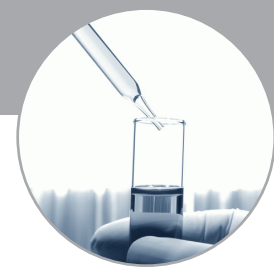

Bioanalysis and Analytical Services Research Group at The Municipal Institute for Medical Research IMIM-Hospital del Mar, Spain

Analytical laboratories involved in health-related research are becoming a fundamental part of the advancement of science in this field. Of particular interest to clinical, legal, toxicological, forensic and environmental matters is the analysis of drugs and medications present in biological fluids of consumers or exposed subjects. The established sensitive and reliable work of sports drug-testing laboratories represents an interesting example of a multidisciplinarity approach toward widespread bioanalytical problems. The experiences reported in this article will be of general interest, especially for analysts studying the determination of substances in biological material.

The Municipal Institute for Medical Research (IMIM) at the Hospital del Mar in Barcelona **(Figure 1)** is a pioneering center in drug addiction treatment and clinical pharmacology in Europe. The Bioanalysis and Analytical Services Research Group's main scientific objective is in the research and development of analytical methods, under strict quality assurance regulations, to facilitate the detection and understanding of the disposition and pharmacology of drugs present in biological fluids. The group possesses all necessary Quality Assurance Accreditations (International Organization of Standardization [ISO]17025, World Antidoping Agency [WADA], Association of Official Racing Chemists [AORC] and Clinical Laboratory by the Government of Catalonia) required to perform this research.

A particular area of interest for the group since 1985 has been the detection of doping agents in sport. The laboratory has been responsible for doping control at several major sports events, including the Olympic, Paralympic, Pan-American and Asian Games, in addition to World and Continental Championships. Its leading position in sport drug testing has been the engine behind multiple research projects carried out by the group. Without diminishing the importance of other activities of the group, we will now expand on our development of new methodologies for drug testing in sport.

Analysis of low-molecular-weight compounds

Most of the compounds included in the list of prohibited drugs in human and animal sports are low-molecular-weight compounds. The detection of their misuse is carried out through analysis in biological samples (mainly urine) of the parent compounds or their metabolites **(Figure 2)**. The metabolism of the compounds must be known in order to identify the best markers for detecting their administration. The main research focus of the group for low-molecular-weight compounds is the study of the metabolic profile of these doping agents in different biological samples.

In antidoping control, screening methods addressing groups of compounds with similar physicochemical properties are applied to all samples to eliminate 'true-negative' specimens. For first-round suspicious samples, a specific confirmation method is applied based on MS. GC or LC coupled to MS are the most commonly used techniques. Due to the stringent sensitivity and specificity requirements, high-performance (HP) chromatography coupled to high-resolution MS or MS/MS is applied for some compounds. The group has made relevant contributions in the development of reliable screening and confirmation methods that are now incorporated into the recommended methodologies for antidoping laboratories worldwide. In particular, the implementation of isotopic ratio MS for 'endogenous-like' anabolic–androgenic steroids and the implementation of sensitive HPLC–MS/MS and capillary GC–MS/MS methods for synthetic anabolic steroids, corticosteroids, diuretics, masking agents, stimulants, β-blockers, β2-agonists and nonsteroidal anti-inflammatory drugs, among others, are noteworthy.

Another focus of research is the development of protocols for differentiating between forbidden and authorized routes of administration. The protocol to discriminate prohibited oral use of salbutamol and authorized inhaled asthma treatment, developed by our group, is based on the different excretion of conjugated metabolites after oral and inhaled administrations and the stereoselectivity of the conjugation process. Studies are in progress

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Quality assurance
Set of activities intended to establish confidence that analytical quality requirements are met

Accreditation
External assessment of a laboratory indicating consistency of procedures to comply with client requirements

FUTURE SCIENCE part of fsg

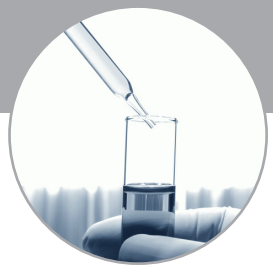


Figure 1. The Municipal Institute for Medical Research IMIM at the Hospital del Mar is part of the Barcelona Biomedical Research Park, where different research organizations are present and can interact. Synergies for the developments of projects involving chemistry, genomics, proteomics, imaging and experimental advanced biology are strongly encouraged. Reproduced with permission from PRBB/Ferran Mateo.

to develop methods to distinguish between legal and forbidden administration routes for corticosteroids, based on the different excretion of Phase I and Phase II metabolites.

Proteins increasing oxygen availability: erythropoietin

Erythropoietin (EPO) is one of the most powerful doping agents and its detection by differentiating recombinant variants from the endogenously produced hormone is one of the most demanding challenges in doping detection. The group has been actively working on this subject for the last decade, with several contributions. On the one hand, our long trajectory in quality assurance allowed us to lead interlaboratory validation of the currently applied method based on isoelectric focusing (IEF). On the other hand, important focus was set on elucidation of the variability of the protein structure, with

Erythropoietin

Glycoprotein, produced mainly by the kidneys, that promotes the maturation of red blood-forming cells in the bone marrow. Its activity is regulated by blood oxygen concentration. At present, erythropoietin of recombinant origin is widely used as a medication for anaemic patients but is also widely abused in sport

Isoelectric focusing

Analytical procedure where separation is mainly based on differences in electric charge. Each substance stops migration through the pH gradient of the gel where the pH coincides with its isolelectric point

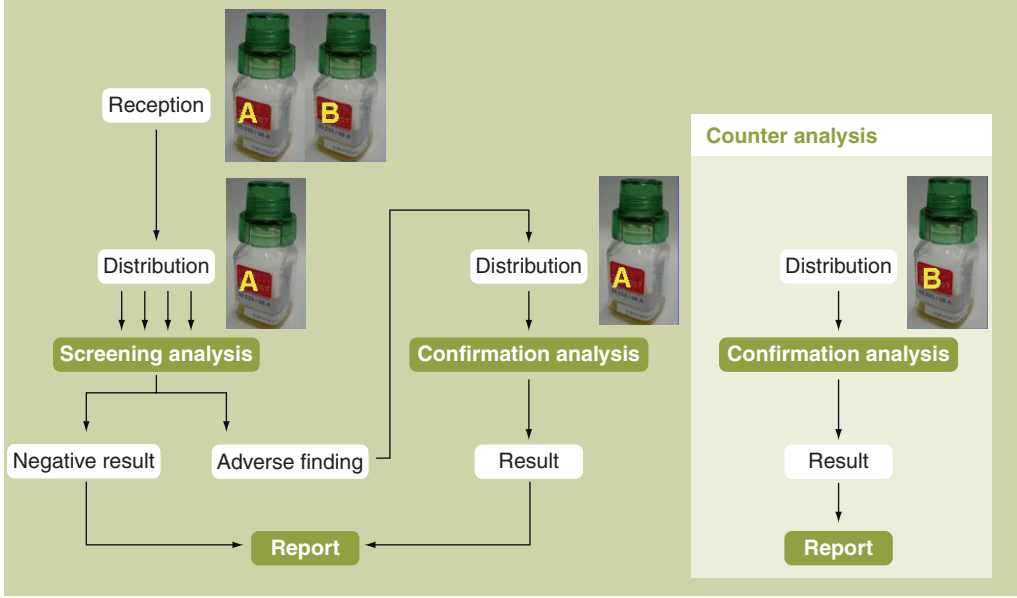
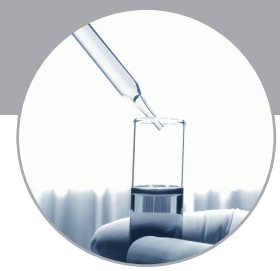


Figure 2. Protocol of analysis of samples for doping control. Two sealed subsamples from the same original urine arrive at the laboratory, where subsample B is stored frozen and subsample A is analyzed. Several methods for initial screening allow the confirmation analyses to concentrate on suspicious samples. In the event of a confirmed adverse analytical finding, the athlete may request and attend a counter-analysis with the preserved, untouched subsample B.



particular attention to the glycans (**Figure 3**), in order that we could prove the difference between recombinant and endogenous hormones.

Recently, the diagnostic presence of the non-human *N*-glycolyl-neuraminic acid (Neu5Gc) in common recombinant variants was confirmed. The presence or absence of this monosaccharide provides a possible way to absolutely identify most of the exogenously produced EPO. Currently, much effort is being devoted to detecting Neu5Gc using the most sensitive techniques available (i.e., nano-LC–MS/MS and miniaturized fluorescence detection).

The development of new antibodies targeting the differences between EPO compounds has also been thoroughly investigated. The development of monoclonal antibodies against Neu5Gc, with sufficient sensitivity to detect the minute concentrations of EPO found in human urine, is still underway. The group has developed specific polyclonal antibodies against a longer-acting EPO analog (novel erythropoiesis stimulating protein) using antigens made up of short peptides containing the differing amino acids in their sequence. Thorough research in immunoaffinity has also led to the development of an immunopurification procedure using antibody-coated microwell plates that allowed adaptation of the current urine IEF protocol to plasma samples, thus opening the field of EPO analysis to this emerging biological fluid in sports drug testing. However, research is still trying to elucidate the structures of human urinary and plasmatic EPO to justify its bizarre electrophoretic behavior.

On the other hand, we identified that, under controlled conditions, antibodies display differing affinity for different EPO glycoforms. This has been used to develop a fast and easy immunoaffinity procedure to detect rEPOs, providing a way to screen all doping-control urine samples (**Figure 4**). This is an important advance with respect to the limited number of EPO IEF analyses being performed due to logistic and cost reasons.

Growth hormone & other proteins

Growth hormone (GH) is also an active playground for the group. Over the last decade, substantial efforts have been put into the development

Growth hormones

Set of related proteins produced by the pituitary gland that stimulate the release of growth factors from the liver and affect the bone turnover rate. A recombinant form of 22 kDa is available for the treatment of patients with hormone deficiency. Although its use is strictly controlled by health authorities, it is believed to be widely abused in sport

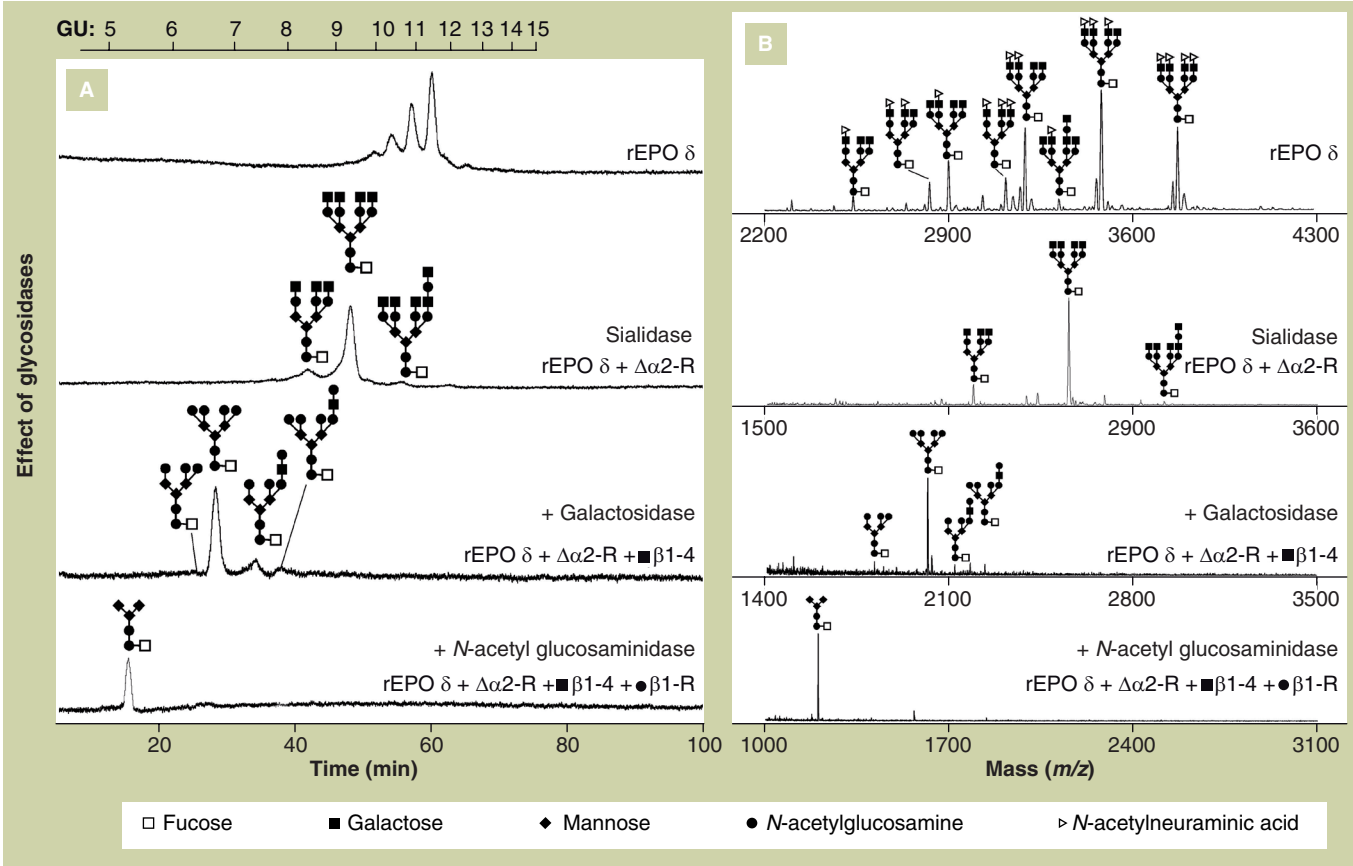
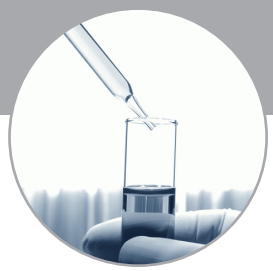


Figure 3. Structural analysis of the *N*-linked glycans from dynepo (epoietin-δ, a recombinant erythropoietin analog).
(A) Normal-phase HPLC profiles and **(B)** corresponding MALDI–TOF mass spectra. The top profile corresponds to the intact released glycans. In the subsequent profiles, downwards sequential exoglycosidase digestions corroborate the initial structural assignments. rEPO: Recombinant erythropoietin.



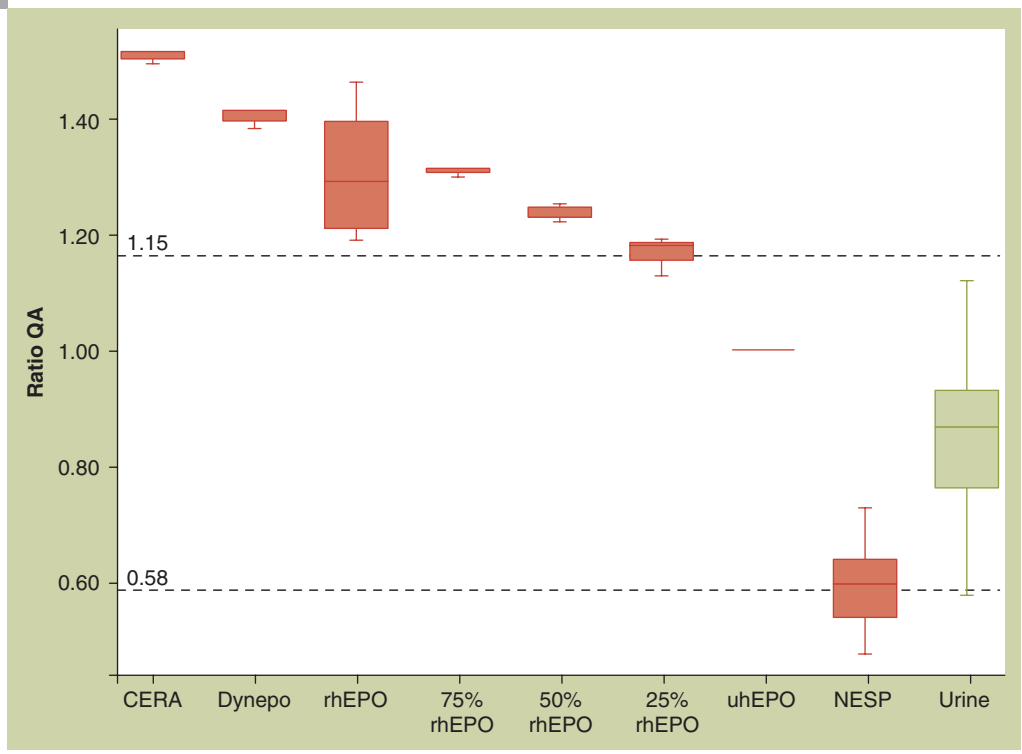


Figure 4. Results obtained for a newly developed screening method applied to different EPO preparations. Values given are the percentage of EPO eluted under acidic conditions from an immunoaffinity-purification microwell plate, normalized with respect to the behavior of the international human urinary EPO standard ('ratio QA'). CERA, dynepo and NESP are mimetics/analogs of rhEPO, which is also analyzed in mixtures (%) with uhEPO. Results obtained with urines from nonconsumers are presented.
CERA: Continuous erythropoietin receptor activator; EPO: Erythropoietin; NESP: Novel erythropoiesis stimulating protein; rhEPO: Recombinant human erythropoietin; uhEPO: Urinary human natural erythropoietin; QA: Quality assurance.

Surface plasmon resonance

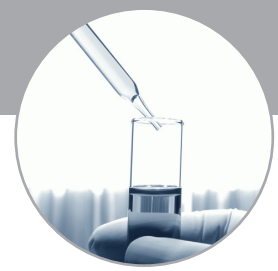
Biosensing technique for studying biomolecular interactions, such as antigen–antibody binding. Biomolecules capable of binding to specific analytes are immobilized, light is then focused to excite the surface plasmons. When the immobilized biomolecules are bound by their ligands, an alteration in surface plasmons on the opposite side of the surface is created. Binding is measured by changes in the light-refractive index

Growth hormone secretagogues

Group of peptide and nonpeptide molecules that promote the release of growth hormone by interaction with the growth hormone secretagogue receptor 1a, fully independent from the receptor used by the common hypothalamic growth hormone-releasing hormone. The natural ligand for this family of compounds is an acylated peptide known as ghrelin

of analytical protocols for this family of compounds (including the main 17.5-, 22- and 20-kDa splice variants, 5-, 12-, 17-kDa proteolytic products and multimers). Given the pulsatile secretion, affected by a variety of stimuli, the low concentrations in circulation and the short half-life, science has not been able to address this hormone until very recently. Based on the concept that the relatively stable endogenous ratio between isoforms should be altered by administration of a single recombinant pharmaceutical (22-kDa rhGH), differential immunoassays have been put together by German and Japanese researchers and our institute has actively participated in both developments. In a Japanese–Spanish project, funded by the WADA, the possibilities of surface plasmon resonance (SPR) for the simultaneous measurement of selected GH isoforms in a single sample were explored. This technique proved extremely useful in the development of the assay that targets the ratio between 22 and 20 kDa. Ultimately, the design of the current SPR instrumentation proved insufficiently sensitive for direct application. However, it was invaluable in understanding the surface-bound behavior of the antibodies. In the German study, SPR contributed to understanding the binding characteristics of the immunoglobulins. In this approach, where the ratio between the 22-kDa and all pituitary variants is determined, precise knowledge of the relative avidity and affinity of each antibody for different isoforms was established, facilitating the precise interpretation of blinded immunoassays. The group also produced several isoforms through solid-phase synthesis or controlled limited proteolysis of the alleged precursor. In this context, the latter is especially relevant, as it represents the first-ever generation of a variant with a structural conformation that could be significantly different from a recombinant variant.

Also related to GH are the synthetic growth hormone secretagogues (GHSs, ghrelin analogs and mimetics) that target the GHS-1a receptor. A protocol based on the displacement of labeled ghrelin from incubations with receptor-expressing cells is being studied. In this



case, the analytical protocol will be in place even before any doping attempt is made using these compounds.

Other peptides and proteins with ergogenic effects, such as hematopoietins, gonadotropins or biologically relevant glycoproteins, also have our interest. In particular, the structural characterization of the glycosylation and, subsequently, the study of how this post-translational modification affects the functionality are active research projects within the group.

Analytical quality & management systems

The increasing demand for doping control and drug testing has led to an increasing number of laboratories becoming involved. As athletes may be tested by different laboratories, the need to standardize the quality of analytical results is obvious. The group has led Quality Management and Proficiency Testing Programs, supported by health-related authorities at both National and European levels since 1986.

Laboratory information management systems (LIMS), have been another of our interests to ensure reliability of analytical laboratories. The group developed a system, specifically (but not exclusively) focused on antidoping laboratories, with the capability of being used in satellite laboratories (i.e., moving the laboratory to another country to perform doping control in major competitions). The initial system was successfully used in the demanding situation of the Olympic Games in Barcelona 1992. Concepts, including analytical batches with predefined quality controls, chain-of-custody from sample to the smallest physical part (i.e., aliquot or extract) and labels for every single test tube needed, for example, were features of the system. In a continuous improvement, a later version was primarily focused on simplicity (database administration, portability, cost and overall expertise required for maintenance) and worldwide applicability. The resulting platform (IMLIMS, now transferred to a web-based interface) is multilingual and covers, in a straightforward way, the most demanding chain-of-custody requirements. It fully understands the many analytical situations encountered in the doping control analytical laboratories and is adaptable to laboratories working in different fields.

Future perspective

Challenges in sports drug testing come from different areas and science will need to cope with them. For instance, methods targeting EPO abuse will divert doping for better oxygenation to the

Computerized laboratory information management system

These systems are becoming essential in order to comply with the strict quality regulations involved in doping control and other analytical laboratory-related areas

Blood transfusion

The introduction of stored blood or red blood cell concentrate into a vein or artery of a subject. Depending on the origin of the blood, the process is known as homologous transfusion (donor is a different subject) or autologous transfusion (donor is the same subject having collected the blood some weeks before)

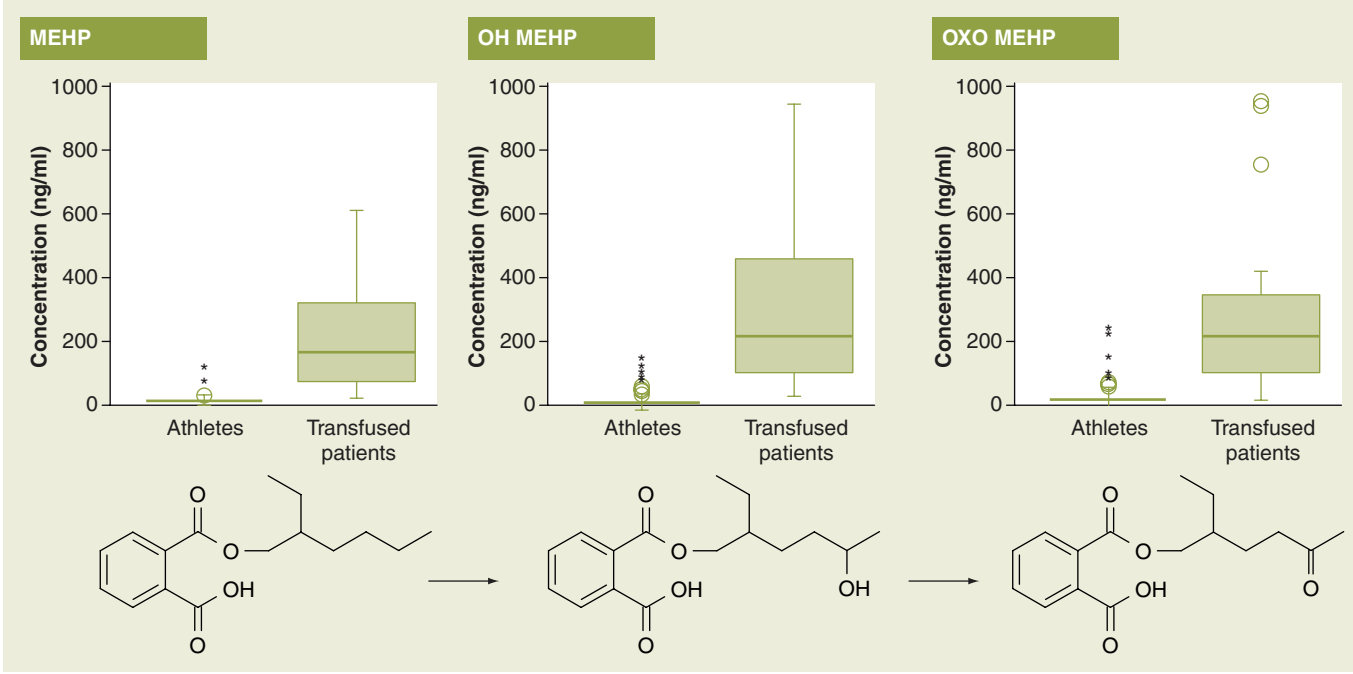
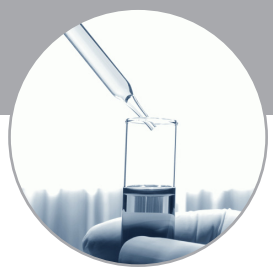


Figure 5. Urinary concentrations di-(2-ethylhexyl)phthalate (DEHP) metabolites in samples collected from competitive athletes for common doping control and in samples collected from patients transfused the previous day. A few samples from athletes (*) are significantly statistically different from the general athletes population but with concentrations close to some of the transfused patients. Either an unusually high exposure to DEHP or a prohibited transfusion could explain these results. Further specific test may address the final outcome.
MEHP: Mono-(2-ethylhexyl)phthalate.



use of blood transfusion, especially autologous transfusion. In this regard, indirect markers, such as the unexpectedly high concentrations of phthalates in urine (leaking from the plastic materials of the bags containing blood) may become useful in identifying potential cheaters **(Figure 5)**.

New analytical advances shall be implemented, mainly on MS and nanotechnologies. Also, permanent surveillance of pharmaceutical developments that may be misused in sport is mandatory. However, the biggest challenge for the next decade may come from the application of so-called gene doping, which gives rise to the *in vivo* expression of a performance-enhancing hormone in commonly nonexpressing human tissues. Along with other teams, our group has pioneered the development of a methodology to address gene doping, using advanced image developments and antisense oligonucleotide technology.

The coming years will also see an increase in the longitudinal follow-up of individual athletes (via their biological passport) to rapidly identify any unnatural deviation of key biochemical parameters. Some of those markers come from blood analysis; however, this fluid is still not routinely obtained in dope testing for logistical reasons. Adapting some marker tests to easier forms of blood collection, such as dried blood spots, will further facilitate its implementation and it is our objective to contribute to this research as well.

Gene doping

The nontherapeutic use of cells, genes, genetic elements or the modulation of gene expression having the capacity to enhance athletic performance. It represents a nonethical use of the emerging possibilities of medical gene therapy

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized this manuscript.

Executive summary

- Antidoping laboratories are accredited by International Organization for Standardization accreditation bodies and by the World Antidoping Agency.
- The majority of doping agents have a molecular mass lower than 1000 Da and are routinely analyzed by a combination of chromatographic and mass spectrometric techniques.
- The recent availability of recombinant pharmaceuticals is shifting the misuse and concomitantly analytical strategies towards proteomics and protein chemistry using either mass spectrometric or immunological methods.
- Differences in glycosylation allow differentiating recombinant from urinary endogenous erythropoietin.
- Well-selected antibodies with differential recognition capabilities for similar isoforms of proteins and hormones are useful for purification, detection and quantitation.
- Downregulation of endogenous growth hormone expression by recombinant growth hormone is the basis of a differential immuno-analytical method.
- Tailored sport drug-testing laboratory information management systems are available to support optimal laboratory performance.
- Future challenges in antidoping testing will include autologous blood transfusion, gene doping and longitudinal follow-up of biochemical markers.

Representative recent publications

■ Low-molecular-weight compounds

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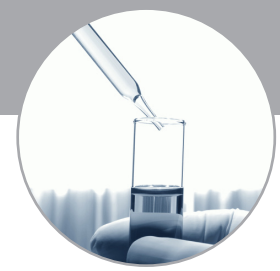

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