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Use of dried blood spots in doping control analysis of anabolic steroid esters

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ABSTRACT

Dried blood spot (DBS) sampling, a technique for whole blood sampling on a piece of filter paper, has more than 50-years tradition, particularly in the diagnostic analysis of metabolic disorders in neonatal screening. Due to the minimal invasiveness, straightforwardness, robustness against manipulation and fastness DBS sampling recommends itself as an advantageous technique in doping control analysis. The present approach highlights the development of a screening assay for the analysis of eight anabolic steroid esters (nandrolone phenylpropionate, trenbolone enanthate, testosterone acetate, testosterone cypionate, testosterone isocaproate, testosterone phenylpropionate, testosterone decanoate and testosterone undecanoate) and nandrolone in DBS. The detection of the intact esters allows an unequivocal proof of the administration of conjugates of exogenous testosterone and its derivatives. Precise, specific and linear conditions were obtained by means of liquid chromatography high resolution/high accuracy mass spectrometry. Sensitivity in the low ppb range was accomplished by the preparation of the methyloxime derivatives of the target compounds. Labeled internal standards (d_3-nandrolone, d_3-nandrolone caproate and d_3-nandrolone undecanoate) were applied to compensate for the broad range in chain length of the esters.

The assay presented here outlines the application of DBS for the analysis of anabolic steroid esters in doping controls for the first time providing great potential to simplify the proof of exogenous administration of testosterone.

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1. Introduction

Dried blood spots (DBS), a sampling technique for whole blood specimens dried on a piece of filter paper, has a more than 50-year long tradition. The first application of this microvolume sampling method using blood from a heel prick was reported in 1963 by Robert Guthrie [1] introducing an assay for the diagnostic investigation of phenylketonuria in newborns. Subsequently, further applications were suggested especially for neonatal screening of metabolic disorders [2–4]. In the last decades DBS analysis by liquid chromatography coupled to mass spectrometry was firmly established and the number of procedures enormously increased. Various applications are described for disease surveillance (e.g. cancer, diabetes), preclinical drug development, toxicological and pharmacokinetic studies, therapeutic drug monitoring (TDM), and metabolomic profiling [4–11]. At this point, however, the DBS sampling technique has never been implemented in routine doping control analysis, although there are a couple of promising application examples [12–16]. Compared to conventional venous blood sampling, the collection of a volume of typically 20 μl of capillary blood from a heel, ear or finger prick is minimally invasive [17,18]. Further benefits involve the fastness including the possibility for automation of the sample processing [10,19], robustness against manipulation [5,9,14] and enhanced stability of the cellulose-fixed analytes at room temperature. Stability improvement may be attributed to the elimination of humidity via dehydration and the inactivation of enzymatic degradation [4,5] as opposed to conventional sample matrices (urine, plasma, serum). This, as well as the small sample volume, results in simplified storage and shipment conditions. However, a few challenges have to be overcome,

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namely the comparably time consuming sample preparation and the extraction of the analytes from the cellulose-based matrix. The small sample volume of typically 20 μl of blood requires high sensitivity of the mass spectrometer and the applied analytical method [9].

The anabolic steroid testosterone is prohibited since 1976 by the International Olympic Committee (IOC) [20] but is still one of the most frequently detected doping agents [21]. Testosterone intake induces an increase of lean body mass and strength as well as a shortened recovery time [22]. Because testosterone is metabolically instable after oral application, prodrugs in form of the steroid esters are administered orally for doping purposes. Additionally sustained release is obtained via esterification of testosterone and its analogues when administered intramuscularly. In the systemic circulation the esters are hydrolyzed by blood esterases and become fully effective. Due to the ester cleavage in blood, concentrations of the intact esters are rather low. Pharmacy offers multiple formulations of the anabolic steroid esters, some of which are approved drugs used in the therapy of hypogonadism [22].

Traditionally the abuse of anabolic agents derived from the endogenous steroid hormone testosterone is screened for by means of the steroid profile (including the ratio of testosterone to epitestosterone (T/E ratio > 4)) in urine. This screening assay poses great challenges for doping control laboratories. The differentiation between the exogenously administered testosterone and testosterone occurring physiologically represents a major difficulty. Therefore suspicious results are verified or falsified by the use of carbon isotope ratio mass spectrometry (IRMS) [23–25].

An enormous benefit is achieved by the application of other sampling matrices such as blood or hair since a direct and unequivocal proof of the intact anabolic steroid esters is possible. A screening assay for testosterone esters in human plasma was presented by Forsdahl et al. [26], others reported the detection of the target analytes in equine serum [27] and human hair [28]. Conventional venous cannula sampling is an invasive method with the need for physicians or phlebotomists for sample collection. To cope with these challenges the present approach highlights the application of the DBS sampling technique in the analysis of anabolic steroid esters in doping control analysis for the first time. A screening method was developed for nandrolone and eight anabolic steroid esters deriving from testosterone, nandrolone and trenbolone using high performance liquid chromatography (HPLC) coupled to high resolution tandem mass spectrometry (HR–MS/MS). The identification and determination of the target compounds was conducted in targeted MS/MS mode.

2. Materials and methods

2.1. Chemicals and reagents

Testosterone acetate, testosterone cypionate, testosterone isocaproate, testosterone phenylpropionate and nandrolone were obtained from Sigma (Schnelldorf, Germany). Nandrolone phenylpropionate was purchased from the National Measurement Institute (Sydney, Australia) and trenbolone enanthate from Steraloids (New Port, USA). Andriol® Testocaps 40 mg were obtained from Essex Pharma GmbH (Munich, Germany). Deuterium labeled nandrolone [29], d_3-nandrolone caproate and d_3-nandrolone undecanoate were synthesized in-house. O-methyl-hydroxylamine hydrochloride 98%, formic acid (analytical grade), undecanoyl chloride (99%), hexanoic anhydride (≥97%), aluminium chloride (≥99.0%) and silica gel (pore size 60 Å, 70–230 mesh) were supplied by Sigma (Schnelldorf, Germany). All solvents (LC grade) were used without further purification.

2.2. Materials

DBS cards (FTA® DMPK C) were obtained from Whatman™ GE Healthcare (Uppsala, Sweden).

2.3. Synthesis of internal standards

The preparation of steroid esters for internal standards starts from a threefold deuterated nandrolone (d_3-nandrolone) taken from an in-house synthesis [29]. Esterification of nandrolone with the acid chlorides or anhydrides of the respective fatty acids was obtained by acid catalysis. In the following the synthesis of d_3-nandrolone undecanoate is shown exemplarily (Fig. 1).

An amount of 75 mg of aluminum chloride (0.56 mmol) was dissolved in 3.0 ml dichloromethane under heat development. After cooling the reaction batch to room temperature, 50 mg of the d_3-nandrolone (0.11 mmol) was added. After stirring for 30 min, 132 μl of undecanoyl chloride (0.59 mmol) was slowly added. The mixture was heated under reflux for 3 h. The reaction was terminated by the addition of crushed ice. Purification included solvent extraction and flush chromatography on a silica column (pentane–ethyl acetate (70:30, v/v)). The combined organic phases were evaporated followed by crystallization of d_3-nandrolone undecanoate. Reaction progress was controlled by thin layer chromatography and mass spectrometry.

2.4. Stock solutions

Standard stock solutions of the anabolic steroid esters were prepared with a concentration of 1 mg/ml in acetonitrile–water (50:50, v/v) and stored at −20 °C. For the tests a cocktail of the esters was prepared at concentration levels of 1 μg/ml, 100 ng/ml and 10 ng/ml by dilution of the stock solutions. Accuracy measurements were performed with two independently prepared stock solutions of nandrolone. As internal standard (IS) a solution was used with d_3-nandrolone, d_3-nandrolone caproate and d_3-nandrolone undecanoate at 50 ng/ml (d_3-nandrolone and d_3-nandrolone caproate) and 10 ng/ml (d_3-nandrolone undecanoate).

2.5. Sample preparation

DBS were prepared with whole blood samples spiked with the cocktail of anabolic steroid esters and extensively mixed prior to spotting an aliquot of 20 μl on a DBS sampling card. After a drying time of at least two hours at room temperature, the whole spots were punched out, transferred into polypropylene tubes and fortified with 5 μl of the IS solution (d_3-nandrolone, d_3-nandrolone caproate, d_3-nandrolone undecanoate). The spots were extracted twice with 500 μl of a mixture of methyl *tert*-butyl ether–2-propanol–methanol (56:24:20, v/v) using an ultrasonic bath for 30 min. The combined supernatants were evaporated to dryness in a centrifuge for 15 min at 60 °C under reduced pressure. The dry residue was resuspended in 50 μl of the derivatization agent (100 mM O-methyl-hydroxylamine in methanol 80%) and the tubes were centrifuged for 10 min at 17,000 × *g*. The supernatant was transferred into a glass tube and heated for 30 min at 80 °C [27]. For LC–MS/MS analysis 5 μl of the derivatized sample were injected.

2.6. Instrumental analysis

LC–MS/MS analysis was performed with a Thermo Dionex Ultimate 3000 liquid chromatograph interfaced to a Q Exactive Plus mass spectrometer (Thermo Scientific, Bremen, Germany), using a heated electrospray ionization (HESI-II) source. The Dionex system was equipped with an Accucore XL C8 analytical column, 3 mm × 100 mm, 4 μm particle size (Thermo Scientific, Bremen,

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