Determination of Synthetic Estrogens in Illegal Veterinary Formulations by HPTLC and HPLC

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Abstract

To determine the actual amount of diethylstilbestrol, hexestrol, and dienestrol in formulations such as pellets and oily injections that are illegally available on the Brazilian market, a simple methanol extraction is used for the analysis of the pellets and an ether extraction with Sephadex columns (for clean-up) is used for the oily injections. High-performance thin-layer chromatography is used for identification (as a qualitative and semiquantitative method), and high-performance liquid chromatography is used for quantitation. The results of the analysis show that all the formulations are not in accordance with the information listed on their labels.

Introduction

Diethylstilbestrol (DES), a synthetic compound with strong estrogen activity, has been widely used as a growth-promoting agent in animal breeding. Since 1979, the use of this drug has been banned in most countries, including Brazil, because of its possible estrogenic and carcinogenic effects on humans (1,2). Hexestrol (HEX) and dienestrol (DIE) are structural analogues of DES that are used for the same purpose.

Although illegal, these substances, particularly DES, are thought to be widely used in Brazil. They are applied as implants or additives to feed. Pharmaceutical formulations of these products, such as pellets and oily injections, are found only on the black market, and consequently, it is not possible to assure either their actual content or the quality of the active principle.

We determined the real amount of these substances in commercially available formulations on the Brazilian market. High-performance thin-layer chromatography (HPTLC) was used for qualitative and semiquantitative analysis and high-performance liquid chromatography (HPLC) was used for quantitation.

Experimental

Chemicals and materials

cis-trans-DES, HEX, and DIE were obtained from Sigma Chemical (St. Louis, MO). Chloroform, methanol, acetone, ethyl acetate, cyclohexane, ethanol, acetonitrile, and dichloromethane were analytical reagent grade (Merck; Darmstadt, Germany). The water used in the HPLC mobile phase was freshly distilled, deionized, and purified in Milli-Q equipment (Millipore; Bedford, MA). Precoated high-performance silica gel GF254 plates (10 × 10 cm, 0.25 mm) (Merck) were used as purchased. Sephadex LH-20 columns were from Sigma.

Preparation of standards

A standard mixture of *cis*- and *trans*-DES (1 mg/mL) was prepared by dissolving the compounds in methanol and allowing the isomerization to equilibrate. Only two peaks were observed by HPTLC and HPLC, and it was assumed that the *cis* and *trans* isomers were present. Standard solutions of HEX and DIE (1 mg/mL) were also prepared in methanol. Working solutions (10 µg/mL) were prepared daily. A methanolic solution of dexamethasone (5 µg/mL) was used as the internal standard.

HPTLC conditions

Sample solutions as well as 0.001% standard solutions of the drugs studied were applied to plates with a PB600 dispenser equipped with a 50-µL Hamilton syringe (Reno, NV) at concentrations of 50, 250, and 500 ng. The solvent system used for the pellets consisted of chloroform–acetone (9:1, v/v); however, for the oily formulation, the solvent systems that consisted of chloroform–acetone (9:1, v/v) and cyclohexane–ethyl acetate–ethanol (77.5:20:2.5, v/v) in a bidimensional development were preferred. The development distances for both solvent systems were 8 cm, and the chromogenic agent was 95% ethanol–concentrated sulfuric acid (9:1, v/v). The plates were air dried, examined under UV light at 254 nm, sprayed with the chromogenic agent, and heated at 110°C for 10 min until a characteristic gray color appeared. The spots were examined under visible and UV light (254 and 366 nm).

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HPLC conditions

A Hewlett-Packard (Palo Alto, CA) 1090M liquid chromatograph equipped with a UV diode-array detector was used. An ODS-Hypersil column (100 × 4.6-mm, 5-µm particle size) (Hewlett-Packard) was used. The mobile phase of methanol–water (60:40, v/v) was isocratic and had a flow rate of 1.0 mL/min. Wavelengths of 240 nm for DES and 230 nm for HEX were used. The injected volume was 10 µL.

Samples

Samples 1–13 were Vi-Gain round pellet formulations (15 mg DES); samples 14–16 were Vi-Gain liquid formulations (60 mg DES); and samples 17 and 18 were Hexettes (15 mg HEX and DES). The quantities mentioned are those indicated on the labels.

Sample treatment

The pellets—three from each lot—were ground and extracted with 10 mL methanol for 20 min with vigorous shaking. From this solution, two aliquots of 100 µL each were taken. The first one was diluted again with 10 mL methanol and used for HPTLC determinations. The second one was added to 1 mL of a methanolic standard solution of dexamethasone (5 µg/mL) and diluted to 10 mL with methanol. This solution was used for HPLC quantitation. All samples were analyzed in replicate (n = 3).

One milliliter of the injectable preparations, as the oily formulation, was diluted in 10 mL diethyl ether, and 1 mL was passed through a 5-cm Sephadex LH-20 column. The drug was eluted with 8 mL dichloromethane–acetonitrile (94:6, v/v). The first 3 mL was discarded, and the remaining 5 mL was collected, evaporated under a gentle stream of nitrogen, and finally resuspended in 1 mL methanol.

Quantitation and validation procedures

The calibration curves for quantitation of DES and HEX were prepared by repeatedly injecting (n = 5) a mixture of these substances in the following concentrations: 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 µg/mL DES and 0.06, 0.125, 0.25, 0.50, 1.0, 2.0, 4.0, 8.0, and 16.0 µg/mL HEX. Linear least-squares equations were used to determine the area ratio of each analyte peak to that of the internal standard.

The precision was determined for each concentration, and these values were used to evaluate the limit of detection (LOD) according to the procedure described by Armbruster and coworkers (3). The limit of quantitation (LOQ) was determined as the lowest concentration at which the coefficient of variation was 15% or less.

Concentrations of 1.6 and 12.8 µg/mL DES and 2.0 and 16 µg/mL HEX were used for the determination of intra- and interday precision (n = 5). Both determinations were performed by different analysts, and the samples were analyzed in two consecutive days for the interday test.

For the recovery study, a standard addition approach was used; that is, six pellets from one formulation (lot 6) were ground and divided into two portions, A and B. Portion A was analyzed (n = 3) according to the procedure described for HPLC. Portion B was added to 15 mg DES, adequately homogenized, and submitted to the same procedure (n = 3). The quantitation of both portions A and B were determined using the calibration curve, and the recovery was calculated from these values.

Results and Discussion

This work was initiated as a result of an apparent failure in an experiment in which bovines were implanted with DES pellet formulations to obtain urine and meat samples for positive controls. Because the results of the experiment were negative and the methodology used for the detection of DES in urine and meat was reliable, we decided to check the actual amount of the drug in the formulations. Therefore, formulations were obtained from different sources during a period of 2 years. Despite the long period used to collect the samples, most of the labels showed the same lot number.

The results of the HPTLC and HPLC analyses of the 18 formulations of DES and HEX are shown in Table I. The claimed contents of all formulations were imprecise. The contents of the oily formulations, as specified by the labels, were particularly confusing in relation to the exact amount per milliliter. The results of the analyses of the three oily injections showed that the whole flask (100 mL) contained no more than 2 mg, and, as stated in the literature (3–6), a much greater concentration than that would be necessary to produce an anabolic effect. Of 13 Vi-Gain pellets analyzed, four had negative results for the

Table I. Results of the Analysis of Commercial Formulations of Synthetic Estrogens

Sample*	HPTLC†	HPLC† (mg per pellet)	% Label claim per pellet
1	DES†	3.970	26.5
2	DES	2.299	15.3
3	DES	1.666	11.1
4	DES	2.987	19.9
5	DES	11.359	75.7
6	ND†	NQ†	–
7	HEX†	16.644	–‡
8	DES	5.568	39.1
9	ND	NQ	–
10	ND	NQ	–
11	ND	NQ	–
12	DES	1.775	11.8
13	DES	1.475	9.8
14	DES (2.0 mg)	NQ	3.33
15	DES (2.0 mg)	NQ	3.33
16	DES (1.5 mg)	NQ	2.50
17	ND	NQ	–
18	ND	NQ	–

* Samples 1–13, Vi-Gain pellets; samples 14–16, Vi-Gain liquid; samples 17 and 18, Hexettes.
† Abbreviations: HPTLC, high-performance thin-layer chromatography; HPLC, high-performance liquid chromatography; DES, diethylstilbestrol; ND, not detected; NQ, not quantitated; HEX, hexestrol.
‡ Label claimed DES was present, but HEX was detected.

analyzed drugs. In one sample in which DES should have been found, HEX was found. The oily formulations were not quantitated by HPLC but semiquantitated by HPTLC; the three samples had DES concentrations much smaller than claimed. The two Hexettes formulations did not contain HEX or DES. The experiment was also conducted to detect the presence of DIE, although none of the samples showed detectable amounts of this drug.

The contents of the active substances in the Vi-Gain pellets measured by HPLC showed amounts that varied from approximately 11% to 75% of the label claim. Sample 7 had no DES but had a considerable amount of HEX (16.64 mg). The results presented in Table I are the mean values of three assays for each sample.

In the HPLC quantitation of DES, only the *trans* peak was considered because the *cis* peak, in all analyzed samples, could not be detected or was shown to be present in negligible amounts that would not add appreciably to the whole amount of the active principle. These findings were also in agreement with those found by Lea and co-workers (7).

The preparations of the oily formulation presented analytical problems because the anabolic portion is soluble in vegetable oils, which complicates its extraction and quantitation. The extraction procedure and the spectrophotometric quantitation described by El-Yazbi and co-workers (8) were tested; however, we failed to reproduce their results. Cameroni and co-workers (9) reported good results using a cyano column for the separation of hormonally active substances by HPLC without previous extraction of the drug from its medium. However, in the present experiment, to protect the analytical HPLC column and to have an adequate residue for HPTLC, we purified the diluted samples using Sephadex columns (10). Even when this procedure was used, a small portion of the oil remained in the extract, which initially caused some difficulty in properly visualizing the drugs with HPTLC. This problem was solved by the use of an adequate solvent system. The one described by Moretti and co-workers (11) was tested in a one-dimensional development; however, the best results were obtained in a bidimensional run with chloroform–acetone and cyclohexane–ethyl acetate–ethanol as the solvent systems. By using these two solvent systems, we separated the remaining oil from *cis*- and *trans*-DES; this separation allowed their semiquantitation.

For the pellets, the first step in the procedure was similar to the one used by De Beer (12), which involved a simple extraction with methanol. Because of the difficulties in preparing pellets with a known amount of DES, the recovery was achieved based on a standard addition approach. The value obtained was 90.5%. Lea and co-workers (7) described a similar method of extraction in which DES tablets were extracted with methanol–water (75:25, v/v), and the mean recovery was 100.8%.

Dexamethasone was used as the internal standard in the HPLC analysis for the following reasons: It had the best absorbance in the range 230–254 nm; its retention time was close to those of DES and HEX in the chosen solvent system; and, most important, it was not likely to be found in this type of formulation. Any other anabolic agent that fit the first two requirements could not be used as the internal standard because of its possible presence in the analyzed formulations.

Table II. Results of Intraday and Interday Analysis of DES and HEX

Substance*	Concentration (µg/mL)	Intraday (% CV)	Interday (% CV)	
			Day 1	Day 2
DES	1.6	12.8	7.5	7.6
DES	12.8	4	4.8	4.9
HEX	2.0	13.4	8.3	8.4
HEX	16.0	4.2	13.4	5.7

* Abbreviations: DES, diethylstilbestrol; HEX, hexestrol.

The results of HPTLC were similar to those of HPLC; the detected substances and their quantities were in agreement for both techniques. The LOD for the studied drugs on HPTLC plates was 20 ng.

The response of the UV detector was found to be linear over the range studied. The retention times for *trans*-DES, *cis*-DES, HEX, and dexamethasone were 5.4, 8.3, 6.1, and 2.9, respectively.

Linear calibration curves were obtained for peak area of the internal standard versus the different concentrations of DES and HEX; the equations and correlation coefficients for DES and HEX were $y = 0.346x + 0.007$, $r^2 = .9998$ and $y = 0.375x + 0.002$, $r^2 = .9998$, respectively.

The LODs for DES and HEX were 0.20 and 0.25 µg/mL, respectively, and the LOQs were 0.40 and 1.0 µg/mL, respectively.

The mean precision values expressed as coefficients of variation were 7.4% for DES (range, 1.2–14.9) and 7.3% for HEX (range, 2.2–13.4). The results of the intraday and interday analyses are shown in Table II.

Conclusion

Although the number of samples was limited because of the obvious difficulties in acquiring them, it is reasonable to conclude that, from the analysis of these pellets and oily injections, the amount actually implanted in bovines to promote quick growth is not high enough to remain in meat as a residue.

References

1. IARC Monograph on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. International Agency for Research on Cancer, Lyon, 1979.
2. M. Metzler. Biochemical toxicology of diethylstilbestrol. *Rev. Biochem. Toxicol.* **6:** 191–220 (1984).
3. D.A. Armbruster, M.D. Tillman, and L.M. Hubbs. Limit of detection (LOD)/limit of quantitation (LOQ): comparison of the empirical and the statistical methods exemplified with GC–MS assays of abused drugs. *Clin. Chem.* **40:** 1233–38 (1994).
4. T.G. Martin, T.W. Perry, M.T. Mohler, and F.H. Owens. Comparison of four levels of protein supplementation with and without oral diethylstilbestrol on daily gain, feed conversion and carcass traits of bulls. *J. Anim. Sci.* **48**: 1026–32 (1979).

5. T.S. Rumsey, H.F. Tyrrell, D.A. Dinius, P.W. Moe, and H.R. Cross. Effect of diethylstilbestrol on tissue gain and carcass merit of feedlot beef steers. *J. Anim. Sci.* **53:** 589–600 (1981).
6. P. Vanderwal, P.L.M. Berende, and J.E. Sprietsma. Effect of anabolic agents on performance of calves. *J. Anim. Sci.* **41:** 978–85 (1975).
7. A.R. Lea, W.J. Kayaba, and D.M. Hailey. Analysis of diethylstilbestrol and its impurities in tablets using reversed-phase high-performance liquid chromatography. *J. Chromatogr.* **177:** 61–68 (1979).
8. F.A. El-Yazbi, M.A. Korany, O. Abdel-Razak, and M.A. Elsayed. Derivative spectrophotometric analysis of oestradiol esters, testosterone and progesterone in oily injections. *Anal. Lett.* **18:** 2127–42 (1985).
9. R. Cameroni, G. Gamberini, V. Ferioli, M.T. Nabel, and G. Coppi. Determination of active principles in pharmaceutical formulations by high-pressure liquid chromatography. *Il Farmaco Ed. Prat.* **33:** 291–300 (1978).
10. C.H. Van Peteghem and G.M. Van Haver. Chromatographic purification and radio-immunoassay of diethylstilbestrol residues in meat. *Anal. Chim. Acta* **182:** 293–98 (1986).
11. G. Moretti, G. Cavina, R. Alimenti, P. Cammarata, and L. Valvo. HPLC analysis of steroid formulations acting as anabolics unlawfully used in veterinary. Proceedings of the Symposium on the Analysis of Steroids, Szeged, Hungary, 1984, pp 471–77.
12. J.O. De Beer. On-line high-performance liquid chromatographic diode array spectrometric analysis of steroidal hormones in illegal preparations. *J. Chromatogr.* **489:** 139–55 (1989).

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