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Hair analysis of several anabolic substances

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1. Introduction

Rising interest of several horse breeder associations in hair analysis of anabolic agents initiated the development of a screening procedure. Uncomplicated sampling and easy storage were the most important considerations in addition to long-term detection of incorporated substances, to choose hair as appropriate specimen to control forbidden medication of horses. Stallions are surveyed and selected for breeding by their physique and assessment of performance at the age of two years. Breeders have the intention to fulfil the requirements of the stallion licensing. For this reason the suspicion of a possible abuse of anabolic compounds arose and the control of substances available as veterinary medicine was envisaged, for example clenbuterol and different esters of nandrolone and testosterone (e. g. decanoate, propionate, phenylpropionate and dodecanoate), see Tab. 1.

Tab. 1: Substances included in the screening procedure.

substance	extraction from hair	detection method	target ion / fragmentation	LOD
clenbuterol	alkaline disintegration	GC-HRMS	335.0695	0.9 pg/mg
nandrolone testosterone	methanol extraction	GC-MS/MS	418.2→194.1 432.3→417.2	0.3 pg/mg 0.3 pg/mg
nandrolone esters: - decanoate - laurate - phenylpropionate testosterone esters: - cypionate - decanoate - phenylpropionate - propionate	methanol extraction	GC-HRMS	 500.3686 528.3999 478.2903 484.3373 514.3842 492.3060 416.2747	 5.0 pg/mg 2.0 pg/mg 1.0 pg/mg 2.0 pg/mg 5.0 pg/mg 0.5 pg/mg 0.1 pg/mg

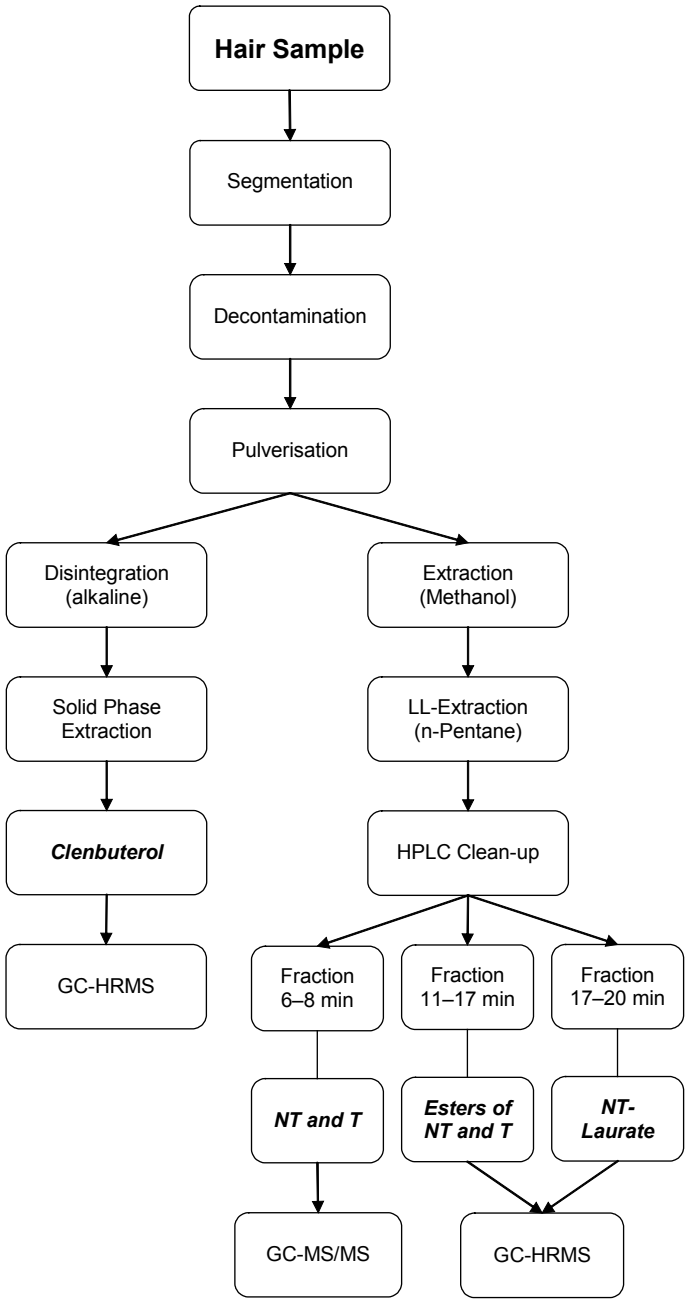

Fig. 1: Scheme of the screening procedure to detect clenbuterol, nandrolone (NT), testosterone (T) and esters of steroid compounds in hair specimens.

2. Experimental

Sample preparation consists of the following parts which had to be optimised: decontamination of the hair strand, extraction of the pulverised material, purification of the raw extract, detection of formed derivatives.

First of all the relevant hair segment had to be chosen according to the requested timeframe or special case. The length of horse hair commonly examined is 6 up to 12 cm representing a growing period of 3 and 6 months, respectively.

Before pulverisation the hair material had to be decontaminated by washing with methanol / water (1:1). 50 mg of hair powder were used for analysis of clenbuterol (Fig. 1). The material was disintegrated with 0.5 M KOH (60°C, ultrasonic bath), the solution was purified by solid phase extraction (cartridges: Oasis HLB, Waters) and the bis-TMS derivatives were analysed by GC-HRMS (AutoSpec, Micromass) [1]. Bambuterol was utilised as internal standard.

To analyse steroid compounds, samples (100 mg) were spiked with testosterone-d_3 and methenolone-enanthate as internal standards and extracted by incubation with 2.5 ml of methanol in ultrasonic bath (5 hours, 50°C). Solutions were evaporated, dissolved in aqueous buffer solution (pH 9, $NaHCO_3/KCO_3$), extracted with n-pentane, and further purification was carried out by HPLC [2] (Fig. 1). Three different HPLC fractions were collected from each sample, separated from 6.0 to 8.0 min (1), 10.9 to 17.2 min (2) and 17.2 to 19.5 min (3). After evaporation to dryness, samples were treated with MSTFA / ammonium iodide / propanethiol to form bis-TMS derivatives.

Fractions (1) containing nandrolone (NT) and testosterone (T) were analysed by GC-MS/MS (AutoSpec, Micromass). Fractions (2) and (3) were analysed by high resolution mass spectrometry to detect steroid esters of nandrolone and testosterone.

3. Results

3.1. Extraction

The analyte clenbuterol is linked to the pigment melanin in hair. The hair material has therefore to be disintegrated to release bound clenbuterol completely.

Esterified substances have to be isolated from hair material using methanol extraction instead of disintegration caused by their instability in alkaline solutions. Further investigations indicated also a lack of stability of 19-norsteroids (nandrolone, norandrost-4-ene-dione and norandrost-4-ene-diol) under alkaline conditions.

Therefore two different extraction procedures have to be performed to examine melanin-linked compounds as well as unstable substances like steroids and esters in hair samples, resulting in an additional workload and amount of specimen.

In the case of an insufficient amount of hair material it is feasible to apply both methods sequently to the same weighted sample. Previous studies showed that clenbuterol is partially extractable from hair specimens by ultrasonic treatment with methanol (5 % of the total quantity, approximately). Nevertheless, analysis of clenbuterol in the hair residue remaining after a methanol extraction is practicable for screening purposes, if necessary.

3.2. Purification

Clenbuterol: Purification of the alkaline solution was carried out by solid phase extraction (automated procedure, Gilson Aspec XL4). Advantages of this method are better reproducibility and less processing time.

Steroid compounds: Several attempts to separate interfering substances from methanol extract of steroid esters using SPE cartridges failed. Liquid-liquid-extraction and HPLC clean-up were therefore applied to isolate the target substances.

3.3. Detection

TMS-derivatives of clenbuterol and steroid esters (HPLC fraction (2) and (3)) were detected by GC-HRMS analysis. Limit of detection of clenbuterol is 0.9 pg/mg; the detection limits of ester compounds range from 0.1 pg/mg (testosterone propionate) to 5.0 pg/mg (nandrolone decanoate) (Tab. 1).

Analytical signals of nandrolone are interfered in SIM mode, that is why fractions (1) containing nandrolone and testosterone were examined by GC-MS/MS technique. Limit of detection is 0.3 pg/mg for both compounds.

4. Conclusions

Our objective was the development of an analytical method to detect several anabolic substances in hair specimens. Importance was attached to achieve appropriate detection limits sensitive enough to analyse low concentrations in hair after possible administration of anabolics. The established procedure has been validated and accredited (DIN EN ISO/IEC 17025) and is applicable for equine and human hair samples as well.

It has to be pointed out that the number of analytes combined into one screening procedure is limited, predominantly due to the specific sample preparation (in particular using HPLC clean-up by splitting into several fractions). Furthermore the available amount of specimen restricts the extent of possible examinations. The minimum required amount of specimen for segmental analyses is 0.5 to 1 g.

Anabolic compounds included in this procedure are clenbuterol, nandrolone (see Fig. 2), testosterone and several esters of nandrolone and testosterone. Further investigations are in progress to integrate additional analytes, e. g. altrenogest and other esters.

References

[1] Schlupp A et al., Equine Vet J. 36(2): 118-22 (2004)

[2] Thieme D et al., Analytica Chimica Acta. 483(1-2): 299-306 (2003)

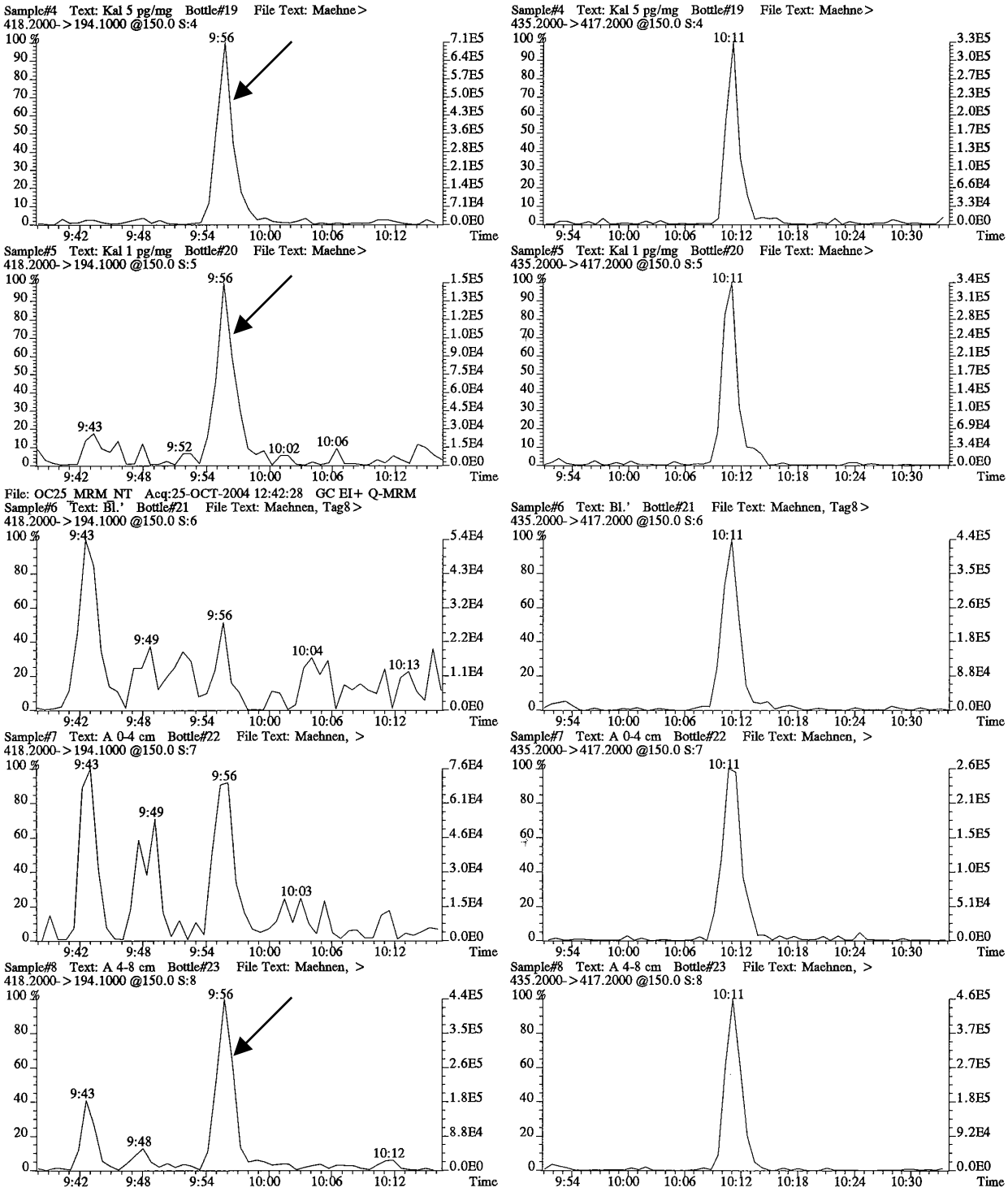

Fig. 2: Detection of nandrolone in horse hair samples by GC-MS/MS. Left column: nandrolone, right column: internal standard (testosterone-d_3). Row 1-2: spiked control samples (5 and 1 pg/mg, respectively); row 3: hair blank; row 4-5: mane hair (length 0-4 cm and 4-8 cm, respectively), taken from a treated gelding 84 days after a single administration of nandrolone precursors (norandrost-4-ene-dione and -diol, 2 mg per kg body weight, transdermal). A nandrolone concentration of 2 pg/mg was measured in the distal hair segment (row 5), and concentration was below the LOD in the segment 0-4 cm (row 4). The analytical findings are consistent with the growth rate of mane and tail hairs of horses (about 2 cm per month).