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Application of high-performance thin-layer chromatography and gas chromatography–mass spectrometry to the detection of new anabolic steroids used as growth promotors in cattle fattening

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Abstract

The misuse of natural and synthetic hormones as growth promotors in cattle fattening, although forbidden within the European Community, is well known. Frequently these hormones are injected into the animal as highly concentrated mixtures (the so-called "hormone cocktails"), which usually stay locally at the site of injection from where they are distributed by a slow diffusion process. The analysis of these injection sites by an HPTLC method following a simple and unselective extraction yields a good picture of the compounds which are misused. Although almost 40 hormone reference standards are run with the sample, unknown spots regularly appear on the HPTLC plates, demonstrating that attempts are continuously made to bypass the laboratory controls by introducing new products on the black market. By continuously gathering HPTLC data for a broad range of hormones which were not yet known to be used in cattle fattening, it was possible to elucidate quickly the identities of two new spots that appeared on the chromatogram in the period between the end of 1992 and early 1993. These new compounds that were found in injection sites were the gestagens delmadinone acetate and algestone acetophenide. Their identities were confirmed by GC–MS analysis.

1. Introduction

The misuse of natural and synthetic hormones as growth promotors in cattle fattening, although forbidden within the European Community (EC), is well known. Among these xenobiotics, orally administered compounds have gained popularity, as they leave no injection sites. Nevertheless, during controls of the cattle carcasses in the slaughterhouses, inspectors of the Ministry of Public Health still frequently encounter injection sites, which generally consist of a piece of inflamed muscle.

The analysis of these injection sites by an HPTLC method following a simple and unselective extraction yields a good picture of the compounds which are misused [6]. Usually amounts in the order of milligrams or more of hormones in an oily matrix, sometimes as mixtures in the so-called "hormone cocktails", are injected into the animal. These large amounts remain at the site of injection, which can be the neck, the back, the udder and even behind the eye socket [1], from where they are distributed by a slow diffusion process (long-term effect). Sometimes, as a result of encapsulation, injected hormones can be found even months after application. Owing to these large amounts of hormon-

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al substances, a considerable risk to the consumer persists [2,3].

Despite the fact that during the analysis almost 40 reference standards are run together with the sample, unknown spots regularly appear on the HPTLC plate, which indicates that continuous attempts are made to bypass the laboratory controls by introducing new products. Therefore, a bank of HPTLC data for a broad range of hormones was collected. By comparing the R_F values and colours with the stored data, the identities of two unknown spots were recently elucidated. Conformation of the identities was carried out by gas chromatography–mass spectrometry (GC–MS).

2. Experimental

2.1. Chemicals, glassware and solvents

Reference materials of 37 anabolics were obtained from different sources. The standards were divided into six mixtures based on the R_F values (see Table 1), in such a way that all constituents of each mixture were clearly resolved in both eluents.

Stock standard solutions of 1 mg/ml of the different reference standards were prepared in methanol and were stored at 4°C. For progesterone, a stock standard solution of 10 mg/ml was used because of its low detectability on the plate. Algestone acetophenide was obtained from Diosynth (Kremer and Louward, Eigenbrakel, Belgium) and delmadinone acetate was a gift from SmithKline Beecham (Louvain-La-Neuve, Belgium).

Methanol, cyclohexane, sulphuric acid, fuming hydrochloric acid and ammonium iodide were purchased from Merck (Darmstadt, Germany), diethyl ether, acetone, chloroform and ethyl acetate from Janssen Chimica (Geel, Belgium), heptafluorobutyric acid anhydride (HFBAA) from Macherey–Nägel (Düren, Germany) and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) and dithioerythritol (DTE) from Aldrich (Milwaukee, WI, USA), were used as received.

2.2. HPTLC analysis

The analysis was based on previously described work [4–6].

Sample preparation

The injection site was cut into pieces with a disposable scalpel and placed in a plastic bag. After adding 5 ml of methanol the bag contents were blended in a Stomacher (Colworth 5) for 5 min. The methanolic extract was then transferred into a glass tube for centrifugation for at least 5 min at 1000 *g*. If the supernatant was clear, it was evaporated under a stream of nitrogen at 40°C. However, if the supernatant apparently contained blood, this supernatant was additionally extracted twice with 5-ml portions of diethylether. After centrifugation, this supernatant was evaporated under a stream of nitrogen at 40°C. The residue was dissolved in 100 μl of methanol.

HPTLC conditions

Separations were carried out on Kieselgel 60 HPTLC plates (10 × 10 cm) (Merck). A 0.75-μl volume of each reference standard mixture was applied to the plate, 1 cm from the edge, on the upper and lower sides of the plate. Of the sample extract, volumes of 0.75 and 1.50 μl were spotted. Development was carried out in one direction in a Camag twin-through chamber at ambient room temperature with 5 ml of chloroform–acetone (90:10, v/v) (solvent system 1) over a distance of 4 cm. After drying the plate in a cold air stream, the spots on the opposite side were developed in 5 ml of cyclohexane–ethyl acetate–methanol (58.5:39.0:5.5, v/v/v) (solvent system 2) over a distance of 4 cm.

Detection

After the second elution, the plate was dried under a cool air stream and sprayed with 10% H_2SO_4 in methanol, then heated for 10 min at 95°C in an oven and examined in daylight and under UV light at 366 nm.

In cases of persistent doubt about the presence of a certain compound in the sample or when the separation between two components was not

sufficient, two-dimensional cochromatography was carried out, as described by De Brabander *et al.* [7].

2.3. GC–MS analysis

Heptafluorobutyrate (HFB) derivatization

A 5-μl volume of the injection site extract was placed in a derivatization vial and concentrated to dryness at 40°C under a stream of nitrogen, then 200 μl of benzene and 50 μl of HFBAA were added. The mixture was heated for 1 h at 60°C and then concentrated to dryness at 40°C under a stream of nitrogen. The final residue was dissolved in 50 μl of hexane and 1 μl was injected into the GC–MS system.

Trimethylsilyl (TMS) derivatization

A 5-μl volume of the injection site extract was placed in a derivatization vial and evaporated to dryness, then 50 μl of a solution containing 20 mg of NH_4I, 40 mg of DTE and 10 ml of MSTFA were added. The mixture was heated for 0.5 h at 60°C. After cooling, 1 μl was injected into the GC–MS system.

Procedure

The analyses were carried out on an HP 5970 mass-selective detector (Hewlett-Packard, Palo Alto, USA) linked to an HP 5890 gas chromatograph equipped with an HP Ultra-2 (5% phenyl–methylsilicone) fused-silica capillary column (25 m × 0.2 mm I.D., film thickness 0.33 μm) and an all-glass moving-needle injection system. The carrier gas was high-purity helium (L'Air Liquide, Liege, Belgium) at a flow-rate of 0.5 ml/min. The injector and interface temperature were maintained at 290°C. The oven temperature was programmed from 200 to 280°C at 5°C/min, the final temperature being held for 10 min (programme 1). To shorten the analysis time, an alternative temperature programme was used, *i.e.*, from 250 to 280°C at 10°C/min, the final temperature being held for 20 min (programme 2). The analyses were performed in the electron impact (EI) mode and the ionization voltage was fixed at 70 eV.

3. Results

The injection sites are routinely screened for the presence of 37 hormones. Their R_F values and the appearance of the spots in daylight and under UV light (366 nm) are listed in Table 1. Although a broad spectrum screening is carried out, new unknown spots sometimes appeared on the HPTLC plates. By continuously collecting HPTLC data for a wide range of hormones which were not yet known to be used in cattle fattening, it was possible to elucidate quickly the identities of two new spots that appeared on the HPTLC plates in the period between the end of 1992 and early 1993. These new compounds that were found in injection sites were the gestagens delmadinone acetate and algestone acetophenide.

Delmadinone acetete (1,6-bisdehydro-6-chloro-17α-acetoxyprogesterone) is a synthetic steroid which possesses progestin activity. In Belgium it is commercially available as an injectable solution for the treatment of hypersexuality of cats and dogs (Tardak; Syntex). Its structure is shown in Fig. 1.

The R_F values relative to β-testosterone and the appearance of the spots in daylight and under UV light (366 nm) are given in Table 2. A study of spots obtained with decreasing amounts showed that the first visible signal was observed with 6 ng per spot on the plate.

The structure of the gestagen algestone acetophenide (16α,17α-dihydroxyprogesterone acetophenide) is shown in Fig. 2 and the HPTLC data are given in Table 2. The detectability on the plate was very low (detection limit 2.5 μg per spot). Therefore, in further routine analysis, 1.5 μl of a standard solution of 25 mg/ml in methanol was spotted.

The identities of these compounds were confirmed by GC–MS. For delmadinone acetate, a mono-HFB derivative was obtained with a molecular ion of $m/z = 598$ and a methylene unit value of 32.13. The spectrum with the presumed molecular formula of the HFB derivative is shown in Fig. 3. The presence of delmadinone acetate in the extract of an injection site was confirmed by GC–MS. The spectrum of the HFB

Table 1
Composition of the reference standard mixtures, R_F values relative to β-testosterone and appearance of the spots in daylight and under UV light (366 nm)

Mixture No.	Components	R_F		Colour	
		Solvent 1	Solvent 2	Daylight	UV (366 nm)
1	Chlorotestosterone	1.33	1.24	Blue	Dark spot with yellow edge
	4-Chlorotestosterone-17-acetate	2.02	1.93	Blue	Dark spot with yellow edge
	β-Estradiol-3-benzoate	1.42	1.54	Orange	Orange
	17β-Trenbolone	1	0.88	Fluorescent yellow	Fluorescent green
	17β-Trenbolone acetate	1.71	2.22	Fluorescent yellow	Fluorescent green
	Mestranol	1.73	1.90	Pink	Light brown
	β-Nortestosterone laurate	1.95	2.09	Brown with blue edge	Red-brown with yellow edge
2	β-Estradiol	0.87	1.22	Orange	Orange
	β-Estradiol-17β-cypionate	1.82	2.02	Orange	Orange
	Medroxyprogesterone acetate	1.73	1.34	Dark blue with yellow edge	Dark brown
	Norethisterone acetate	1.82	2.22	Light-grey-purple	Light orange
	Diethylstilbestrol	1.20	1.63	Grey-purple	Purple
	β-Testosterone-17β-cypionate	1.96	1.93	Purple	Red with yellow edge
3	17β,19-Nortestosterone	0.93	0.90	Brown with blue edge	Red-brown with yellow edge
	17α-Methyltestosterone	1.04	1.02	Yellow-orange	Fluorescent yellow with orange core
	Megestrol acetate	1.73	1.29	Heavy green	Dark green with yellow edge
	Progesterone	1.89	1.63	Yellow	Fluorescent green
	Testosterone isocaproate	2.04	2.05	Purple	Red with yellow edge
	17β,19-Nortestosterone-17-decanoate	2.07	2.12	Brown with blue edge	Red-brown with yellow edge
4	Boldenone	0.89	1.29	Brown	Chestnut brown
	Chlormadinone acetate	1.89	1.34	Petroleum green	Dark orange
	Diethylstilbestrol dipropionate	1.33	1.66	Light purple	Faded purple
	Testosterone propionate	2	1.83	Purple	Red with yellow edge
	Testosterone enanthate	2	1.95	Purple	Red with yellow edge
	Estradiol phenylpropionate	1.98	2	Orange	Orange
5	Stanozolol	0.38	0.49	Purple-brown	Purple
	β-Testosterone	1	1	Purple	Red with yellow edge
	Norethisterone	1.29	1.24	Light grey	Light orange
	Ethinylestradiol	1.18	1.56	Bright pink	Dark with orange edge
	Testosterone phenylpropionate	1.93	1.85	Purple	Red with yellow edge
	Estradiol valerate	1.82	2	Orange	Orange
6	Fluoxymesterone	0.27	0.41	Light grey-brown	Chestnut brown
	Methylboldenone	0.62	0.51	Red-purple	Chestnut brown
	17α-Acetoxyprogesterone	1.64	1.15	Purple with yellow edge	Bright red with yellow edge
	Melengestrol acetate	1.73	1.29	Brown	Brown with yellow edge
	Vinyltestosterone	1.33	1.37	Grey-brown	Fluorescent yellow
	17α-Hydroxyprogesterone caproate	1.89	1.76	Purple with yellow edge	Bright red with yellow edge

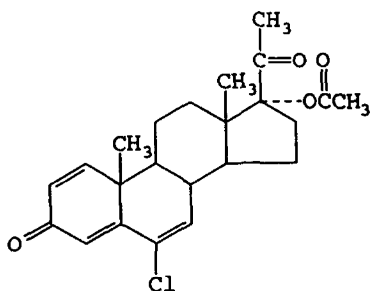

Fig. 1. Structure of delmadinone acetate.

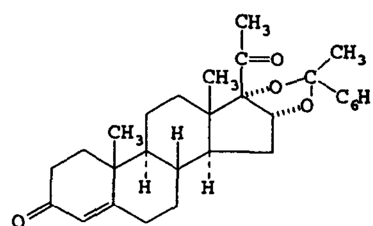

Fig. 2. Structure of algestone acetophenide.

derivative is shown in Fig. 4 and corresponds to that of the reference standard.

Algestone acetophenide could be injected into the GC–MS system without derivatization but derivatization was preferred because of the higher specificity. Derivatization with HFBAA yielded a mono-HFB derivative, the spectrum and presumed molecular structure of which are shown in Fig. 5. The molecular ion was at $m/z = 644$ and the retention times were 26.66 and 16.87 min for temperature programmes 1 and 2, respectively. Trimethylsilyl derivatization yielded a mono-TMS derivative. The spectrum and the presumed structure of the derivative are shown in Fig. 6. The molecular ion was at $m/z = 520$ and the retention times were 34.82 and 24.76 min for temperature programmes 1 and 2, respectively. Methylene unit values of these derivatives could not be determined because no suitable alkanes ($>C_{34}$) were available.

The presence of algestone acetophenide in the extract of an injection site was confirmed by GC–MS. The spectra of algestone acetophenide-mono-TMS and algestone acetophenide-mono-HFB in a routine sample are shown in Figs. 7 and 8, respectively.

The use of delmadinone acetate and algestone

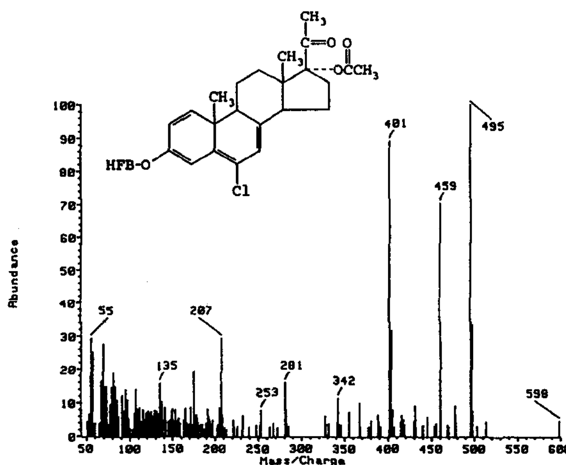

Fig. 3. Spectrum of the HFB derivative of delmadinone acetate.

Table 2
HPTLC data for delmadinone acetate and algestone acetophenide

Compound	R_F [a]		Colour	
	Solvent 1	Solvent 2	Daylight	UV (366 nm)
Delmadinone acetate	1.59	1.19	Brown	Chestnut brown
Algestone acetophenide	1.99	1.83	Faded yellow	Faded beige with blue shine

[a] Relative to β-testosterone.

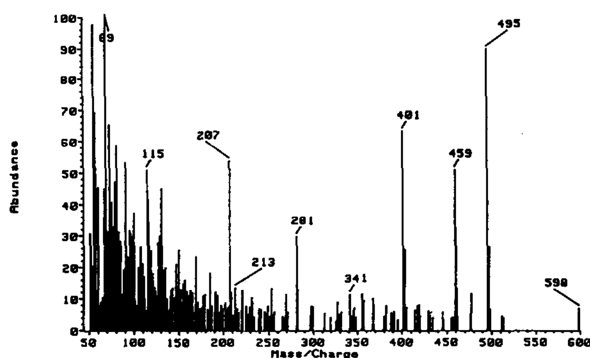

Fig. 4. Spectrum of HFB derivative of delmadinone acetate in the extract of an injection site.

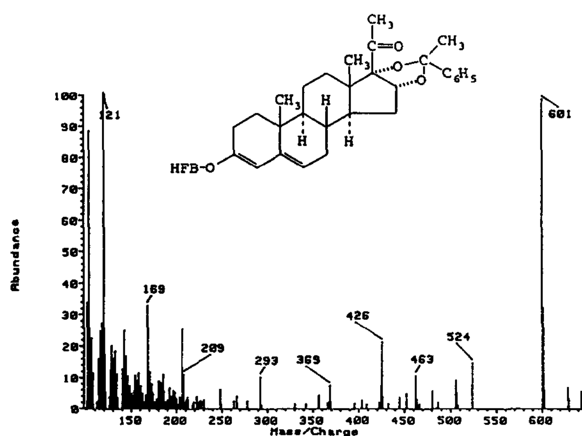

Fig. 5. Spectrum of the HFB derivative of algestone acetophenide.

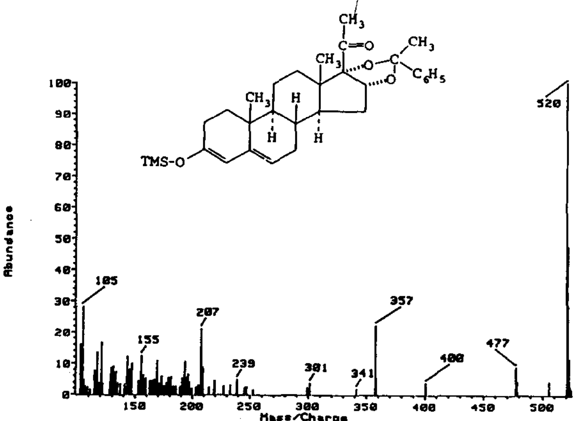

Fig. 6. Spectrum of the TMS derivative of algestone acetophenide.

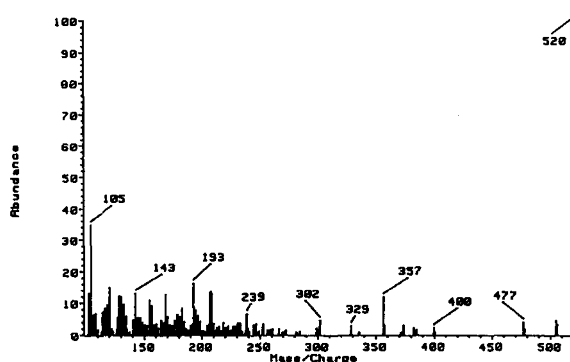

Fig. 7. Spectrum of TMS derivative of algestone acetophenide in the extract of an injection site.

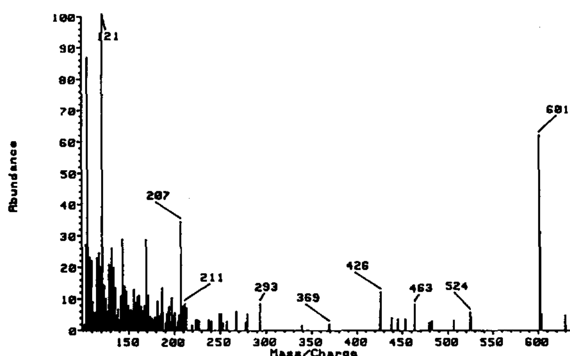

Fig. 8. Spectrum of HFB derivative of algestone acetophenide in the extract of an injection site.

acetophenide as cattle fattening agents has not yet been reported.

4. Conclusions

In the routine analysis of injection sites, HPTLC has been shown to be a very efficient multi-screening method for the presence of anabolic agents. This technique has also been shown to be very useful for the detection of new products administered illegally. By comparing the HPTLC data for new spots with a data bank of a large number of hormones, rapid information can be obtained as to the identity of new products. Confirmation by GC–MS gave decisive answers about the identities of the compounds.

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