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New drugs and methods of doping and manipulation

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The issue of doping in sport is multifaceted. New drugs not only with anabolic properties such as selective androgen receptor modulators, synthetic insulins, blood doping with erythropoietins or homologous and autologous blood transfusions but also with sample manipulation have necessitated sensitive, comprehensive and specific detection assays allowing for the identification of cheats. New methods based on mass spectrometry, flow cytometry and immunological techniques have been introduced and improved in the past years to support and enhance the antidoping fight. Although numerous approaches are successful and promising, these methods still have some shortcomings.

Introduction

The issue of doping in sport has been closely related to the developments of drugs invented and prepared to cure severe diseases. Anabolic agents such as anabolic androgenic steroids were developed and employed, for example, for the treatment of testosterone deficiencies or wasting diseases, and recombinant human insulin has been utilized to correct symptoms of *diabetes mellitus*. The misuse of these and many other drugs has been prohibited by the International Olympic Committee (IOC), World Anti-Doping Agency (WADA) and national antidoping organizations, and banned drugs and methods of doping are categorized in 12 groups listed in the annually released Prohibited List [1]. Doping using these drugs was proven by means of state-of-the-art sports drug-testing assays and assumed numerous times substantiated by the confessions of convicted athletes and results of house searches. Novel drugs and methods of doping and manipulation have recently become evident, which require new, complementary and more comprehensive doping control strategies.

Drugs misused in sports are commonly analyzed and identified using chromatographic/mass spectrometric techniques, which allow for the determination of approximately 95% of all target analytes [2–4]. Few drugs, such as recombinant human erythropoietin (EPO) and analogs [5–7] as well as growth hormone (hGH) [8,9] necessitated immunological methods to enable the discrimination between endogenously produced hormones and their modified recombinant counterparts. Because of the dynamic nature of pharmaceutical markets and new methods of doping and manipulation, existing sports drug-testing procedures have constantly been improved, expanded and complemented to cope with the growing threat of performance manipulation in elite, professional and amateur sport. New candidates presenting high risk for misuse in sport are compounds with anabolic [10] and growth promoting properties based on drugs such as selective androgen receptor modulators (SARMs) and synthetic insulin analogs, respectively [11]. In addition, methods of doping conducted to increase the oxygen transport capacity by means of stimulated erythropoiesis and homologous or autologous blood transfusion as well as new urine adulteration strategies employing proteases to prevent adverse analytical findings in doping controls were revealed recently. In particular with regard to doping scandals in Germany and Spain in 2006 and 2007, the need for intelligent and comprehensive out-of-competition testing has, once more, become evident and outlined the necessity of preventive doping research, that is, early method development for emerging drugs with potential for misuse in sports. Selected strategies currently employed by doping control laboratories to ban the cheats and frauds in sports are summarized in the present short review.

Selective androgen receptor modulators (SARMs)

Ever since the importance of serum testosterone levels for muscle strength and physical performance became evident, the development of tissue-selective and orally bioactive anabolic agents has

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been a pharmaceutical challenge [12–15]. To counteract the age-related functional decline in men, not only symptoms of frailty but also muscle wasting, numerous derivatives of anabolic androgenic steroids were synthesized and clinically tested. Although beneficial effects such as improved lean body mass and muscle strength were observed, clinical concerns about potential side effects including benign prostatic hypertrophy and occult prostate cancer limited the utility of these substances. Hence, numerous research groups focused on the development of nonsteroidal, tissue-selective anabolic agents with significantly reduced androgenic side effects, and in 1998, a first series of SARMs was described by Dalton *et al.* [16]. These compounds were followed by various classes with SARMs activity, which are currently categorized into propionanilides (a), tricyclic quinolines (b), tetrahydroquinolines (c) and bicyclic hydantoins (d) as illustrated in Figure 1. Major advantages of these drug candidates are enormous tissue-selective anabolic properties combined with considerably reduced side effects commonly associated with steroid replacement therapies such as gynecomastia, decreased levels of HDL cholesterol and hepatic toxicity [15]. This is, at least in part, because these drugs do not undergo the metabolic reactions of testosterone and analogous compounds, that is, are not aromatizable or substrates for 5α-reductases [17]. Pilot studies with a lead drug candidate (*R*)-3-(4-acetylamino-phenoxy)-*N*-(4-cyano-3-trifluoromethyl-phenyl)-2-hydroxy-2-methyl-propionamide (Ostarine®, GTx Inc., Memphis, TN) stressed these facts when a group of elderly volunteers gained an average of 1.3 kg of muscle mass with concomitantly improved muscle strength within a period of 12 weeks of treatment with Ostarine. The potential of SARMs for being misused in sports is considered very high in particular, since anabolic agents have represented the most frequently detected class of drugs misused in sports for more than two decades. Hence, doping control laboratories are obliged to implement detection assays capable of detecting most advanced representatives of these new therapeutics. Until now, no SARM is commercially available but few have passed phase-II clinical trials.

For the purpose of preventive doping control strategies and earlier discovery of drugs that did not complete clinical approval, screening protocols have been developed that allow the detection of selected SARMs and their putative metabolic products in urine specimens. One approach is based on common targeted analyses expanded to structurally related drugs and degradation products. Urine sample aliquots of 2 mL are prepared using solid-phase extraction (SPE) followed by an analysis based on liquid chromatography–tandem mass spectrometry (LC–MS/MS) utilizing electrospray ionization (ESI). Drugs of known structure and composition are recorded employing targeted screening tools (e.g. multiple reaction monitoring) that enable detection limits below 1 ng/mL, but because of the virtually unlimited variety of possible structures and metabolic products of SARMs, precursor ion scan analyses are applied simultaneously. Focusing on common nuclei of known SARM structures and corresponding product ions generated under collision-induced dissociation (CID) conditions, unknown analogs and metabolites become detectable,

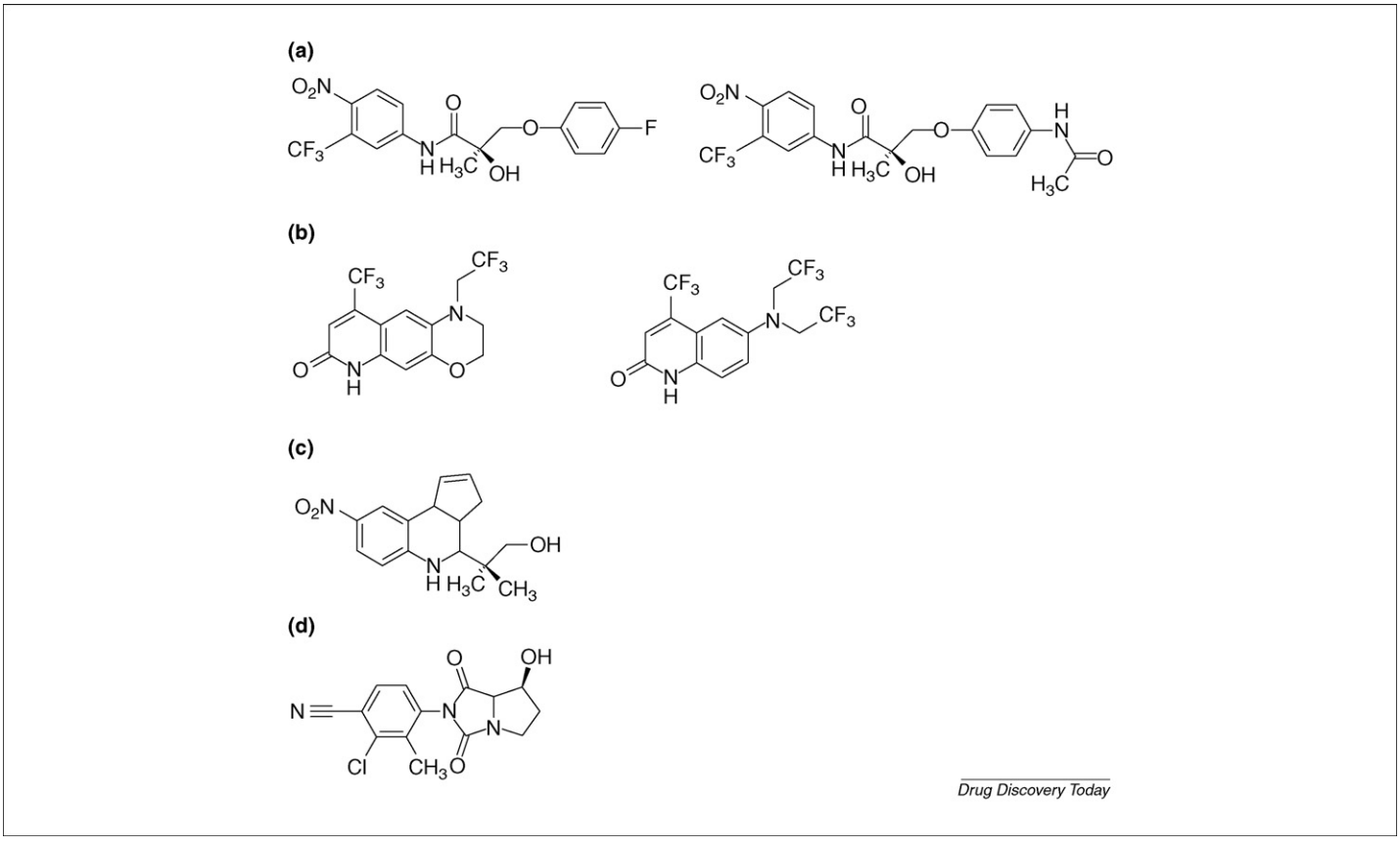

FIGURE 1

Structures of selected SARMs of different chemical classes: (a) arylpropionamides (left: Andarine® and right: Ostarine®), (b) quinolines (left: compound 8 and right: LGD-2226), (c) tetrahydroquinoline and (d) bicyclic hydantoin (BMS-564929).

which enables the detection of a wide range of SARM drugs [18]. Until now, approximately 4000 doping control urine specimens were tested for the presence of propionanilide-derived SARMs but no adverse analytical finding, that is, positive doping control result according to the regulations of the WADA, was reported. Alternatively, recently introduced androgen bioassays, with [19] or without [20] mass spectrometric characterization of analytes, allow the sensitive detection of designer anabolic agents and novel drugs with anabolic properties. Specimens are applied to yeast-based *in vitro* bioassays either as such or after chromatographic fractionation. In particular the latter yields an informative biogram that is matched with a simultaneously recorded high-resolution mass spectrum. Thus, unknown or new drugs will be detected and characterized utilizing accurate masses and database searches.

Recombinant modified insulins

The use of insulins in sports has always been allowed to athletes suffering from *diabetes mellitus* [4] but was banned in 1999 for all other sportsmen as a reaction to the assumed performance influencing the effects of insulin and health issues potentially arising from its misuse. In contrast to the medical reasons, athletes have been tempted to abuse insulins in order to accelerate recovery and loading of glycogen stores, and anabolic [21] as well as anticatabolic properties of hyperinsulinemic clamps were suspected [22], and various reports on insulin misuse have been published in particular in strength sports [23–26]. Moreover, house searches and confessions of athletes as well as team members substantiated the widespread misuse of insulins in elite sports [27,28]. The fact that so far no athlete has been sanctioned for the misuse of insulins is because of the lack of doping control analyses efficiently and specifically targeting these peptide hormones.

Recombinant modified insulins such as Lispro (Humalog®) or Apidra (Glulisine®) are structurally different from endogenously produced insulin and provide improved injection-to-onset profiles, that is, faster bioavailability of therapeutics and/or facilitated controllability in terms of dosage and duration of effect [29–32]. Lispro is referred to as a rapid-acting insulin bearing a set of amino acids that is identical to that of human insulin. It differs in the primary structure of the B-chain where the positions of proline (B_{28}) and lysine (B_{29}) are switched causing a reduced tendency to selfassociation and, thus, faster bioavailability. In case of Apidra, two amino acid residues are substituted resulting in a molecular weight different from that of Humalog or human insulin.

Insulins undergo extensive metabolism but minor amounts of intact protein/drug are excreted into the urine. New assays based on purification and concentration techniques employing antibody/antigen reactions and sensitive as well as specific LC–MS/MS instruments have recently led to methods allowing the unequivocal detection of recombinant modified insulins in doping control plasma and urine specimens [33,34]. Target analytes were isolated from respective matrices (1–2 mL of serum/plasma or 5–10 mL of urine) using sepharose-linked monoclonal antibodies specified against the N-terminal region of human insulin. Thereby, all C-terminally modified recombinant and numerous animal insulins are captured along with human insulin. After the elution of target analytes, concentration of solutions is accomplished using SPE and evaporation, which is followed by the reconstitution for LC–MS/MS identification. Liquid chromatography enables the separation of insulins from remaining interfering matrix residues, and analytes are identified using characteristic product ions (e.g. y_3 and $y_3 - y_1$) generated from multiply charged molecules under CID conditions. Compound-specific data at great sensitivity is obtained that allows the determination of 0.5 and 0.05 ng/mL of all insulins in plasma and urine, respectively [3]. Here, product ions derived from the C-terminus of the B-chain possess particular significance because of a proline-directed dissociation, and the capability of mass spectrometry to measure simultaneously and differentiate structurally; closely related analytes represent one of the most important advantages over commonly employed immunological detection methods. These commonly suffer from cross-reactivity and, hence, low specificity to particular antigens with smallest structural differences. A reliable distinction, for instance, between human insulin and Lispro or porcine insulin is hardly accomplished.

Despite the establishment of efficient methods to determine recombinant modified insulins, the issue of detecting the misuse of recombinant human insulin has not been solved yet. At present, promising approaches based on the analysis of profiles comprising intact insulin and its major urinary metabolites are under investigation. Apart from intact human insulin, three degradation products, namely, des-B30, des-B24-30 and des-B25-30 were characterized in urine aliquots of healthy and diabetic persons [35]. Depending on the route of insulin to enter the human blood stream (i.e. secretion from pancreatic β-cells or subcutaneous injection), the metabolic pathways vary, leading to differing metabolite patterns. Measuring these patterns might provide a future tool to determine the abuse of insulin for enhancing physical performance and accelerating regeneration.

Blood doping

Blood doping in sport has many facets and includes techniques such as the abuse of EPO as well as homologous (donor and recipient are different) and autologous (donor and recipient are identical) blood transfusions, all of which are aimed at artificially increasing the amount of erythrocytes and, thus, the oxygen transport capacity of the blood of athletes. These methods of doping have been revealed numerous times in endurance sports such as cycling and crosscountry skiing ever since the respective tests were introduced in 2001 and 2002.

Recombinant human EPO (rhEPO) is therapeutically administered in cases of anemia associated with chronic renal failure, cancer, AIDS, and also in pre- and postoperative periods in surgical patients [36] because of its stimulating effect on red blood cell production. Its clinical importance has been demonstrated manifold and numerous biosimilars and mimetic agents have been investigated and developed recently [37,38]. rhEPO is a highly glycosylated protein with 165-amino acid residues with an approximate molecular weight of 30 kDa [39]. rhEPO is commonly produced in Chinese hamster ovary cells, baby hamster kidney cells as well as human tumor cells [40]. Recombinant EPO differs from human EPO in glycosylation pattern and glycan size [39,41], which leads to differing isoelectric focusing (IEF) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) behavior and thus, options for the distinction between natural

and recombinant EPO. In sports drug testing, rhEPO is commonly differentiated in urine from its natural counterpart utilizing IEF and immunoblotting [42]. Aliquots of 20 mL of urine are concentrated followed by IEF separation of proteins employing a pH gradient of 2–6. Subsequent double blotting and chemiluminescence detection allow for sensitive and specific visualization of urinary EPO. However, samples with high protein content can complicate the focusing process, resulting in images that are hard to evaluate [43,44]. Recently improved sample preparation procedures include the pretreatment of the retentate of urine samples using immunoaffinity purification either with immunoaffinity chromatography [5,45], immunoprecipitation [46] or ELISA well-plate assays (C. Reichel, unpublished). In particular the latter technique was convincing because of its efficiency and simplicity allowing a considerable improvement of IEF images. In Figure 2a, the effect of ELISA-based purification on shapes of EPO isoform bands is presented exemplarily.

A complementary approach for the detection of recombinant EPO utilizing SDS-PAGE was presented early 2007 [46] based on the observations that endogenously produced EPO from serum and urine [47] as well as recombinant EPO and human urinary EPO are differentiated [48] using one-dimensional SDS-PAGE. The average molecular weight of recombinant EPO is approximately 30 kDa but it can be differentiated from endogenously produced urinary EPO by its migration on SDS gels [49] substantiating and confirming suspicious results of IEF analyses. Recombinant rat EPO was suggested as an internal standard (M. Kohler *et al.*, unpublished) as it is produced in insect cells, which are neither able to produce complex glycan structures nor to add sialic acid at the end of carbohydrate chains. As the amino acid sequence is highly similar to human EPO and the glycosylation different from EPO produced in vertebrate cells, the molecular weight is much smaller (approximately 20 kDa) but the protein is still recognized by anti-EPO antibodies allowing for conventional immunoblotting detection. The difference in migration between natural urinary or recombinant EPO and the internal standard on immunoblot images enables the distinction between endogenously produced EPO and synthetic analogs as illustrated in Figure 2b. This procedure proved particularly helpful in cases of ambiguous test results obtained from IEF analysis, as found for instance in lane 6 of Figure 2a that was proven negative as shown in lane 3 of Figure 2b. Moreover, the issue of EPO biosimilars might successfully be addressed combining both approaches, IEF and SDS-PAGE. The enormous amount of new EPO biosimilars [50] such as DynEpo [51] potentially complicates the distinction of recombinant EPO preparations from human urinary EPO using the conventional IEF approach only. Hence, additional information as obtained by SDS-PAGE might provide the required item to unequivocally distinguish endogenously produced EPO from recombinant analogs.

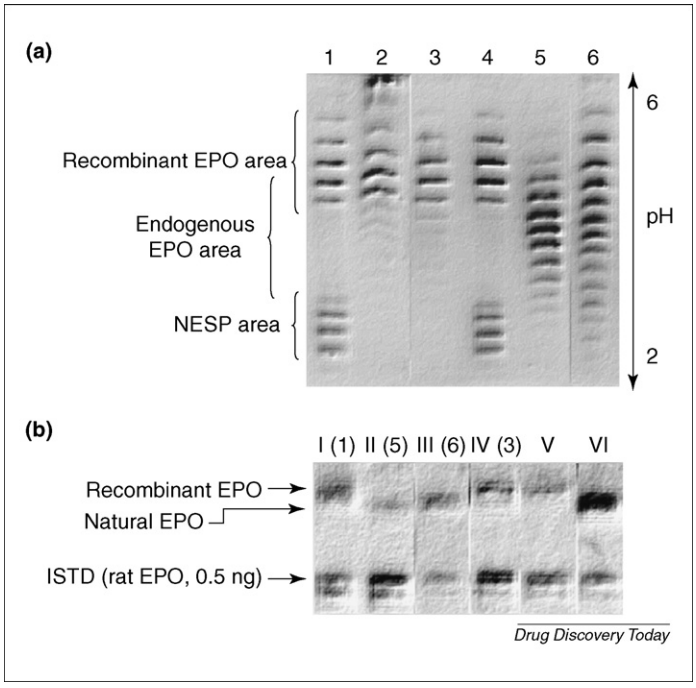

FIGURE 2

(a) IEF images of pure reference standards (lanes 1 and 4) of rhEPO (0.1 ng) and a hyperglycosylated analog-termed NESP (novel erythropoiesis-stimulating protein, 0.12 ng), a urine sample tested positive without (lane 2) and with (lane 3) ELISA pretreatment, a negative (lane 5) and a suspicious but negative (lane 6) doping control sample. (b) SDS-PAGE images of a pure reference standard of rhEPO (lane I, 0.1 ng), a negative specimen (lane II), a sample considered suspicious after IEF analysis but yielding a negative test results in SDS-PAGE (lane III, identical sample as shown in (a), lane 6), a positive specimen (lane IV, identical sample as shown in (a), lanes 2 and 3), a positive QC sample spiked with 0.2 ng of rhEPO (lane V) and a reference standard of natural EPO (0.15 ng, lane VI). The lower bands represent the internal standard (rat EPO, 0.5 ng) with different isoforms, and their distances to bands resulting from endogenously produced EPO differ significantly from those obtained from recombinant human EPO. Both images (a and b) were prepared using AlphaEase FC 3.0 Software (Biozym Scientific, Oldendorf, Germany).

In contrast to the misuse of erythropoiesis-stimulating drugs, homologous and autologous blood transfusions, which are clinical strategies to counteract and correct acute anemia, are considered prohibited *methods* of doping. Although autologous blood transfusions are not yet conclusively detectable, homologous blood transfusions can be determined in doping control samples utilizing the uniqueness of red blood cell surface antigen patterns. The membranes of erythrocytes bear numerous complex oligosaccharides and rhesus (Rh) polypeptides that compose a virtually unique and identifying set of parameters. The genetically defined combination of at least 45 independent antigens (e.g. C, c, E, e, K, k, Fya, Fyb, Jka, Jkb, etc.) characterizes an individual's erythrocytes and allows the unambiguous discrimination of those from a second population, which would be present after homologous blood transfusion [52,53]. Nelson *et al.* introduced this reliable method of doping testing based on the detection of blood group antigens utilizing flow cytometry [54], which has recently enabled the determination of several positive blood specimens. A technique termed signal amplification allowed for an improved separation of weak or heterozygously expressed antigens to facilitate and

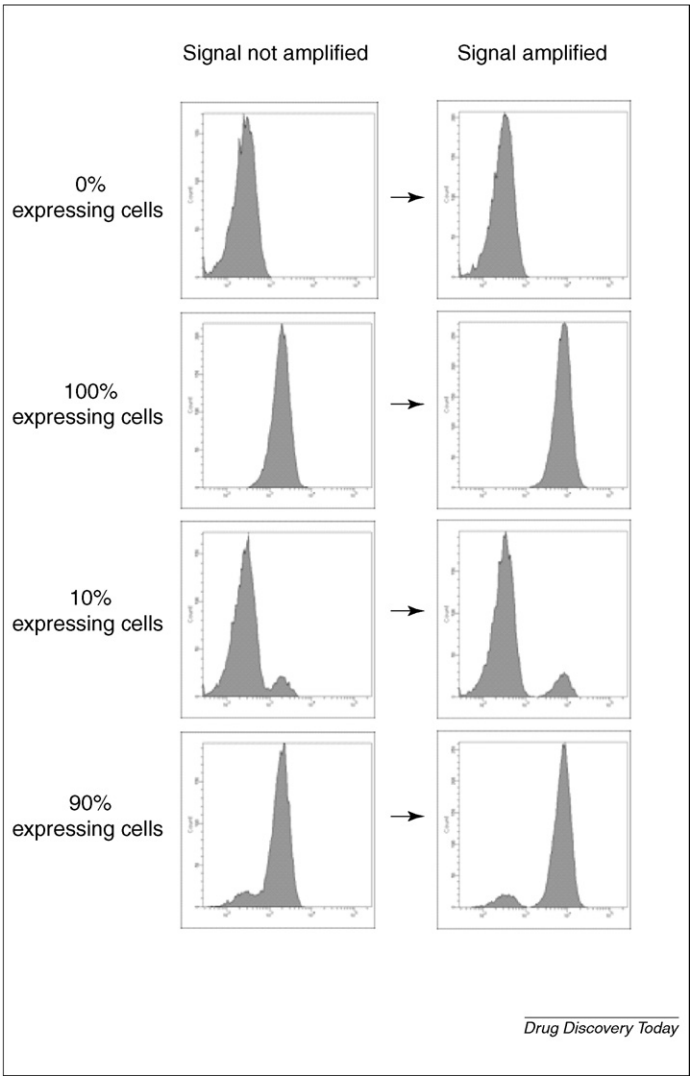

FIGURE 3

Result of a flow cytometry analysis without (left) and with (right) signal amplification. Jkb-nonexpressing cells are not influenced (i.e. shifted in fluorescence intensity on the *x*-axis) while expressing cells are tagged by antibodies and differentiated from nonexpressing cells. The discrimination between the two populations in mixed samples is demonstrated with 10 and 90% Jkb-expressing erythrocytes.

enhance the interpretation of histograms [55] resulting from blood analyses. Using this technique, mixed red blood cell populations in homologous blood transfusion samples containing 0.3–2.0% of donor blood were unequivocally determined because of an improved separation of different species of red blood cells. This is illustrated in Figure 3, which shows the expression analysis of antigen Jkb with and without signal amplification. Two populations of erythrocytes were clearly separated in mixed blood specimens demonstrating the capability to discriminate red blood cells from different donors.

Sample manipulation

Doping offences and resulting sanctions are not limited to a proven drug abuse or use of prohibited methods but also include all facets of manipulation. Urine substitution [56], urine dilution and refusal of a doping control sample as well as attempts to adulterate specimens with highly oxidative chemicals [57] have been reported. Very recently, a method based on the introduction of 'rice grains' into the urethra has become public. Athletes stated that administered peptide hormones such as EPO were not detected in doping control samples because of the masking properties of these 'grains'. As low molecular weight compounds such as endogenously produced steroids were not affected, drug-testing authorities assumed that these 'grains' are protease granules, which athletes employed to impede common urine drug tests. If a granule containing, for instance, 200 μg of proteases is flushed into the doping control sample during urine collection, respective enzymes can efficiently digest urinary proteins and eliminate any kind of target analyte such as EPO or insulins without affecting other urinary parameters that would have raised suspicion of manipulation.

Several analytical approaches have been employed to cope with this new issue of urine manipulation [58,59]. A semiquantitative determination of protease activity was suggested to indicate elevated urinary levels. In case of significantly increased concentrations of urinary proteases (>15 μg/mL), subsequent studies based on urine concentration and SDS 1D-gel electrophoresis were performed, which provided evidence for the presence or absence of proteins normally excreted into the urine such as uromodulin, albumin, etc. In addition, proteases (e.g. trypsin, chymotrypsin, papain, etc.) were visualized using conventional coomassie blue staining if their concentrations are greater than 5–10 μg/mL. More recently, the utility of zymogram gels was demonstrated, which allowed the visualization of low abundant urinary protease activity. Conventional polyacrylamide gels preconditioned with low amounts of immobilized and gel-entrapped bovine casein enabled the separation and staining of remaining urinary proteins, while in-gel renaturation of proteases caused the degradation of casein yielding colorless bands on the gel. Examples of regular doping control samples with noticeable low protein content (lanes 2–8), blank urine specimen (lane 9) and aliquots fortified with bovine trypsin or chymotrypsin (lanes 10 and 11, respectively) are depicted in Figure 4. The presence or absence of protein bands and protease activity is readily observed, and concentrations of proteases higher than 20 μg/mL cause large 'traces of burning' (lanes 10 and 11) indicating either a severe health issue or urine manipulation.

If suspicion arises from the absence of urinary proteins and the presence of bands possibly resulting from proteases, detected proteins and/or proteases are identified by means of commonly employed proteomics strategies including enzymatic digestion and LC–MS/MS bottom-up sequencing. Proteases and other proteins are characterized by proteotypical peptides, and the determination of the species of origin (e.g. human, animal or plant) by characteristic and unique amino acid sequences is mandatory. This enables a differentiation between human and xenobiotic proteases, with the presence of the latter indicating a doping offence. So far, several suspicious urine specimens were subjected to the detection assay for protease manipulation, but none of these samples was tested 'positive'. This outlines the fact that the protein content of urine specimens can be very low even without proteolytic degradation and, in addition, that this strategy of sample manipulation might have been abandoned by cheating athletes.

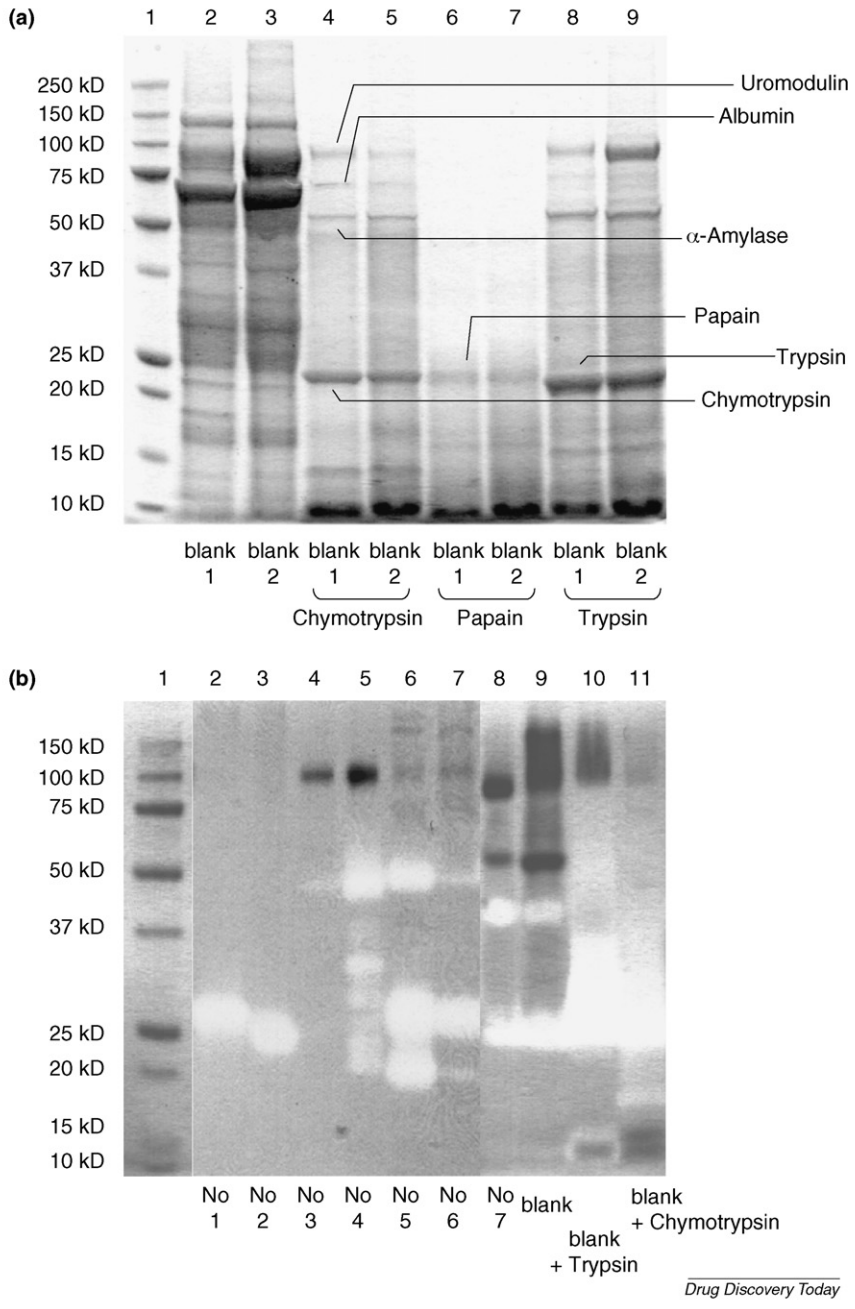

FIGURE 4

Images of SDS 1D-gel electrophoreses of blank urine specimens and aliquots fortified with proteases. (a) SDS-PAGE and (b) zymogram gel, both stained with coomassie blue. Selected urinary proteins and added proteases (20 μg/mL each) were visualized and subsequently characterized using commonly employed proteomics strategies.

Conclusion

Doping in elite sport has many facets and it has been a constant competition between cheating athletes and doping control authorities. New drugs and methods of doping and manipulation constantly arise and represent new challenges for drug-testing laboratories, which have reacted by expanding and improving the screening methods to provide detection tools as comprehensive, reliable and sensitive as possible (Table 1). However, few issues of doping still remain unsolved and represent future tasks to enable fair competition and clean sports. Those include, for instance, autologous blood transfusions, recombinant human insulin and new drugs, mimetics or biosimilars with virtually identical structures to naturally occurring agents. The fact that autologous blood transfusion requires blood donation, storage and reinfusion might provide indicators that can be utilized to uncover doping practices. The introduction of so-called blood passports will become mandatory for professional cyclists from 2008, and frequent blood analyses shall allow the detection of manipulation with EPO and blood transfusions. The administration of recombinant human insulin might be identified by means

TABLE 1

Methods for the detection of doping and manipulation

Drugs/method of doping	*Matrix*	Detection strategy	Refs
SARMs	Urine	LC–MS/MS of parent compounds and/or metabolites; bioassay with mass spectrometric characterization	[18–20]
Modified recombinant insulins	Urine	LC–MS/MS of parent compounds and/or metabolites; metabolite pattern determination	[33–35]
EPO and biosimilars	Urine	IEF, SDS-PAGE	[42–46]
Homologous blood transfusion	Blood	Flow cytometry; detection of more than one erythrocyte population by means of specific sets of antigens	[54,55]
Sample manipulation with proteases	Urine	Detection of protease activity; detection of xenobiotic proteases using proteomics approaches and zymography	[58,59]

of altered metabolite patterns that arise from subcutaneous applications of insulin to healthy athletes. Moreover, the relation of biosynthetically related substances, that is, proinsulin, C-peptide and insulin, might enable a screening for altered plasma and/or urine levels. Finally, comprehensive screening tools for new or unknown drugs with anabolic properties need to be implemented and expanded utilizing most modern analytical instruments and bioassays that represent the only common aspect of all anabolic drugs: the ability to activate specific target receptors.

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