gas Chromatography−Tandem Mass Spectrometry. Samples (2 μL) were injected, in the splitless mode, into a TRACE GC 2000 gas chromatograph (Thermo Quest, Italy), equipped with an AS 2000 autosampler (Thermo Quest, Italy). The GC system was interfaced to a Finnigan GCQ plus ion trap mass spectrometer (San Jose, CA). Chromatographic separation was performed by using a capillary column (HP-5 MS; cross-linked 5% phenyl-methylpolysiloxane; column length 30 m × 0.25 mm with a 0.25-μm film thickness) from Agilent Technologies (Palo Alto, CA). The GC temperature program was as follows: initial temperature was 120 °C for 2 min, then a temperature program 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. 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 electronic impact ionization mode. For collision-induced dissociation in MS^2, helium was used as collision gas. Other MS^2 instrumental conditions for the trimethylsilyl derivatives of 17α-ethinylestradiol and the internal standard are indicated in Table 1.

Table 1. Main Ion Trap Mass Spectrometer Parameters for MS^2 Detection of 17α-Ethinylestradiol

substance	parent ion *m/z*	isolation time (ms)	excitation voltage (V)	excitation time (ms)	product ions *m/z*
17α-ethinylestradiol	425	12	0.90	30	397, 407 (**t**[a])
testosterone d_3 (I. S.)	435	12	0.90	21	330, 420 (**t**)

[a] **t** denotes target ion.

In vivo Study. Seven calves of the Rubia Gallega (4) and Holstein (3) breeds (approximately 3 months old) weighing 99 ± 2 kg were put in individual cages. Animals had free access to hay and water. Two animals were used as untreated controls. The rest were treated with anabolic doses of ethinylestradiol (61.3 mg/mL in a mixture of arachis oil and benzyl alcohol; 2 mg of 17α-ethinylestradiol/kg of body weight) via intramuscular injection. Hair samples of all animals were taken before the treatment, and after the treatment for three months. Approximately 1 g of hair was cut each time from the flanks of the animals by using electric clippers (Oster, Boca Raton, FL).

RESULTS AND DISCUSSION

Validation of the Method. To ensure the confidence of the results obtained by the analysis of samples arising from treated animals, a validation of the analytical method was carried out.

The linearity of the method over the concentration range was determined by adding to 500 mg of hair (17α-ethinylestradiol free) 20, 40, 60, 80, and 100 μL of the 17α-ethinylestradiol (0.1 ng/μL) and 50 ng of testosterone-d_3. Each sample was repeated three times. The linear regression equation was $y = 0.14088x - 0.00235$ ($r = 0.998$).

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

The overall recovery ($n = 9$) was 74% at 4 ng/g, 90% at 12 ng/g, and 94% at 20 ng/g.

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

Sample Pretreatment. Hair is a complex structure made in the hair follicle. During its formation in hair follicles substances are incorporated in the matrix following different mechanisms (*12*), and being retained there for months. For the extraction of the analyte from hair some aspects of the extraction procedure have to be kept in mind, such as the type of drug to be analyzed, the relative distribution of metabolites, and the final detection method.

17α-ethinylestradiol could be extracted efficiently by using the extraction method described here. Alkaline digestion enabled the total destruction of the keratinized fiber, providing a complete release of the steroid in a short period of time. Good extraction recoveries could be achieved (more than 70% in all cases).

Sample Cleanup. Diphasic dialysis has been used in the extraction/purification step. This technique was used for the extraction/purification of clenbuterol present in bovine hair (*14*).

The use of this technique enables extraction of 17α-ethinylestradiol present in the alkaline digestion medium and the purification of the steroidl in the same step. This way, the

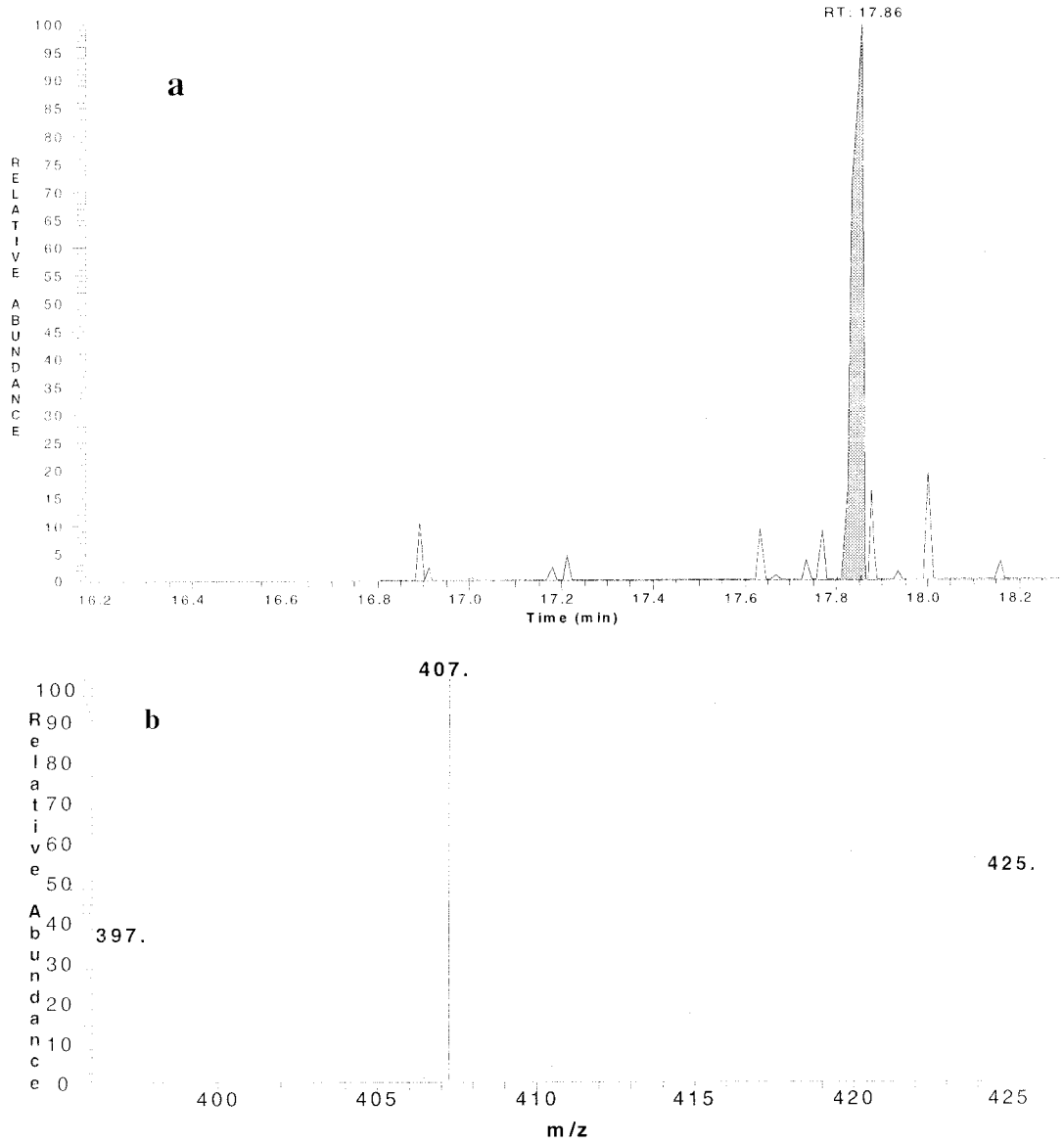

Figure 1. Detection of 17α-ethinylestradiol in the hair of calves. (a) Chromatogram from a calf hair (from Calf 2) sampled 42 days after intramuscular injection of 2 mg of 17α-ethinylestradiol/kg of body weight. (b) Mass spectrum of the above chromatogram, used for the detection: *m*/*z* 425 (parent ion) and 397, 407 (daughter ions of *m*/*z* 425).

drug is extracted from the aqueous medium because of its solubility in the extraction solvent (dichloromethane), and by virtue of its molecular weight, because only low-molecular-weight substances (such as ethinylestradiol) can cross the dialysis membrane without any need for an additional purification step. No substance present in the digestion alkaline medium that could interfere with the detection of the steroid was extracted. Also, the performance of this method allows a good recovery (94% at 20 ng/g; $n = 9$).

Gas Chromatography−Tandem Mass Spectrometry. For the performance of the MS^2 analysis using an ion trap analyzer, the criterion used for the selection of the parent ion was that the ion selected should have the higher *m*/*z* ratio, to obtain the greatest selectivity. Also, this ion should have the highest intensity to increase the sensitivity. Thus, the parent ion chosen for 17α-ethinylestradiol was *m*/*z* 425.

The fragmentation of the precursor ion was performed by collision-induced dissociation (CID) with helium molecules; but first the suitable isolation of this ion had to take place. This is done by applying to the endcap electrodes of the ion trap an isolation waveform voltage during an optimum time (isolation time). The main parameters used for the subsequent fragmentation of the parent ion are the excitation voltage and the excitation time. The excitation voltage and excitation time selected were those which made it possible to obtain a spectrum with abundant fragment, and the presence of a minimal, but representative, amount of the parent ion. Thus, three ions were selected to perform the analysis in the SIR mode. The main instrumental optimum parameters used for identification of 17α-ethinylestradiol and the internal standard are provided in Table 1.

For the confirmation of 17α-ethinylestradiol residue in the hair of cattle, the revised version of Commission Decision 93/256/EC (*20*) was used. When $GC-MS^n$ is employed as a confirmatory method at least two transition ions have to be monitored. The relative intensities must correspond to those of the standard analyte, either from calibration standards or from spiked samples at comparable concentrations.

Application to Treated Cattle. To evaluate the usefulness of the method for detecting 17α-ethinylestradiol in the hair of cattle, and other aspects related to incorporation of the drug into the matrix, five animals of the Rubia Gallega (with white and yellow coats) and Holstein (with black coat) breeds were

Table 2. Concentration of 17α-Ethinylestradiol in Hair of Calves Treated with the Anabolic Steroid (ng/g)

days	calf 1 (black)	calf 2 (black)	calf 3 (white)	calf 4 (yellow)	calf 5 (yellow)	calf 6 (black)	calf 7 (yellow)
0[a]	–[b]	-	-	-	-	-	-
7	-	2.01	-	-	10.73	-	-
14	18.63	1.53	-	10.00	10.25	-	-
28	2.48	5.43	19.87	11.60	7.15	-	-
42	6.39	16.46	17.97	12.07	3.45	-	-
56	3.64	13.83	3.86	7.43	4.84	-	-
70	4.13	3.33	3.89	3.96	3.06	-	-
84	-	4.89	3.91	9.14	8.78	-	-
98	4.39	5.42	-	2.92	2.82	-	-

[a] Sample taken before the treatment. [b] - indicates below the limit of detection

treated. Two calves (one of each breed) were used as experimental controls; and during the entire study no 17α-ethinylestradiol was detected in the hair of these animals (Table 2).

One mechanism proposed for the incorporation of drugs into hair matrix is that in which the substances present in the blood stream cross the matrix cells plasma membrane (in the growing hair) at the base of the hair follicle. Once the drug is in the cell, it will dissociate depending upon its pk_a value (*21*). Depending upon the lipid solubility of the substance and the pH of the medium, the drug will distribute in different cell compartments. Drugs are retained in the keratinized fiber by virtue of their interaction with other cell components such as melanin, proteins, etc (*12*).

The growth rate of hair is approximately 6−7 mm per month, whereas the thickness of the skin is between 2.5 and 3 mm for a three-month old calf. Also, during the anagen period (the period of the cycle of hair growth when drugs can be incorporated into the fiber), the follicle is located at 75−85% of the skin's depth(*13*). Once ethinylestradiol present in the blood stream is incorporated in the hair fiber under the animal's skin, a delay in the detection of the drug is expected, because of the time that passes between this incorporation and the appearance of the hair at the animal's skin surface (7 or 14 days after the treatment, Table 2).

17α-ethinylestradiol could be detected in the hair of all treated animals up to three months after the administration of the drug. Figure 1 shows a chromatogram and the mass spectrum of 17α-ethinylestradiol, detected 42 days after the treatment.

The small concentration of 17α-ethinylestradiol detected in hair can be explained in terms of the physiochemical properties of the drug and the permanent replacement of hair. The influences of melanin affinity, lipophilicity, and membrane permeability on drug incorporation were studied by Nakahara et al. (*22*). Substances such as steroids have quite low incoporation rates into hair. This is due to the low membrane permeability of these neutral drugs based on the pH gradient between blood and the acidic matrix, despite their high lipophilicity. Second, the concentration of the xenobiotic in hair decreases with time because of the natural replacement of the hair of these animals. Despite these disadvantages, the methodology in this work using an ion trap mass spectrometer analyzer enabled the detection of low quantities of 17α-ethinylestradiol.

It has been suggested that pigmentation may play an important role in the incorporation of certain drugs into hair (*12, 23, 24*). This would be due to the presence of melanin which is responsible for the color of pigmented tissues such as hair (*25*). It is believed that a type of adsorption occurs between the particular drug and the melanin polymer (and/or melanin granules), in which forces such as charge-transfer reactions (the melanin being the electron receptor), as well as electrostatic forces occur. Other interactions, such as hydrophobic and ionic attraction, may also take place. Drugs entrapped by melanins originate a pemanent concentration gradient into the melanocyte which will be higher for drugs with high melanin affinity (*26*).

The relation between the concentration of 17α-ethinylestradiol detected and hair color was also studied. Statistical evaluation of differences in sample frequencies has been done by goodness-of-fit Chi square analysis (χ^2), with a significance level (α) of 0.05. The results of the test suggest that there are not any significant statistical differences between hair color and hair assay outcomes. It is expected that the steroid may be bound to lipids or proteins inside the keratinized hair.

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