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Detection of SARMs in doping control analysis

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ABSTRACT

The class of selective androgen receptor modulators (SARMs) has been the subject of intense and dedicated clinical research over the past two decades. Potential therapeutic applications of SARMs are manifold and focus particularly on the treatment of conditions manifesting in muscle loss such as general sarcopenia, cancer-associated cachexia, muscular dystrophy, *etc*. Consequently, based on the substantial muscle- and bone-anabolic properties of SARMs, these agents constitute substances with significant potential for misuse in sport and have therefore been added to the Word Anti-Doping Agency's (WADA's) Prohibited List in 2008. Since then, numerous adverse analytical findings have been reported for various different SARMs, which has underlined the importance of proactive and preventive anti-doping measures concerning emerging drugs such as these anabolic agents, which have evidently been misused in sport despite the fact that none of these SARMs has yet received full clinical approval. In this review, analytical data on SARMs generated in the context of research conducted for sports drug testing purposes are summarized and state-of-the-art test methods aiming at intact drugs as well as diagnostic urinary metabolites are discussed. Doping control analytical approaches predominantly rely on chromatography hyphenated to mass spectrometry, which have allowed for appropriately covering the considerable variety of pharmacophores present in SARMs such as the non-steroidal representatives ACP-105, BMS-564929, GLPG0492 (DT-200), LG-121071, LGD-2226, LGD-4033/VK 5211, ostarine/enobosarm, RAD-140, S-40503, *etc*. as well as steroidal compounds such as MK-0773 and YK-11.

1. Introduction

The utility of anabolic agents as therapeutics in addressing conditions of muscle wasting, particularly in cases of different forms of cachexia (Dalton et al., 2013; Srinath and Dobs, 2014; Bhasin et al., 2006; Cadilla and Turnbull, 2006; Chen et al., 2002, 2005; Gao and Dalton, 2007; Mohler et al., 2009; Zilbermint and Dobs, 2009), has been considered frequently in the past. In addition, the management of sarcopenia and frailty (O'Connell and Wu, 2014; Siparsky et al., 2014; Kilbourne et al., 2007) as well as hypogonadism (Coss et al., 2014) by means of anabolic agents has been shown to receive continuously growing interest, supported and fueled by the constantly expanding information on new drug entities commonly referred to as selective androgen receptor modulators (SARMs). To date, no SARM has yet received full clinical approval. Nevertheless, due to the enormous muscle- and bone-anabolic properties of SARMs as evidenced in numerous preclinical and clinical studies (Dalton et al., 2013; Choi and Lee, 2015; Zhang and Sui, 2013), the potential for misuse in the context of amateur and elite sport has been recognized and led to the inclusion of SARMs into the World Anti-Doping Agency's (WADA's) Prohibited List (World Anti-Doping Agency, 2016a) in 2008. First adverse analytical findings (AAFs) for SARMs were reported in 2010 (Grata et al., 2011; Starcevic et al., 2013; Cox and Eichner, 2017) and since the number of AAFs for this class of compounds has been constantly increasing (World Anti-Doping Agency, 2016b), indicating the relevance of doping control analytical procedures enabling the comprehensive detection of SARMs misuse.

Most sports drug testing approaches allowing to efficiently analyze for SARMs are based on multi-analyte low- or high-resolution mass spectrometric methods (Thevis et al., 2015a, 2016, 2011a). These methods largely necessitate the knowledge about the drugs' composition and metabolic fate in humans, but

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especially the information on metabolic biotransformation is, to date, rather limited in the scientific literature. Therefore, drug candidates (where available) have commonly been subjected to in-depth chromatographic-mass spectrometric studies in order to provide analytical data for the evaluation of subsequent *in vitro* or animal metabolism studies. From these, preliminary target analytes for human sports drug testing procedures can be construed until confirmed or substituted by analytes derived from authentic drug elimination study specimens offering *e.g.* superior detection windows. An emerging alternative strategy to the aforementioned mass spectrometric approaches enabling comprehensive initial testing for anabolic agents in sports has been the use of androgen receptor-based bioassays. While currently not routinely employed in doping control laboratories, advantages and limitations of this methodology for sports drug testing purposes have been assessed as recently summarized elsewhere (Cadwallader et al., 2011; Bailey et al., 2016; Campana et al., 2015). In this review, an overview about mass spectrometric data of selected SARMs of steroidal and non-steroidal pharmacophores is presented, and principles and performance characteristics of corresponding routine doping control analytical assays are discussed.

2. Mass spectrometry of SARMs

The class of drug candidates referred to as SARMs includes a considerable variety of chemical core structures, which can generally be categorized into steroidal and non-steroidal compounds (Table 1). Within these categories, drug entities of substantially different physico-chemical properties exist, which have necessitated comprehensive studies concerning the substances' ionization and collision-induced dissociation (CID) behaviors to generate the information required for the analytes' implementation into existing routine sports drug testing programs or for establishing dedicated and SARM-specific analytical assays.

Mass spectrometric data and detection methods for selected arylpropionamide-, quinolinone-, tetrahydroquinoline, and hydantoin-derived SARMs such as the compounds **1**–**6**, **7** and **10**, **11**, and **15**, respectively (Fig. 1), have been the subject of comprehensive reviews before (Thevis and Schänzer, 2008; Thevis, 2010). Hence, in this report, mass spectral data of SARMs and corresponding metabolites comprising of phenyl-oxadiazole-, tropanol-, pyrrolidinyl-benzonitrile-, diarylimidazolidinedione-, and steroid-related pharmacophores such as RAD140 (**17**), ACP-105 (**18**), LGD-4033 (**20**), GLPG0492 (**30**), and MK-0773 (**34**) as well as YK-11 (**37**), respectively, are discussed.

2.1. RAD140

RAD140 (2-chloro-4-(((1R,2S)-1-(5-(4-cyanophenyl)-1,3,4-oxadiazol-2-yl)-2-hydroxypropyl)amino)-3-methylbenzonitrile, Fig. 1, **17**) was first referred to as a new drug entity with SARM-like properties in 2011 (Miller et al., 2011). Owing to the presence of a 1,3,4-oxadiazole moiety (Trifonov and Ostrovskii, 2006), RAD140 is readily ionized using positive electrospray ionization (ESI). The protonated molecule $[M+H]^+$ of **17** is found at *m/z* 394 and dissociates under CID conditions into three major product ions observed at *m/z* 223, 205, and 172 (Fig. 2a), the formation of which was suggested to result from a charge-driven dissociation process as illustrated in Scheme 1 (Thevis et al., 2013). Following protonation of **17**, the oxadiazole residue was shown to be eliminated inclusive of the 2-positioned substituent, yielding the abundant product ion at *m/z* 223, attributed to the protonated species of 2-chloro-4-((2-hydroxypropylidene)amino)-3-methylbenzonitrile. This ion was shown to undergo further dissociation processes including the loss of water and a chlorine radical, resulting in additional diagnostic product ions at *m/z* 205 and 170 (Scheme 1). Moreover, a product ion characterizing the 4-(1,3,4-oxadiazol-2-yl)benzonitrile residue of **17** is given at *m/z* 172, which was proposed to represent the counterpart to *m/z* 223 as supported by the analysis of a first generation analog to RAD140. Due to the presence of a 2-phenyl-1,3,4-oxadiazole moiety instead of a 4-(1,3,4-oxadiazol-2-yl)benzonitrile residue in the RAD140 analog, a product ion decremented by 25 Da was found accordingly at *m/z* 147 (Thevis et al., 2013).

Complementary, Sobolevsky et al. studied RAD140 using negative ESI (Sobolevsky et al., 2013). Following deprotonation, the precursor ion of RAD140 $[M-H]^-$ at *m/z* 392 rapidly eliminates acetaldehyde (44 Da), yielding the product ion at *m/z* 348 as depicted in Fig. 2b. Further abundant and/or diagnostic product ions are observed at *m/z* 321, 175, 165, and 127, attributable to the loss of HCN (27 Da) from *m/z* 348 and the formation of deprotonated 4-chloro-indole-5-carbonitrile, deprotonated 4-amino-2-chloro-3-methylbenzonitrile, and deprotonated terephthalonitrile, respectively. This information was subsequently utilized to study *in vitro*-as well as *in vivo*-derived biotransformation products. The analyses of metabolism study samples demonstrated a substantial metabolic stability of RAD140 and only two monohydroxylated species of yet unconfirmed structure were observed. Due to an increment of the drug candidate's product ion at *m/z* 175, which was tentatively assigned to the 2-chloro-3-methylbenzonitrile moiety of RAD140 (*vide supra*), by 16 Da, hydroxylation at this residue was proposed (Sobolevsky et al., 2013). Using liquid chromatography (LC)-tandem mass spectrometry (MS/MS) employing a triple quadrupole (QqQ) instrument, administration study urine samples were analyzed in multiple reaction monitoring (MRM) mode. Both the administered drug as well as the monohydroxylated metabolites were detected using diagnostic precursor-product ion pairs following enzymatic hydrolysis of respective glucuronic acid conjugates. Since unconjugated metabolites were not observed, the implementation of RAD140 into routine doping controls either necessitates targeting the intact conjugates (when applying direct urine injection procedures) or the inclusion of the deconjugated analytes into methods that employ hydrolytic sample work-up steps. According to Sobolevsky et al., aiming at RAD140 rather than its hydroxylated metabolites is favored due to more abundant signals observed in the pilot drug elimination study (Sobolevsky et al., 2013).

2.2. ACP-105 and AC262536

The SARM-like properties of the tropanol-based compounds such as AC262536 and ACP-105 (Fig. 1, **18** and **19**) were first reported in 2008 (Piu et al., 2008; Schlienger et al., 2009), justifying a preventive and proactive consideration of these substances in an anti-doping context. Due to the alkaline nature of most tropine derivatives (Prankerd, 2007), positive ESI efficiently produces protonated species of both SARMs, which dissociate into various diagnostic product ions under CID conditions as illustrated in Fig. 3a and b. The dissociation pattern of ACP-105 was studied in great detail (Thevis et al., 2013), suggesting distinct routes of product ion formation that are applicable also to the structural analog AC262536. As exemplified with AC262536 in Scheme 2, protonation of the analytes is followed by comprehensive fragmentation of the tropine moiety upon collisional activation, yielding product ions that indicate the immediate as well as stepwise elimination of the azabicyclo[3.2.1]octan-3-ol residue. The formation of the product ions at *m/z* 169 and 195 for instance are plausibly explained by losses of cycloheptadienol (110 Da) and cyclopentenol (84 Da), respectively, while the ions at *m/z* 235 and 179 as well as *m/z* 261 and 181 are proposed to result from consecutive eliminations of acetaldehyde (44 Da) plus butene

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