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II. **Solheim SA**, Levernæs MC, Mørkeberg J, Juul A, Upners EN, Nordsborg NB, Dehnes Y. Dried blood spot analysis of testosterone esters is applicable in doping analysis. *Draf*t.

III. **Solheim SA**, Mørkeberg J, Juul A, Freiesleben SY, Upners EN, Dehnes Y, Nordsborg NB. An intramuscular injection of mixed testosterone esters does not acutely enhance strength and power in recreationally active young men. *Submitted to European Journal of Applied Physiology*

IV. **Solheim SA**, Jessen S, Mørkeberg J, Thevis M, Dehnes Y, Eibye K, Hostrup M, Nordsborg NB. Single-dose administration of clenbuterol is detectable in Dried Blood Spots. *Submitted to Drug Testing and Analysis*

Abstract

Testosterone esters and clenbuterol are among the most frequently used doping substances in elite and recreational sports. Direct detection in urine and blood samples is hampered by the costs of collection, transportation and analysis, and the rapid hydrolysis of testosterone esters in blood. Indirect detection of testosterone by the 'Athlete Biological Passport' (ABP) steroidal module is limited by both the associated costs and confounding factors. Therefore, the present thesis aimed to improve the time- and cost-efficiency in doping analysis by evaluating 1) the applicability of dried blood spots (DBS) as a complementary sample matrix and 2) whether the hematological module of the ABP can be used to indicate doping of testosterone and thereby increase detection, given the erythropoietic effect of testosterone, and 3) by determining the most cost-efficient anti-doping testing program based on detection windows and performance-enhancing effects.

In *Paper I-III*, DBS, urine and blood samples from men receiving two intramuscular injections of Sustanon® 250 (n = 9) or placebo (n = 10) in a randomized, placebo-controlled design were analyzed for direct and indirect detection of testosterone esters and assessment of serum levels of reproductive hormones. In *Paper III*, the performances in countermovement jump, 30-s all out cycle sprint and one-arm isometric elbow flexion were measured before and 24 h after the first Sustanon® injection. In *Paper IV*, DBS and urine samples from 6 healthy men receiving a single oral dose of 80 μg clenbuterol were collected and analyzed for detection of clenbuterol.

Paper II and *IV* demonstrated that the DBS assays allow for detection up to 14 days after an intramuscular injection of 250 mg Sustanon®, and for at least 3 days after an oral ingestion of 80 μg clenbuterol, with 100% specificity. Further, preliminary data suggest that DBS-sampling is well accepted by athletes. Additionally, *Paper IV* showed that clenbuterol can be detected for at least 10 days in urine after ingestion of 80 μg of drug. *Paper I* demonstrated that some hematological biomarkers are affected by testosterone administration, and that the largest changes occur 3-10 days after an injection. *Paper III* showed that a single injection of testosterone esters do not enhance human performance acutely in a countermovement jump test, a one-arm isometric elbow flexion test nor a 30-sec cycle sprint test.

In conclusion, the DBS analyses of testosterone esters and clenbuterol appear to have sufficient specificity and sensitivity to be implemented in routine doping control in elite and recreational sports. Given the longer detection windows for clenbuterol in urine, urine is expected to remain as the preferred sample matrix for clenbuterol analysis. However, the implementation of DBS sampling could improve time- and cost-efficiency while reducing intrusiveness, and thereby allow for higher frequency of testing, or testing of a large number of athletes in a short time, with the aim of increasing detection and deterrence. Further, changes in markers in the hematological module could be indicative of testosterone doping, and should be considered an additional tool for targeted follow-up sample collection and confirmatory analysis. Moreover, since testosterone did not have any acute performance-enhancing effects in power/strength exercises, athletes are likely not to have an advantage if administering a single dose of testosterone esters immediately before or during a competition in power/strength sports.

Resumé

Testosteronestere og clenbuterol er blandt de mest anvendte dopingstoffer i elite- og motionsidræt. Direkte detektion i urin- og blodprøver er forbundet med betydelige prøveopsamlingsomkostninger, strenge krav til transport og analyse, samt hurtig nedbrydning af testosteronestere i blod. Denne afhandling havde til formål at forbedre kosteffektiviteten af anti-dopingtestning gennem undersøgelse af 1) anvendeligheden af en komplementær prøveopsamlingsmatrix; dried blood spots (DBS) og 2) hvorvidt det hæmatologiske modul i 'the Athlete Biological Passport' (ABP) kan bruges til at indikere testosterondoping og derved forbedre detekteringen, givet testosterons stimulerende effekt på produktionen af røde blodceller, og 3) ved at give konkrete forslag til testning baseret på sporingstid og præstationsfremmende effekter.

I *Studie I-III* blev DBS, urin- og blodprøver fra mænd, der modtog to intramuskulære injektioner af Sustanon® 250 (n = 9) eller placebo (n = 10) i en randomiseret, placebokontrolleret design, analyseret for direkte og indirekte (ABP) påvisning af testosteronestere og måling af reproduktionshormoner i serum. I *Studie III* blev præstationen i countermovement jump, 30-sekunders cykelsprint og isometrisk albuefleksion målt før og 24 timer efter den første Sustanon®-injektion. I *Studie IV* blev DBS og urinprøver fra 6 raske mænd, der modtog en enkelt oral dosis af 80 μg clenbuterol, opsamlet og analyseret for påvisning af clenbuterol.

Studie II og *IV* viste, at analyser af DBS kan påvise doping op til 14 dage efter en intramuskulær injektion af 250 mg Sustanon® og i mindst 3 dage efter en oral dosis af 80 μg clenbuterol, med 100% specificitet. Endvidere viste *Studie IV*, at clenbuterol kan påvises i mindst 10 dage i urin efter en oral dosis à 80 μg. *Studie I* viste, at de hæmatologiske markører i ABP påvirkes af testosteronadministration, og at de mest markante ændringer finder sted 3-10 dage efter en injektion. *Stude III* viste, at en enkelt injektion af testosteronestere ikke forbedrer præstationen akut i countermovement jump, 30-sekunders cykelsprint eller isometrisk albuefleksion.

Samlet set ser det ud til, at DBS-baserede analyser af testosteronestere og clenbuterol har tilstrækkelig specificitet og sensitivitet til at kunne implementeres i dopingkontrol i elite- og motionsidræt. I betragtning af den længere detektionstid for clenbuterol i urin, forventes urin at fortsat være den foretrukne prøvematrix til at detektere clenbuteroldoping. Dog vil implementering af DBS reducere prøveopsamlingstiden, og derved muliggøre en højere testfrekvens samt testning af mange atleter på kort tid. Endvidere kan ændringer i markører i det hæmatologiske modul indikere testosterondoping, og bør betragtes som et ekstra værktøj til sporing af testosterondoping herunder målrettet testning. Da testosteron ikke havde nogen akut præstationsfremmende effekt i styrke-/kraftøvelser, vil atleter sandsynligvis ikke have en fordel, hvis de administrerer en enkelt dosis testosteronestere umiddelbart før eller under en konkurrence i styrke-/kraftidrætter.

1. Introduction

Many of the performance- and body image-enhancing drugs used by athletes and nonathletes fall within the category of lean mass builders that enhance muscle growth, reduce body fat or a combination thereof. Specifically, questionnaires, interviews and anti-doping testing figures show that anabolic agents are some of the most prevalent substances used by athletes[1–3] and people engaging in recreational exercise training.[3–5] In 2017, 44% of all adverse analytical findings in the in World Anti-Doping Agency's (WADA) anti-doping and management system, ADAMS, were substances under Section 1, 'anabolic agents', on the Prohibited List.[2] This includes substances such as the anabolic androgenic steroid (AAS) testosterone and its synthetic analogues, as well as the anabolic agent clenbuterol. Moreover, anecdotal and empirical evidence indicate that doping, particularly with clenbuterol, is an increasing problem among females. In Denmark, 26% of the female fitness customers who delivered a positive doping test between January 2014 and 2019 tested positive for this substance, which is likely to have particular appeal to women because it does not cause the androgenic side effects associated with AAS. Thus, detection of these substances is of high relevance within anti-doping. However, the current methods are challenged by the related costs from sample collection to analysis, as well as the athletes' experience of the sample collection process.[6] Therefore, the present thesis will focus on the further development of both current detection methods and alternative methods, with the aims of improving time-and-cost efficiency within anti-doping, making the process more athlete-friendly and increasing the likelihood of catching dopers.

The following sections will introduce possible ergogenic effects and adverse side-effects of testosterone and clenbuterol, how doping with these substances can be detected, current challenges for doping control sample collection and analysis, as well as future possibilities.