By-products of Steroid Synthesis: a Cause of Interferences in Thin-layer Chromatography Residue Analysis*

Felix Smets and Gaspard Pottie
Instituut voor Hygiene en Epidemiologie J. Wytsmanstraat 14, B-1050 Brussels, Belgium

Hubert F. De Brabander and Peter Batjoens
Department of Veterinary Food Inspection, Laboratory of Chemical Analysis, Universiteit Gent, Casinoplein 24, B-9000 Ghent, Belgium

Lutgart Hendriks
Dr. L. Willems-Institute, Universitaire Campus, B-3590 Diepenbeek, Belgium

Dirk Courtheyn
Ryksontledingslaboratorium, Braemkasteelstraat 59, B-9050 Gentbrugge, Belgium

Bernard Lancival and Philippe Delahaut
Centre D'Economie Rurale, Rue du Point du Jour 8, B-6900 Marloie, Belgium

Since the late 1980s all of the laboratories involved in high-performance thin-layer chromatography (HPTLC) control of hormonal residues in kidney fat, have occasionally detected a green fluorescent spot with similar R_F values and colour to those observed for methyltestosterone (MT). This spot (product) could lead to false positive results for MT and was thus named 'le faux méthyl' (the false methyl) by a French-speaking colleague. All of the samples with a false methyl spot also contained a relatively high concentration of progesterone. Differentiation of this product from methyltestosterone can be performed in three ways: firstly, extra HPTLC on reversed-phase plates, secondly, extra purification of the extract with HPLC prior to HPTLC and thirdly, gas chromatography–mass spectrometry. This interference was identified as 20β-hydroxyprogesterone, a by-product of progesterone. The problem of the false methyl was not only linked with the TLC characteristics of MT but also to the progesterone used as standard. Some laboratories used an analytical-reagent grade standard and others used commercial progesterone powders as standards (*e.g.*, obtained in crude form from pharmaceutical companies). The commercial-grade progesterones showed two spots in comparison with the analytical standard that showed just one spot. As the false methyl was observed not only in kidney fat and meat samples, but also in illegal hormone cocktails, it was concluded that we had detected a by-product of an illegally used 'natural progesterone'.

Keywords: *Thin-layer chromatography; high-performance liquid chromatography; gas chromatography–mass spectrometry; hormonal residue; kidney fat; progesterone; by-product; commercial-grade standard*

Introduction

In Belgium seven field laboratories perform the control experiments on the illegal use of anabolic steroids. Biological samples of animal origin *e.g.*, urine, meat, kidney fat, injection sites, plasma and faeces are analysed by using appropriate methods, *i.e.*, thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), immunoassay (IA) and gas chromatography–mass spectrometry (GC–MS). An important type of sample is the kidney fat sample, which is analysed by TLC as a screening and confirmation technique, used in conjunction with GC–MS in some laboratories.[1]

In most instances TLC is the last step in the detection and identification in the anabolic residue analysis procedure. It is important that the results are reliable. This means that possible interfering compounds have to be removed *via* a good clean-up procedure. Because of the complexity of the biological materials, the purification step is of a great importance.

A solid phase extraction (SPE) method was developed by the National Reference Laboratory (NRL) and some field laboratories. This method is used for screening purposes.[2] More specificity was obtained by using HPLC purification in combination with 2D-HPTLC. This became the recommended method for confirmational analysis.[3,4] To obtain the most reliable result by using TLC, the well defined quality criteria described previously have to be fulfilled.[5,6] The two most important criteria discussed are: firstly, the R_F value of the analyte should agree with the R_F value characteristic of the standard material (a variation of 3% is accepted) and secondly, the visual appearance of the analyte should be indistinguishable from that of the standard material.

Lately the laboratories involved in the HPTLC control of hormonal residues in kidney fat, regularly detected a spot with a similar R_F value to that of methyltestosterone (MT) that exhibited an almost identical fluorescence colour. Depending

---

* Presented at The Second International Symposium on Hormone and Veterinary Drug Residue Analysis, Oud St-Jan, Bruges, Belgium, May 31–June 3, 1994

on the concentration, a slightly different colour and R_F value was observed and research was started to determine the exact identity of this spot. In this paper we report on a technique performed to isolate and identify this interference, first called 'the false methyl', in addition to performing the GC–MS method.

Experimental

Apparatus

A homogenizer (Ultra Turrax from Janke and Kunkel, Staufen, Germany, 20000 rpm), a water-bath, a centrifuge equipped with 450 ml centrifugation tubes (Beckman), a mechanical extractor (Stomacher, from Sheward Medical, London, UK), a rotary vacuum evaporator (Rotavapor, from Büchi, Flawil, Switzerland), an N_2 evaporator (*e.g.*, Vapotherm from Labor Technik Barkey, Bielefeld, Germany), 100 ml and 250 ml extraction flasks, a solid-phase extractor (Baker, Deventer, The Netherlands), chromatographic tanks and an ultraviolet (UV) transilluminator (λ = 366 nm) were used. The sample applicator used was a semi-automatic Camag Linomat IV, Camag, Muttenz, Switzerland. The LC system consisted of a Series 4 pump (Perkin-Elmer, Norwalk, CT, USA), an ISS-100 auto-injector (Perkin-Elmer), an automatic switching valve, a Model 440 UV detector (Waters, Milford, MA, USA) and a Model 202 fraction collector (Gilson, Worthington, OH, USA). The gas chromatograph–mass spectrometer used was an ITS 40 ion trap (Finnagan MAT, San Jose, CA, USA). HPTLC plates were obtained from Merck (Darmstadt, Germany).

Reagents, Reference Compounds and Solutions

All solvents were of analytical-reagent grade or LC grade and were obtained from Merck, diethyl ether was obtained from Gifrer & Barbezat (Decines, France).

N-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) was obtained from Macherey Nagel (Düren, Germany) and iodotrimethylsilane (ITMS) and dithioerythritol (DTE) from Pierce (Rockford, IL, USA). The progesterone standard was obtained from Sigma (St. Louis, MO, USA) and commercial progesterone powders were obtained from Diosynth (The Netherlands), Akzo (The Netherlands), and Profarma (Belgium). The MT standard and the internal standards Equilenine and Spironolactone were obtained from Steraloids (Wilton, NY, USA).

Solutions

Stock standard solutions were prepared in ethanol at a concentration of 1 mg ml^{-1}. For routine control purposes, working standard solutions were prepared by diluting the stock standard solutions to 50 ng µl^{-1}. Buffer solutions of sodium acetate (0.04 mol l^{-1}) pH 5.2 and sodium carbonate (10%) pH $\leqslant$ 10.25, were used.

The following solvent systems were used to develop the HPTLC plates: 1, hexane–diethyl ether–dichloromethane (25 + 45 + 30); 2, chloroform–acetone (90 + 10); 3, cyclohexane–ethyl acetate–ethanol (60 + 40 + 2.5); 4, chloroform–hexane–acetone (50 + 40 + 10); 5, toluene–methanol–water (5 + 75 + 20); and 6, hexane–dichloromethane–acetonitrile (80 + 20 + 10).

LC Columns

A semipreparative C_{18}, Ultrasphere ODS [pore size, 80 Å (8 nm) particle size, 5 µm] column (250 mm × 10 mm i.d.) was obtained from Beckman Instruments (San Ramon, CA, USA), C_{18} pellicular ODS (particle size 37–53 µm) guard column (30 mm × 4.6 mm i.d.) from Whatman (Maidstone, UK) and a C_{18} MCH-10 cartridge (particle size 10 µm) pre-column (30 mm × 4.6 mm i.d.) from Varian.

Silica (Si) and aminopropyl (NH_2) SPE columns (Bond Elut columns) were obtained from Varian (Harbor City, CA, USA). Before use, all columns were conditioned with the appropriate solvents, as described later.

Solid-phase Extraction (Screening Procedure)

The over-all scheme of the method used for screening is given in Fig. 1.

Kidney fat is cut into small pieces, 25 g are weighed into a 250 ml poly(propylene) flask and 50 ml of sodium acetate buffer (0.04 mol l^{-1}, pH 5.2) are added. The fat sample is then melted in a microwave oven (1 min on high power, 4 min on low power). The sample is homogenized by using an Ultra Turrax (1 min on high speed, 1 min on low speed) or a Waring Blender (1 min on high speed, 1 min on low speed) or in the flask by using a Stomacher (5 min). A 125 ng amount of Equilenine and 50 ng of Spironolactone (internal standards) are added. After extraction with 50 ml of hot methanol, the mixture is centrifuged at 13000*g* for 10 min and the supernatant is filtered through cotton wool in a separating funnel. De-fatting of the methanolic extract is carried out using 1 × 25 ml of hexane and this hexane phase is later discarded. Further extraction is performed using 1 × 100 ml of diethyl ether. This phase is washed with 15 ml of H_2O and then evaporated to dryness.

The SPE Si-column is preconditioned with 1 × 25 ml of hexane. The dry ether extract is dissolved in 500 µl of chloroform and 5 ml of hexane are added just before the extract is loaded onto the column.

The Si column is washed with 5 ml of hexane. Next, the Si column is placed on top of a NH_2 column and the combined columns are washed with 5 ml of hexane. A 5 ml volume of chloroform–acetone (4 + 1) are used to elute the column. The eluate is evaporated to dryness under a stream of N_2 at 60 °C.

HPLC as Clean-up

For confirmatory purposes a second purification procedure can be used. The primary methanolic extraction of the kidney fat and the liquid–liquid extractions are similar to those in the

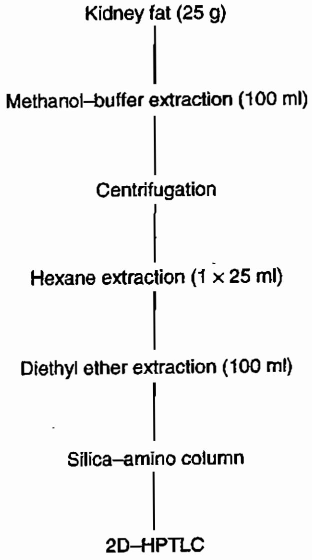

Fig. 1 Over-all scheme of the screening procedure.

screening method but to achieve a sufficient degree of separation a gradient elution on a semipreparative column was applied. Full details of this procedure are described in a previous paper.[4,7]

HPTLC

For screening purposes the extract is separated on a precoated silica gel plate (10 × 10 cm) following the 4 × 4 elution technique.[1]

The dry residue obtained after SPE, is dissolved in 30 μl of ethanol and 10 μl is spotted onto the plate. Chromatographic development is performed with the solvent systems 1 and 2 or 3 and 4 (listed earlier). After fluorescence induction, the spots were detected by viewing by transillumination under UV radiation ($\lambda = 366$ nm) and were identified by comparing the R_F values and fluorescence colours with those of the reference standards.

GC–MS

The derivatization reagent mixture used is MSTFA–ITMS–DTE (1000 + 2 + 2). Extracts should be evaporated to dryness and then stored in a desiccator prior to derivatization.

To the tube containing the extract, 25 μl of the reagent mixture are added and heated at 60 °C for 15 min. The tube is allowed to cool and then 1 μl is injected (in the splitless mode) onto the GC–MS instrument.

The analyses were carried out on a Finnigan ITS 40 ion trap in the full-scan mode. The GC column used was a DB-5 fused silica (30 m × 0.25 mm i.d.) with a 0.25 μm film thickness and a carrier gas (helium) flow rate of 1 ml min^{-1}. The temperature settings used are as follows: injector, 260 °C; transfer line, 300 °C; oven, programmed from 100 to 250 °C at 16.7 °C min^{-1} and from 250 to 300 °C at 4 °C min^{-1}, the final temperature of 300 °C being maintained for 3.5 min.

Results and Discussion

SPE–TLC

It has already been shown in an earlier study[7] that the results are influenced by the kind of matrix that is analysed and also by the method that is used. HPLC, for instance, results in a better purification of the extract, enhancing the possibility of detecting more residues of interest and thus the method becomes more specific.

As this technique was very time consuming for screening purposes and in some cases the HPLC-column was blocked by the extract, the described SPE method was elaborated. With SPE there is less risk of column blockage and only one fraction is eluted for further separation by TLC. It was in using this SPE–TLC screening technique that over the last few years the field laboratories regularly detected a false MT spot. Indeed, depending on the concentration and the TLC-elution circumstances, this spot can be very similar to methyltestosterone, even after 2D-co-chromatography. The first effort to obtain a differentiation between the two spots by performing reversed-phase TLC on C_{18} plates was successful. The MT spot and the unknown spot were well separated in the first direction [see Fig. 2(*b*)].

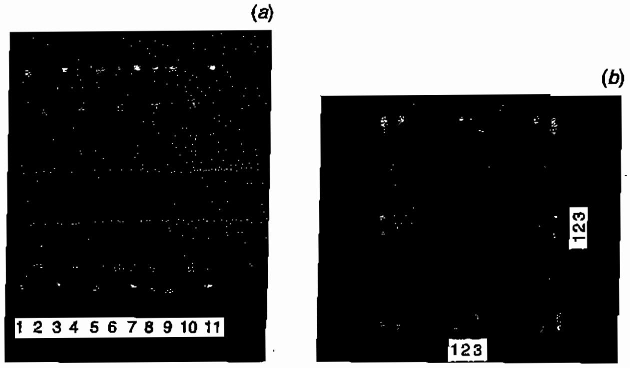

Fig. 2 (*a*), Silica HPTLC of the different standards: lanes 1, 5 and 9, MT standard; and lanes 3, 7 and 11, PA4; lane 2, $P_{Diosynth}$; lane 4, P_{Akzo}; lane 6, $P_{Profarma}$; lane 8, $P_{Illegal}$; and lane 10, P_{Sigma}. (*b*), Reversed-phase C_{18} co-chromatography of an illegal cocktail. Reference lanes: 1, 20β-hydroxyprogesterone; 2, progesterone; and 3, methyltestosterone.

One of the control laboratories related the false methyl to the progesterone standard they used as a reference. Every laboratory involved in the regulatory control experiment was asked to supply the NRL with their standard. This resulted in a series of various analytical progesterone standards in addition to the three commercial powders and an unknown powder used on the illegal market which was sampled by the inspection. The TLC results are shown in Fig. 2(*a*).

Two of the commercial powders showed the false methyl spot at different concentrations and the unknown powder showed the same spot at a much higher concentration. The next step in our research was the isolation and identification of this compound.

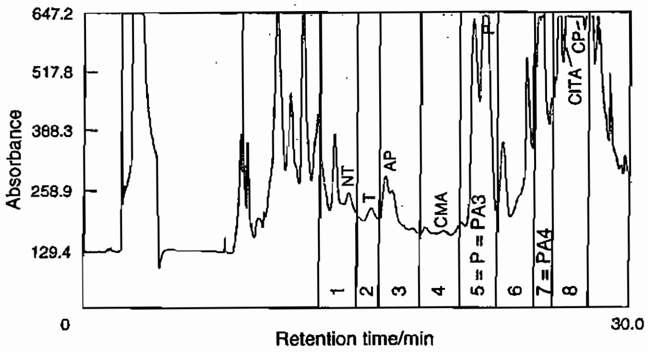

Fig. 3 UV chromatogram ($\lambda = 254$ nm) of a kidney fat sample with selected windows.

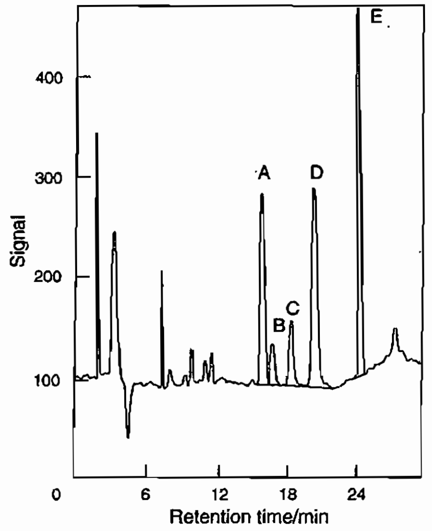

Fig. 4 UV-chromatogram of the HPLC elution of some standards of interest: A, AP; B. MT; C, MEGA; D, 20α-hydroxyprogesterone; and E, 20β-hydroxyprogesterone. Retention time: A, 16 min 13.0 s; B, 17 min 11.9 s; C, 18 min 41.5 s; D, 20 min 44.4 s; and E, 24 min 42.5 s.

HPLC–TLC

The extract of an appropriate fat sample was purified by using HPLC and fraction collection was performed as shown in Fig. 3. Eight different fractions were collected so that the most important part of the elution process could be analysed further. The same elution procedure was performed for the different standards (see Fig. 4) and also for an oil illegally used suspension (cocktail) containing a high concentration of progesterone and the second compound (false methyl) (see Fig. 5). In fraction 7 we isolated and successfully detected the green fluorescent spot, and confirmed this by comparison with the same fractions of the injected standard and dilution of the cocktail.

GC–MS

The HPLC fraction that we identified by TLC as the 'second P-spot' was analysed by GC–MS after derivatization with MSTFA. As for the TLC detection we analysed that same fraction of a false positive MT fat sample, a P-standard containing the second spot and the earlier mentioned cocktail. We isolated the appropriate fraction and obtained the mass spectrum, as shown in Fig. 6, which was the same in the three samples.

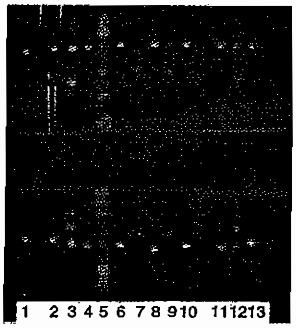

Fig. 5 HPTLC of a cocktail, PA4 standard and MT-standard: lanes 3 and 12, illegal cocktail; lanes 2, 6, 8 and 11, PA4; lanes 1, 4, 10 and 13, MT-standard; lane 5, kidney fat; lane 7, P_{Akzo}; and lane 9, $P_{Illegal}$.

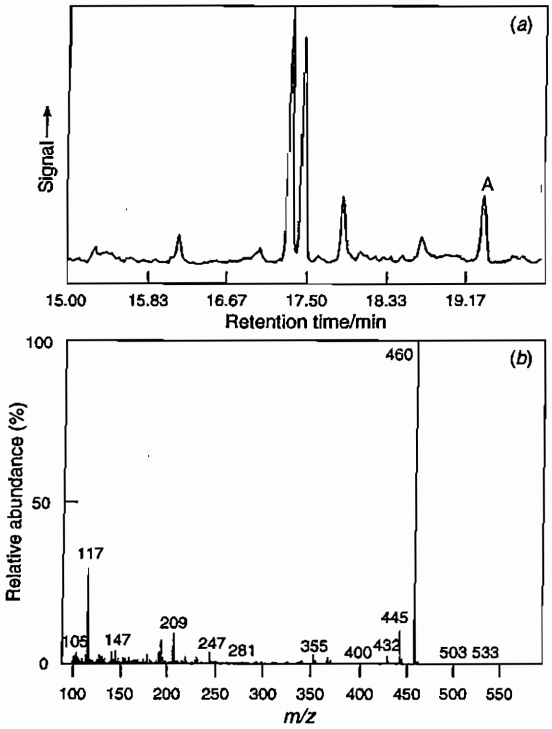

Fig. 6 GC–MS results of a compound (A) eluted in HPLC-fraction 7.

Until now, the only conclusion that we could make was that the second P-spot was probably an impurity in the progesterone standard. This unknown compound has a M^+ ion of 460 after silylation with MSTFA. If a di-TMS ws formed, the molecular mass (*M*) of the unknown must be 316. After a dBase search in our list of standards we selected 20 compounds with *M* 316. After further research into the chemical structure (to find progesterone analogues), five products that we had as reference material in our laboratory were selected (see Table 1).

Fig. 7 shows the GC–MS results of the PA4 standard and the corresponding spectrum in a fat sample. The only product that corresponded to the HPLC, as well as the TLC and the GC–MS results was 20β-hydroxyprogesterone. These results were confirmed in different kidney fat samples and also in some meat samples.

The conclusion that the interference was probably a by-product of progesterone, is therefore confirmed because a relatively high concentration of progesterone was detected in all samples.

Conclusions

The problem caused by the by-product of progesterone was first detected in the control laboratories after using the SPE–TLC as screening method.

Table 1 Chemical nomenclature of the selected P-analogues

P-Analogue	Systematic name
PA1	5α-pregnane-3,20-dione
PA2	5β-pregnane-3,20-dione
PA3	4-pregnen-20α-ol-3-one
PA4	4-pregnen-20β-ol-3-one
PA5	5-pregnen-3β-ol-20-one

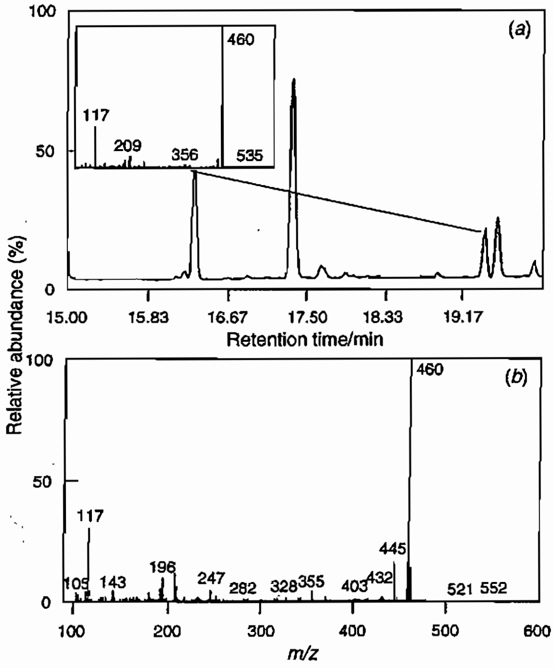

Fig. 7 GC–MS results of the (*a*) PA4 standard and (*b*) the corresponding fraction of a kidney fat sample.

By using a HPLC clean-up procedure prior to HPTLC we could increase the specificity of the technique and differentiate between methyltestosterone and the false methyl.

Identification and confirmation of the results obtained by using the other techniques could be performed using GC–MS. The first conclusion of this study is that all control laboratories must be aware of the by-products of anabolics used as illegal preparations in cattle fattening. These by-products can, as in the case of progesterone, be resorbed by the organism and produce interference problems in the residue analysis of biological samples. Moreover the false methyl discussed may be an indication of illegal treatment with natural hormones.

Secondly the use of a combination of different techniques, for purification as well as for detection is the best way to obtain the most reliable result. In our study, the HPLC clean-up procedure prior to HPTLC, in combination with GC–MS was the tool with which we solved the lack of specificity of the screening method.

The final and significant conclusion is that the exposed problem of possible interferences caused by commercial steroids' by-products, reminds us the problem of not only the purity but also of the availability of certified reference standards and reference materials.

This study is the result of the collaboration of five laboratories, all members of the working group 'analysis of anabolics in kidney fat'.

The authors thank the technical teams in each laboratory M. Delval-Deridder, A. Deligne-Debbaut (Instituut voor Hygiene en Epidemilogie) and A. Hoevenaeghel (Universiteit Gent) for their co-operation in the development and application of the chromatographic and application techniques.

References

1 De Brabander, H. F., Smets, F., and Pottie, G., *J. Planar Chromatogr.-Mod. TLC*, 1988, **1**, 369.
2 De Brabander, H. F., Hendriks, L., Smets, F., Delahaut, P., Batjoens, P., Leyssens, L., Pottie, G., *Proceedings of the Euroresidue II: Conference on Residues on Veterinary Drugs in Food* Veldhoven, The Netherlands, 1993, 211.
3 Smets, F., Benelux Economische Unie SP/LAB/h(90)18.
4 Smets, F., Vandewalle, M., Z.L.U.F., 1982, 175, 29.
5 EC directive 93/256, 1993.
6 De Brabander, H. F., Smets, F., and Pottie, G., *J. Planar Chromatogr.-Mod. TLC*, 1991, **4**, 52.
7 Smets, F., Vanhoenackere Ch., Pottie, G., *Anal. Chim. Acta*, 1993, **275**, 147.

Paper 4/03392B
Received June 6, 1994
Accepted July 27, 1994