Journal of Pharmaceutical and Biomedical Analysis 101 (2014) 66–83

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Review

Analytical approaches for the detection of emerging therapeutics and non-approved drugs in human doping controls

Mario Thevis[a,*], Wilhelm Schänzer[b]

[a] Center for Preventive Doping Research – Institute of Biochemistry, German Sport University Cologne, Am Sportpark Müngersdorf 6, 50933 Cologne, Germany
[b] European Monitoring Center for Emerging Doping Agents, Cologne/Bonn, Germany

ARTICLE INFO

Article history:
Received 3 March 2014
Received in revised form 5 May 2014
Accepted 6 May 2014
Available online 23 May 2014

Keywords:
Doping
Sport
Mass spectrometry
Emerging drugs
Stamulumab
Anti-myostatin antibody

ABSTRACT

The number and diversity of potentially performance-enhancing substances is continuously growing, fueled by new pharmaceutical developments but also by the inventiveness and, at the same time, unscrupulousness of black-market (designer) drug producers and providers. In terms of sports drug testing, this situation necessitates reactive as well as proactive research and expansion of the analytical armamentarium to ensure timely, adequate, and comprehensive doping controls. This review summarizes literature published over the past 5 years on new drug entities, discontinued therapeutics, and 'tailored' compounds classified as doping agents according to the regulations of the World Anti-Doping Agency, with particular attention to analytical strategies enabling their detection in human blood or urine. Among these compounds, low- and high-molecular mass substances of peptidic (*e.g.* modified insulin-like growth factor-1, TB-500, hematide/peginesatide, growth hormone releasing peptides, AOD-9604, *etc.*) and non-peptidic (selective androgen receptor modulators, hypoxia-inducible factor stabilizers, siRNA, S-107 and ARM036/aladorian, *etc.*) as well as inorganic (cobalt) nature are considered and discussed in terms of specific requirements originating from physicochemical properties, concentration levels, metabolism, and their amenability for chromatographic-mass spectrometric or alternative detection methods.

1. Introduction

With the constantly increasing knowledge about biochemical mechanisms at cellular and molecular levels, more and more options for pharmacological interventions have been identified that suggest new paths to desired therapies potentially allowing cure for severe if not fatal diseases. The flipside of such research is the misuse potential offered by a subset of new drug candidates, especially those that promote muscle growth, stimulate erythrocyte production, or enhance physical stamina and athletic performance *via* other routes [1]. Such drug candidates have been offered and sold *via* Internet-based providers for years, despite the lack of clinical approval and, in some cases, discontinuation of their development due to severe side effects. The targeted 'clientele' of such offerings is composed of recreational as well as professional athletes, with the latter ones being at risk of violating regulations established by the World Anti-Doping Agency (WADA) [2] These regulations as presented in WADA's Prohibited List include a category of substances dedicated to particularly such compounds, *i.e.* 'non-approved for human use'/discontinued drug candidates, referred to as S0. In order to enable comprehensive doping controls, accredited laboratories update, expand, and improve their portfolio of analytical assays, most of which rely on chromatographic-mass spectrometric approaches [3,4]; however, the implementation of new compounds into sports drug testing protocols requires a substantial amount of information including therapeutic dosage, pharmacokinetics, metabolism, and elimination. Moreover, specific physicochemical properties might necessitate dedicated sample collection and transport conditions, sample preparation or analytical procedures to ensure the required sensitivity and specificity to detect the target analyte with appropriate limits of detection (LODs) [5] With the constraints in budget, time, sample volume(s), laboratory staff and instrumentation, sports drug testing laboratories however are urged to combine as many detection assays as possible without compromising the necessary analytical requirements, preferably by using and expanding existing analytical approaches. Hence, test menus need to be rationally arranged and their fitness-for-purpose as appropriate initial testing procedure has to be demonstrated.

* Corresponding author at: Institute of Biochemistry – Center for Preventive Doping Research, German Sport University Cologne, Am Sportpark Müngersdorf 6, 50933 Cologne, Germany. Tel.: +49 221 4982 7070; fax: +49 221 4982 7071.
E-mail address: thevis@dshs-koeln.de (M. Thevis).

http://dx.doi.org/10.1016/j.jpba.2014.05.020
0731-7085/

While formerly drug classes dictated the composition of analytical assays, nowadays the available analytical equipment commonly governs the employed test strategies [3]. To date, routine doping control matrices are urine, serum and blood, occasionally complemented by alternative matrices such as hair potentially providing supporting evidence. The collection protocols follow stringent regulations and require trained doping control officers/phlebotomists; transport times and conditions have to be controlled and documented especially in case of blood samples for the Athlete Biological Passport (ABP), where also time limits for transport and analysis apply. In addition, sample storage (urine and serum) has to be ensured for up to 10 years to allow for re-testing if requested.

In the present review, literature published between 2009 and 2013 concerning emerging, 'designer', and discontinued drugs is discussed in the context of human doping controls. Challenges arising from structural feature of substances are presented and metabolite identification and detection strategies are outlined for a representative selection of compounds covering low- and high molecular mass analytes of non-peptidic, peptidic, and ribonucleic acid composition.

2. Compounds affecting skeletal muscle performance

Due to the substantial number of compounds with evident or presumed impact on skeletal muscle physiology and/or performance, the substances considered in the following are divided into the categories of low and high molecular mass products.

2.1. Low molecular mass substances

2.1.1. Ryanodine receptor-calstabin-complex stabilizers (Rycals)

Studies on cardiac arrhythmia as well as sarcopenia (as defined as the age-related loss of muscle mass, force, and exercise capacity) and muscular dystrophy have revealed the relevance of the ryanodine receptor 1 (RyR1) and its Ca^{2+}-channel complex building partner molecule calstabin-1 (FK506 binding protein 12, FKBP12) with regard to normal skeletal and cardiac muscle function. Substantial research on mechanisms of post-translational modifications has been conducted in animal models and, more recently, also in humans indicating particularly S-nitrosylation and (hyper)phosphorylation of RyR1 as main factors of the aging-, disease-, or exercise-induced functional impairment of myocytes [6–8]. A potential therapy is based on benzothiazepine-derived drug candidates such as the first- and second-generation therapeutics JTV-519 and S107 (Fig. 1a, **1** and **2**) [9], which have been shown to reduce muscle fatigue and improve exercise capacity in laboratory rodents by restoring the RyR1-FKBP12 complex. Consequently, the relevance of such compounds for sports drug testing was recognized and detection assays for the intact drugs and/or *in vitro* generated metabolites in blood and urine were established.

The mass spectrometric behavior of JTV-519 and S-107 was studied *in extenso* using electrospray ionization (ESI) and collision-induced dissociation (CID) [10] as well as electron ionization (EI) [11] employing high resolution/high accuracy mass spectrometry, stable isotope labeling and, in case of ESI-CID, H/D-exchange experiments. By means of the obtained information, test methods for urine [10,11] and plasma [12] were developed enabling the detection of the intact molecules at LODs of 0.1–6 ng/ml. In case of blood plasma, peak concentrations of the drug candidates after therapeutic dosing were expected at approximately 40 ng/ml, which was well within the detection window of the developed test method. In the absence of data on the metabolism and (renal) elimination of the benzothiazepines, urine samples were subjected to enzymatic hydrolysis followed by liquid–liquid extraction (LLE) of the target analytes and subsequent detection by means of liquid chromatography–(tandem) mass spectrometry (LC–MS/MS) or gas chromatography–mass spectrometry (GC–MS). In order to further complement the analytical approach with putative metabolites, phase-I and phase-II metabolic reactions were simulated for S-107 *in vitro*, yielding predominantly N- and S-oxygenated species as well as N- and O-demethylated metabolites. Moreover, glucuronic acid conjugates of the intact drug and its O-demethylated phase-I metabolic product were identified representing viable targets for future doping controls [13]. Moreover, the development of next-generation benzothiazepine-derived compounds needs further consideration, *e.g.* with regard to the phase-II clinical trial drug candidate referred to as ARM036 (Aladorian, Fig. 1a, **3**) [14], the product ion mass spectrum of which is presented in Fig. 1b.

2.1.2. Selective androgen receptor modulators (SARMs)

Non-steroidal selective androgen receptor modulators (SARMs) have been subject of extensive preclinical and clinical trials since the first-in-class compounds were identified in 1998, predominantly aiming at the treatment and prevention of sarcopenia, osteoporosis, and disease-related losses of skeletal muscle mass, strength, and function [15,16]. Moreover, the potential utility of SARMs in cardiology has been discussed [17], and the substantial interest in new drug entities with SARM-like properties is still on the incline according to recent reviews [16,18] and publications on advances in SARM-related research [19–21]. With the increasing amount of possible non-steroidal and steroidal SARM drug candidates, examples of which are illustrated in Fig. 2 (**4**–**13**), the portfolio of compounds potentially misused in sports is expanded accordingly [22,23] and detection assays plus ample information on metabolism and elimination are vital for appropriate doping controls. Consequently, studies focusing on the metabolism of SARMs and possibilities to detect intact as well as metabolized SARMs have been initiated and continued, and the relevance and necessity of adequate test methods was demonstrated with the first adverse analytical findings (AAFs) for SARMs in 2010 and the following years [24,25]. The analytical assays for SARMs have been established for plasma [12,26], dried blood spots (DBS) [27], and urine targeting either the intact substances (plasma and DBS) or main metabolites (urine) as identified and characterized in *in vitro* [28] and *in vivo* studies [29–31]. Despite modest structural similarities between some SARMs comprising *e.g.* a 4-substituted aniline moiety, a substantial heterogeneity of pharmacophores is present in currently investigated SARMs including (amongst others) arylpropionamide, quinolinone, tropanol, tetrahydroquinoline, hydantoin, thiophene, phenyl-oxadiazol, and steroid derivatives (Fig. 2, Table 1). Hence, various projects have been required providing insights into main metabolic pathways and the mass spectrometric behavior of identified and characterized target compounds.

All SARMs recently studied in a doping control context demonstrated good or excellent ionization properties using electrospray, thus supporting the sensitive detection of these compounds and related metabolic products in sports drug test samples employing LC–MS/MS-based strategies [3,32]. Arylpropionamide-derived SARMs were among the first category of emerging anabolic agents investigated with ESI-MS/MS, EI-MS(/MS), and under *in vitro* and *in vivo* metabolism conditions. Substantial agreement between results of *in vitro* and *in vivo* studies was observed, and post-administration study urine samples of the arylpropionamide-derived SARMs S-4 and S-22 (Fig. 2, **4** and **5**, respectively) predominantly yielded the glucuronic acid conjugates of the intact drugs and corresponding mono-hydroxylated metabolites as viable analytes for routine doping controls [29,30] with LODs for the intact drug candidates found below 1 ng/ml. Complementary, LODs of 0.05–20 ng/ml [27,33] and 10 ng/ml [26] were determined in DBS and plasma, respectively, for the intact therapeutics. Substance