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Disintegration of hair samples vs. methanol extraction – Influences on the analysis of anabolic steroids

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Introduction

Hair analysis of anabolic steroids is mainly applied on horse samples for purposes of breeding control. The method to analyse hair samples consists of the following steps: decontamination of the hair strand, extraction of the pulverised material with methanol, purification of the raw extract using LL-extraction and HPLC-Clean up, formation of TMS-derivatives, detection by GC-HRMS [1]. Anabolic compounds included in the standard procedure are clenbuterol, 19-nortestosterone (NT), testosterone (T) and several esters of both of these steroids.

Investigations have been carried out to optimise the initial extraction of the hair material. An ideal procedure is characterised by the complete release of incorporated substances combined with the separation of interfering compounds. Several procedures are applicable performed either by extraction (organic solvents, buffer solutions) or by digestion of hair samples (alkaline, acidic, enzymatic, reductive pulping). The suitability of different treatments depends on the substance-specific properties and the kind of binding in hair. Therefore, the stability of the analytes under conditions required for digestion and extraction, respectively, has to be taken into consideration. Due to the usage of methanol for the standard procedure, incorporated steroids are extracted unchanged from hair. Therefore, it is possible to analyse the applied parent compounds itself, e. g. the entire esters of NT and T after intramuscular injection. However, the resulting methanol extract contains fatty impurities, and a time-consuming sample preparation is necessary. In contrast, the alkaline digestion of hair results in cleaner solutions, but the detection of the esters of steroids is prevented due to the hydrolysis under alkaline conditions.

Different methods were tested and compared with the methanol extraction, concerning sensitivity, recovery, feasibility and efficiency. Disintegration utilising the reductive agent tris(2-carboxyethyl)phosphine hydrochloride (TCEP) has been described for hair analysis [2] and was therefore of particular interest.

Experimental

20 mg of the pulverised hair specimen were processed. Calibration samples were prepared

using blank material spiked with NT and 19-norandrost-4-ene-3,17-dione (4-Dione). The positive hair sample originated from a gelding and was taken after the administration of NT prohormones. T-d_3 was added as internal standard (IS). Hair samples were treated in an ultrasonic bath at 50°C using 1 mL of methanol, methanol/water (10:1, v/v), and 25 mM TCEP solution (aqueous), respectively. As described below, the different solutions were subsequently extracted with n-pentane, and the organic phases were separated for further purification.

Methanol and methanol/water: Extracts resulting after a 4 hour-incubation were evaporated, dissolved in a buffer solution (1.2 M of $NaHCO_3$ and K_2CO_3, pH 9) and extracted with n-pentane.

TCEP: Samples digested by 1 mL of 25 mM TCEP (for 2 hours) were prepared in three different ways as follows:

Experiment I: LL-extraction of the entire solution with n-pentane.

Experiment II: Separation of aqueous solution from the hair residue; LL-extraction of the solution with n-pentane; subsequent extraction of remaining hair material with methanol for 2 hours; purification of the methanol extract (see above).

Experiment III: Addition of 2.5 mL methanol to the TCEP digest; separation of the solution from the hair residue; LL-extraction with n-pentane; subsequent extraction of remaining hair material with methanol; purification of the methanol extract (see above).

The n-pentane extracts were evaporated and further purification was carried out by HPLC fractionation (details described in [1]). The collected fractions were evaporated to dryness under nitrogen, and derivatised using a mixture of MSTFA / ammonium iodide / propanethiol. The formed TMS derivatives were analysed by GC-HRMS (AutoSpec, Micromass). Monitored ions: 401.2332, 416.2567 (4-Dione), 403.2489, 418.2723 (NT), 435.3115 (IS). Stability of steroids against TCEP was checked by incubation of standards without any hair material (2 hours, 50°C). Several 19-norsteroids as well as different esters of NT and T were investigated.

Results and Discussion

Extraction with methanol (standard procedure): Using methanol as extracting agent, lipophilic compounds unlinked to melanine are removable from hair. The structure of steroids and their esters remain unchanged during treatment, but there is no information about the actual content in the hair material and the extracted portion. 4-Dione and NT were detected in the hair sample taken from a treated gelding, at a concentration of 190 pg/mg and 14 pg/mg, respectively.

Extraction with methanol/water (10:1, v/v): A mixture of methanol and water was applied with the intention to improve the yield of the extraction, due to the swelling of hair fibres.

However, concentrations measured in hair samples did not significantly differ from results obtained after methanol extraction.

TCEP (25 mM): Examination of stability against TCEP solution at 50°C gave no indications for degradation neither of 19-norsteroids nor of esters of NT and T.

The incubated hair material was not completely disintegrated after a period of 2 hours in ultrasonic bath. Rather, a swelling of the hair fibres was observed. However, the investigated compounds have been shown to be adsorbed to residual specimen in the aqueous TCEP solution. Recoveries of 4-Dione and NT were lower than 10% in the separated TCEP solution in experiment II (fig., 2nd row), and the subsequent methanol extract of the hair residue gave an amount of 35 to 50% (fig., 3rd row). This result was observed both in the spiked control samples and in the positive hair sample, and therefore was probably not caused by an incomplete digestion. In contrast, the estimated concentrations are similar to the standard method if the entire solution is extracted without separation of the hair residue (experiment I, recovery about 40%; fig., 1st row). Therefore it is supposed that using TCEP, hair texture is broken and incorporated substances are getting unfixed, but lipophilic compounds remain adsorbed to the surface of the residual hair material.

To reduce polarity of the aqueous TCEP digest, in experiment III 2.5 mL methanol was added after the 2 hours-incubation. Following, 19-norsteroids were solubilised to an amount of 40 to 50% (fig., 4th row). The additional quantity of 10 to 30% was released by a subsequent extraction of the remaining material with methanol.

The results indicate that the residue of the TCEP digest has to be subsequently extracted with methanol for a complete separation of lipophilic compounds. Further investigation is necessary to evaluate reproducibility and detection limits of the method.

Conclusions

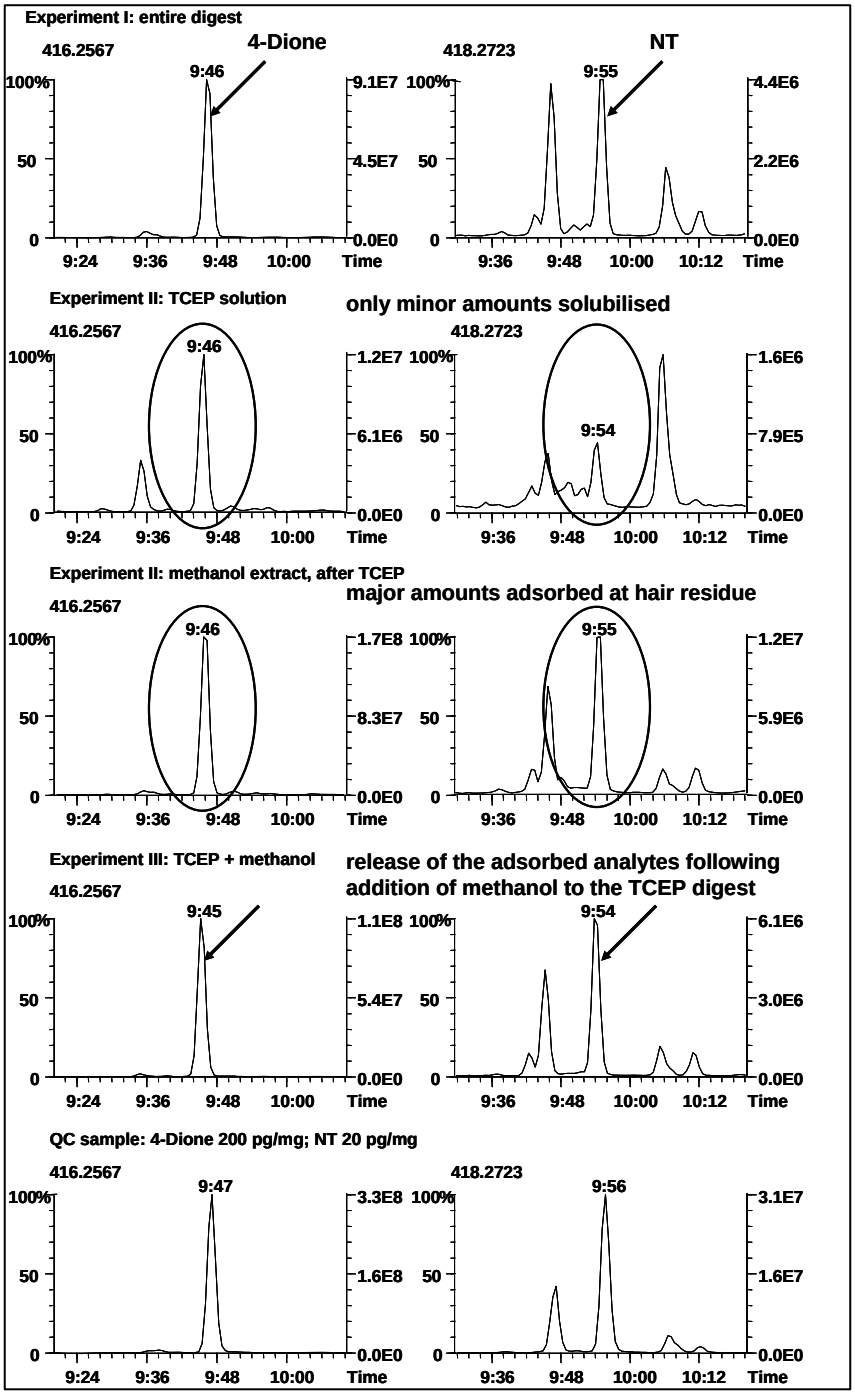
The stability of several 19-norsteroids and esters of NT and T against TCEP solution was confirmed (25 mM TCEP, at 50°C for 2 hours). The TCEP digest of hair samples contained less fatty impurities compared to the methanol extract, resulting in improved S/N ratios. Separation of lipophilic analytes was incomplete using only the TCEP disintegration, caused by an adsorption of the released substances to the hair residue. To solubilise the complete amount and therefore exceed the recovery of the standard procedure, a subsequent extraction of the remaining hair material with methanol was necessary.

Nevertheless, TCEP disintegration seems to be a potential alternative to the methanol extraction of hair specimen, due to less matrix interferences of the resulting digest, which enables further optimisation and simplification of the sample preparation.

References

[1] Anielski P, Thieme D, Schlupp A, Grosse J, Ellendorff F, Mueller RK. (2005) Detection of testosterone, nandrolone and precursors in horse hair. *Anal Bioanal Chem* **383**, 903-908.

[2] Nielen MW, Lasaroms JJ, Mulder PP, Van Hende J, van Rhijn JH, Groot MJ. (2006) Multi residue screening of intact testosterone esters and boldenone undecylenate in bovine hair using liquid chromatography electrospray tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 830, 126-134.



Figure

Detection of 19-norandrost-4-ene-3,17-dione (m/e 416, left column) and 19-nortestosterone (m/e 418, right) in the tail hair of a treated gelding, in comparison with a spiked control sample (5th row). Results of different sample preparations after disintegration with TCEP are represented (see chapter results).