Short communication

Planar Chromatography of Steroid Hormones and Anabolics

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Abstract

Simultaneous separation of eleven steroid hormones and synthetic anabolics: progesterone, trenbolone acetate, melengestrol acetate, 17-ß-estradiol, 19-nortestosterone, fluoxymesterone, norethandrolone, 4-chloro-δ-1-methyl testosterone, clostebol acetate, 6-ß-hydroxymethandienone and oxymetholone, was performed on HPTLC plates, 10 × 10 cm, silicagel 60 F_{254} (Merck) by horizontal elution in chloroform-acetone mobile phase. The investigated steroids were successfully visualised under UV light (254 nm), and after spraying with an ethanolic solution of *p*-toluenesulphonic acid. The efficacy of chromatographic system was checked using simulated real samples for some of the examined steroids, melengestrol acetate and trenbolone acetate, usually misused as growth promoters in cattle and stored unchanged in animal tissue.

Keywords: steroid hormones, steroid anabolics, HPTLC

1. Introduction

The misuse of steroid hormones and anabolics is nowadays present not only in humans (doping in sport), but also in animals. The use of steroid hormones and anabolics has been banned in the European Union since February 2001. This prohibition includes 17-β-estradiol, progesterone, testosterone, trenbolone acetate, melengestrol acetate and zeranol. A lot of studies show that their use can cause toxicological, immunotoxic and genotoxic effects.[1,2] The consequences of short-term and long-term use of these substances must be taken into account, as well as their effect in the nutrition chain.

Most of synthetic steroid anabolics are poorly metabolised, unlike natural steroid hormones,[3] so they are stored unchanged in the liver, kidneys, muscles and fat tissue in animals. In humans they are excreted mostly in urine.

The analysis of steroid hormones and anabolics is often performed by gas chromatography (GC) and high performance liquid chromatography (HPLC), as well as gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography-mass spectrometry (HPLC-MS).[4–7] Planar chromatography (PC), because of its advantages such as: simplicity, inexpensive analysis, selectivity, precision and versatility can be a convenient method for the analysis of those compounds.

2. Experimental

The main aim of this work was to determine optimal conditions for efficient simultaneous separation and detection of steroid hormones and synthetic steroid anabolics by means of planar chromatography. For this purpose progesterone (Sigma, +99%), trenbolone acetate (Sigma, +98%), melengestrol acetate (Bio Chemica, +97%, HPLC grade), 17-β-estradiol (Sigma, +98%), 19-nortestosterone, fluoxymesterone, oxymetholone (Sigma, > 97%), norethandrolone, 4-chloro-δ-1-methyltestosterone, clostebol acetate and 6-β-hydroxymethandienone (LGC Promochem, Germany) were examined.

The samples were applied on HPTLC glass plates, 10 × 10 cm, silicagel 60 F_{254} (Merck) using Nanomat 4 (Camag). Separation was obtained by horizontal elution in chloroform-acetone mobile phase (80 + 20, v + v, p.a., Merck). Visualization of investigated steroid hormones and synthetic anabolics was achieved:

- Under UV light at 254 nm. All examined compounds, except of melengestrol acetate, appeared as grey or dark blue spots on a green background.
- Plates were also sprayed with a 20% ethanolic solution of *p*-toluenesulfonic acid (p.a. Merck) and heated for 20 min at 105 °C. The examined steroids appeared as yellow-orange and pink-violet spots. It was not possible to detect clostebol acetate and oxymetholone in this way.

Homogenized bovine liver aliquot (2 g) was spiked with 100 μL fluoxymesterone methanolic solution (concentration 2 mg mL^{-1}) and 100 μL melengestrol acetate (trenbolone acetate) methanolic solution (concentration 5 mg mL^{-1}). Homogenized liver spiked with fluoxymesterone only was used as a blank.

Isolation of anabolics was performed by using solid phase extraction. After adding 10 mL of methanole (p.a. Merck), samples were vortexed for 30 min, and then centrifuged for 15 min at 4000 rpm. Supernatants were evaporated to dryness at 50 °C under the stream of nitrogen. One mL of deionised water and 20 μL phosphoric acid (ρ = 1.4 g mL^{-1}, p.a., Merck), has been added to each sample, which then were applied on cartridges Nexus Bond Elut Straight (6 mL, Varian), washed with 1 mL of deionised water and then eluted with 1 mL of methanole.

HPLC conditions were the following: injection system: Shimadzu SIL-10 Advp; column Varian C18, Chromsep SS, 250 mm × 4.6 mm, 5 μm; detector Shimadzu SPD-M 10 Avp UV 240/280 nm; pump Shimadzu LC-10AT; mobile phase: methanol:water (55:45, v:v); flow: 1.5 mL min^{-1}; column temperature: 40 °C; sample volume: 20 μL; software: SHIMADZU CLASS-VP Version 5.03.

3. Results and Discussion

The obtained chromatograms are shown in Figures 1 and 2. Chromatographic conditions of PC analysis and data for hR_f values for the examined steroid hormones and anabolics are shown in Table 1.

Table 1. hR_f values for steroid hormones and synthetic steroid anabolics. Migration distance: 8 cm, elution time 30 min, concentration 5 mg mL^{-1}, amount applied 1 μL.

No	Compound	hR_f value
1	Progesterone	76.25
2	Trenbolone Acetate	58.75
3	Melengestrol Acetate*	64.95
4	17-β-Estradiol	52.50
5	19-Nortestosterone	56.25
6	Fluoxymesterone	17.50
7	Norethandrolone	63.75
8	4-chloro-δ-1-methyltestosterone	59.97
9	Clostebol Acetate**	84.61
10	6-β-Hydroxymethandienone	19.23
11	Oxymetholone**	66.66

* no identification under UV light at 254 nm.
** no identification with *p*-toluenesulfonic acid.

The suitability and efficacy of the chromatographic system were checked by analysis of an extract of animal tissue obtained after spiking with melengestrol acetate and trenbolone acetate. Fluoxymesterone (concentration 2 mg mL^{-1}) was used as internal standard. The derived extracts were analyzed by HPTLC under the same chromatographic conditions (Figure 3). Estimated hR_f values for examined anabolics are shown in Table 2.

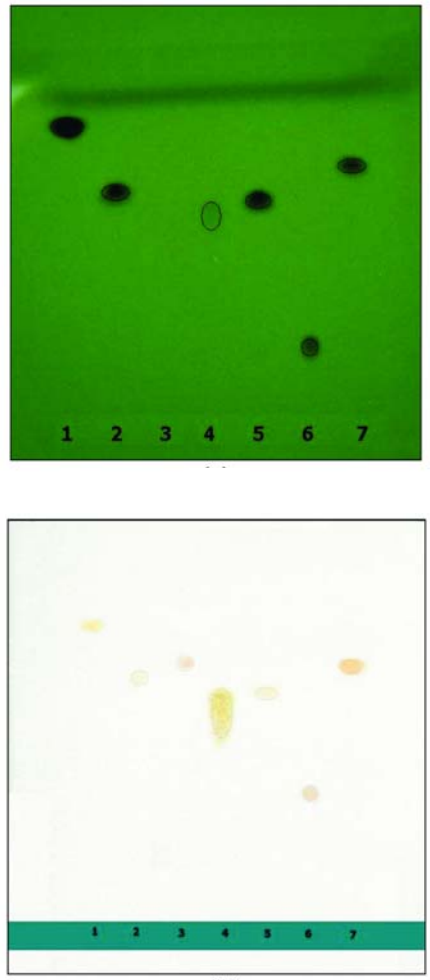

Figure 1. Developed HPTLC plates with steroid hormones and synthetic steroid anabolics visualised under UV light at 254 nm (above) and after spraying with *p*-toluenesulfonic acid (below).

The results were confirmed using HPLC on a C18 reversed-phase using a diode array detector. The chromatograms of liver extracts obtained by HPLC are shown in Figure 4.

4. Conclusions

(a) Simultaneous separation of eleven steroid hormones and synthetic steroid anabolics was efficiently obtained on HPTLC plates with UV detection and by spraying with *p*-toluenesulfonic acid.
(b) Melengestrol acetate can be identified only after spraying with *p*-toluenesulfonic acid, while clostebol acetate and oxymetholone could be identified only under UV light.

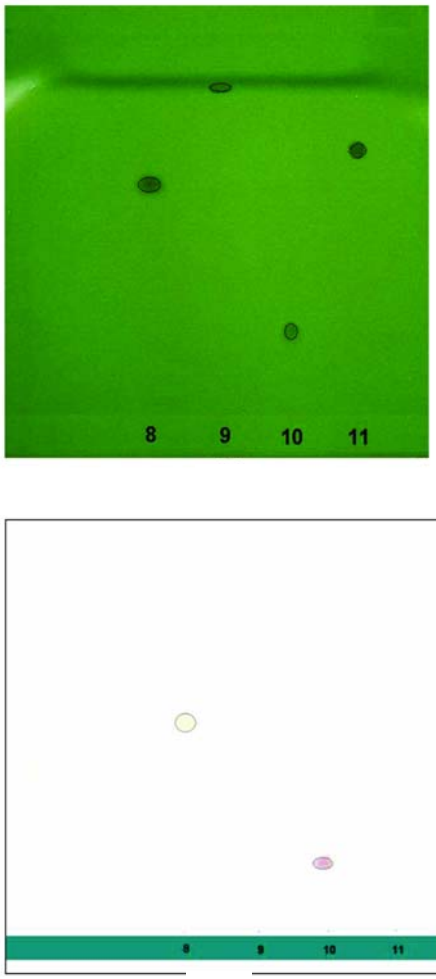

Figure 2. Developed HPTLC plates with steroid hormones and synthetic steroid anabolics visualised under UV light at 254 nm (above) and after spraying with *p*-toluenesulfonic acid (below).

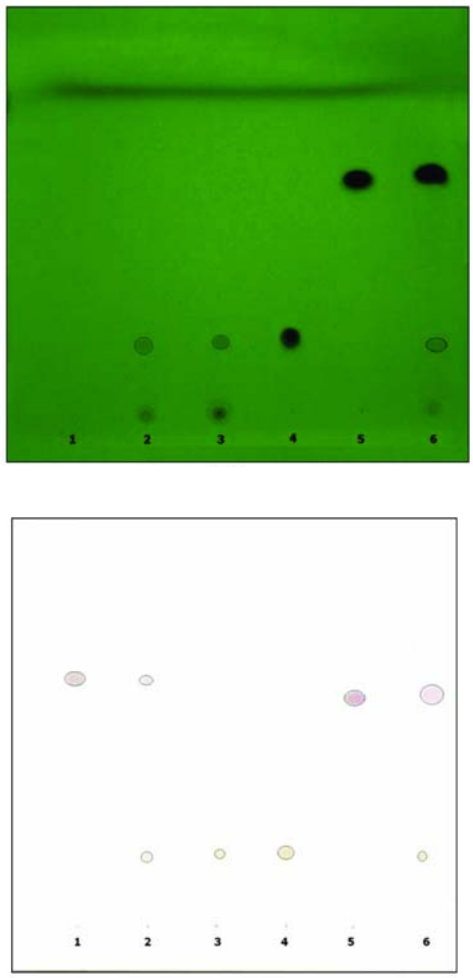

Figure 3. Liver extract chromatogram obtained by HPTLC: (above) UV 254 nm; (below) *p*-toluenesulfonic acid.

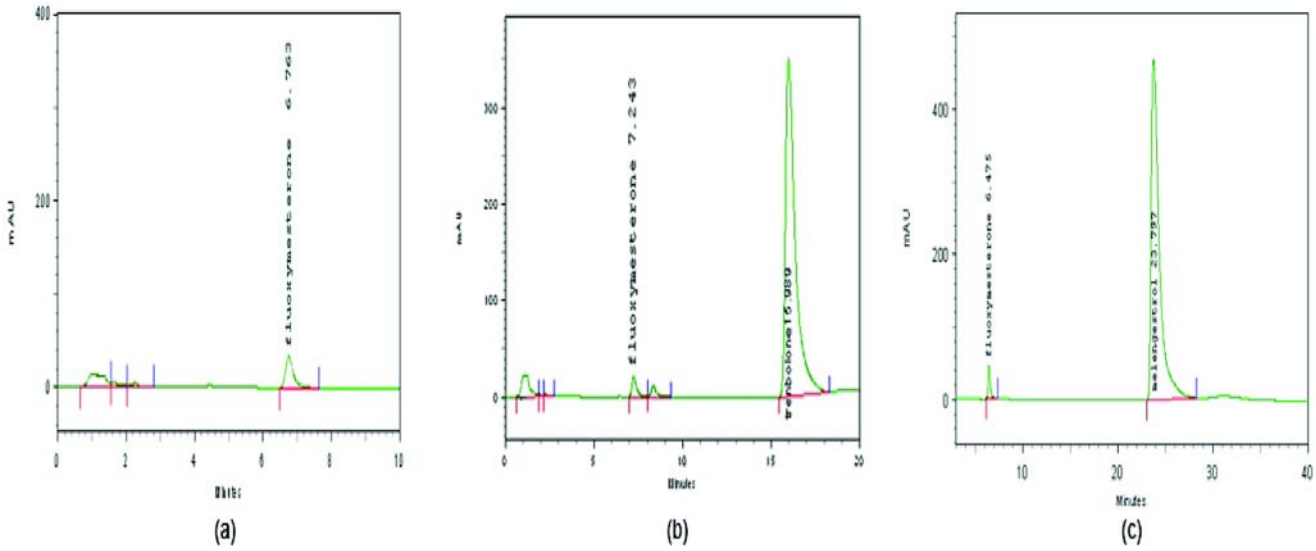

Figure 4. Chromatograms of liver extracts obtained by HPLC: (a) blank; (b) trenbolone acetate; (c) melengestrol acetate.

Table 2. hR_f values of liver extracts. Migration distance 8 cm, elution time 32 min.

No	Compound	Amount applied	Concentration (mg mL^{-1})	hR_f value
1	Melengestrol Acetate standard	4 μL	5	53,66
2	Liver extract with melengestrol acetate	4 μL		53,56 19,48*
3	Liver extract blank	2 μL		19,49*
4	Fluoxymesterone standard	1 μL	2	19,51*
5	Trenbolone acetate standard	1 μL	5	60,90
6	Liver extract with trenbolone acetate	2 μL		60,97 19,43*

* Fluoxymesterone as internal standard

(c) Isolation of melengestrol acetate and trenbolone acetate from spiked (simulated) liver samples was performed by solid phase extraction. The extraction efficiency was acceptable for quantitative and semiquantitative analysis.

(d) The proposed chromatographic system and extraction method can be used as a reliable screening test for identification of melengestrol acetate and trenbolone acetate, which are illegally used as growth promoters in animals.

5. Acknowledgement

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6. References

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Povzetek

Opravili smo hkratno ločitev enajstih steroidnih hormonov in sinteznih anabolikov: progesterona, trenbolon acetata, melengestrol acetata, 17-*β*-estradiola, 19-nortestosterona, fluoksimesterona, noretandrolona, 4-kloro-*δ*-1-metil testosterona, klostebol acetata, 6-*β*-hidroksimetandienona in oksimetolona s tenkoplastno tekočinsko kromatografijo na ploščah 10 × 10 cm, s silikagelom 60 F_{254} (Merck) s horizontalno elucijo z mobilno fazo kloroform-aceton. Detekcijo smo opravili z obsevanjem z UV svetlobo (254 nm) in s pršenjem z etanolno raztopino *p*-toluensulfonske kisline. Učinkovitost kromatografskega sistema smo preverili s simuliranimi vzorci, v katerih smo določali melengestrol acetat in trenbolon acetat, katerih uporaba za pospeševanje rasti v živinoreji je prepovedana in ki se lahko kopičijo v živalskih tkivih.