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Automated Sample Preparation

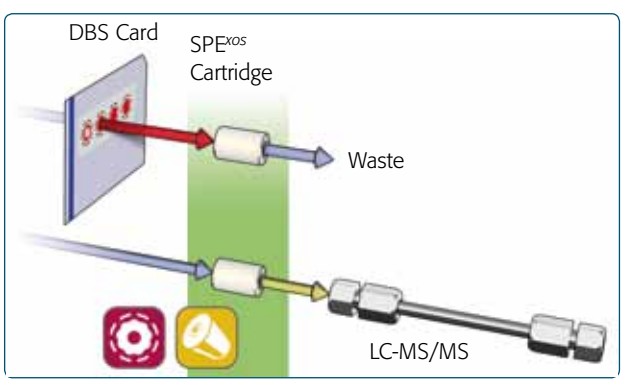
Efficient Doping Control

Automated determination of nicotine and its metabolites in blood

In the race to keep up with new and suspected doping agents, the Centre for Preventive Doping Research at the German Sport University Cologne, Germany is upgrading their laboratory with performance enhancing technologies and automation: The institute is betting on a fully automated Dried Blood Spot (DBS)-LC-MS/MS analysis system.

By Guido Deussing

The pressure to achieve record-breaking results in sports has become extremely intense, advances in technologies that can effectively guide an athlete to peak performance has made achievement of new records an almost routine occurrence. However, even after all aspects of an athlete's training regime have been optimized and the seemingly endless hours of preparation have been put in, some athletes still cannot achieve the desired results. This is when the temptation to use performance enhancing substances rears its head.



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Principle of Flow Through Desorption (FTD) of a DBS card (left hand side).

Not every chemical means of enhancing physical or mental performance is currently listed as illegal. But the World Anti-Doping Agency (WADA) has a watchful eye on many substances that they suspect are being used to gain an unfair advantage in sports. In 2015, the watch list sported names of active pharmaceutical ingredients such as Bupropion, Phenylephrine, Phenylpropanolamine, Pipradol, and Synephrine, along with stimulants such as caffeine and nicotine that are naturally present in coffee and tobacco.

Doping with nicotine – more than just a suspicion

In 2011, the German daily Süddeutsche Zeitung reported that scientists at the University of Lausanne, Switzerland, had found elevated levels of nicotine in urine samples from athletes from various disciplines.

"Nicotine doesn't improve stamina or muscle power, but it affects the brain and places the athlete in a different state of mind", says the pharmacologist Fritz Sörgel, a recognized doping expert and Head of the Institute of Biomedical and Pharmaceutical research (IBMP) at the University of Nuremberg, Germany. In those sports, in which reaction time and concentration are especially important to performance, an increased nicotine level could help athletes gain an advantage. While smoking tobacco could have significant detrimental health and performance effects, e-cigarettes or chewing tobacco could present an attractive alternative without the negative side-effects.

The same applies to snuff, an orally consumed form of tobacco, which is widely used in Norway and Sweden. "The suspicion is that snuff is being abused for doping purposes", states Professor Mario Thevis from the Centre for Preventive Doping Research at the German Sport University Cologne, Germany. No clinical studies or other well-founded data were available concerning the use or effects of nicotine as a performance enhancing drug even though WADA had placed nicotine on the watch list as a suspected doping agent. Prof. Thevis and his colleagues in Cologne along with a scientist from the National Veterinary Institute of the Department of Chemistry in Uppsala, Sweden set out to develop a "fast and inexpensive" method of analysis that would enable the determination of nicotine and its metabolites while also providing insight on how they had been introduced into the body [1]. During their search to find the most suitable analysis technique for their purposes, Dried Blood Spot (DBS) analysis in combination with online solid phase extraction (SPE) and LC-MS/MS soon emerged as the most promising solution.

World Anti-Doping Agency (WADA)

The World Anti-Doping Agency (WADA) is a foundation initiated by the International Olympic Committee to promote, coordinate and monitor the fight against the use of performance enhancing drugs in sports. The agency's key activities include scientific research, education, development of anti-doping capacities, and monitoring of the World Anti-Doping Code, whose provisions are enforced by the UNESCO International Convention against Doping in Sport. WADA was founded in 1999, it is an international non-governmental organization (NGO) headquartered in Montreal, Canada. Wada organizes world-wide campaigns against the use of doping in sports. These employ urine tests, blood tests and other tests as required by medical indications. Currently around 30 laboratories world-wide are authorized by WADA to analyze the required replicate samples (A and B samples) for traces of prohibited substances and for signs that prohibited methods have been used, such as, for example blood doping. The prohibited list is updated annually and it serves as the international reference for identifying substances and methods prohibited in all sports that fall under the World Anti-Doping Code (WADC). Individual countries have national anti-doping agencies such as the USADA in the US, UKAD in the United Kingdom, AFLD in France and NADA in Germany. *Source: WADA, Wikipedia*

Suggested Reading

OPEN ACCESS ARTICLE!

L. Tretzel, C. Görgens, H. Geyer, A. Thomas, J. Dib, S. Guddat, V. Pop, W. Schänzer, M. Thevis, **Analyses of Meldonium (Mildronate) from Blood, Dried Blood Spots (DBS), and Urine Suggest Drug Incorporation into Erythrocytes**, International Journal of Sports Medicine · DOI10.1055/s-0036-1582317, (https://www.thieme-connect.com/products/ejournals/abstract/10.1055/s-0036-1582317?lang=de)

Cost and speed are the deciding factors

According to the scientists, DBS has proven itself many times over, for example in pre-clinical pharmaceutical research; for monitoring of active therapeutic agents; in forensic toxicology; as well as in studies of metabolic disorders. Meanwhile, several examples were also published on DBS being used in doping analysis. According to Mario Thevis and his colleagues, DBS offers a number of benefits when compared with standard strategies for blood sampling: DBS is minimally invasive – a simple finger prick is enough to withdraw a sufficient sample volume (20 µL) for the analysis. Just a few drops of blood absorbed on a suitable, cellulose based medium is all that is needed for a successful determination of the compounds of inter-

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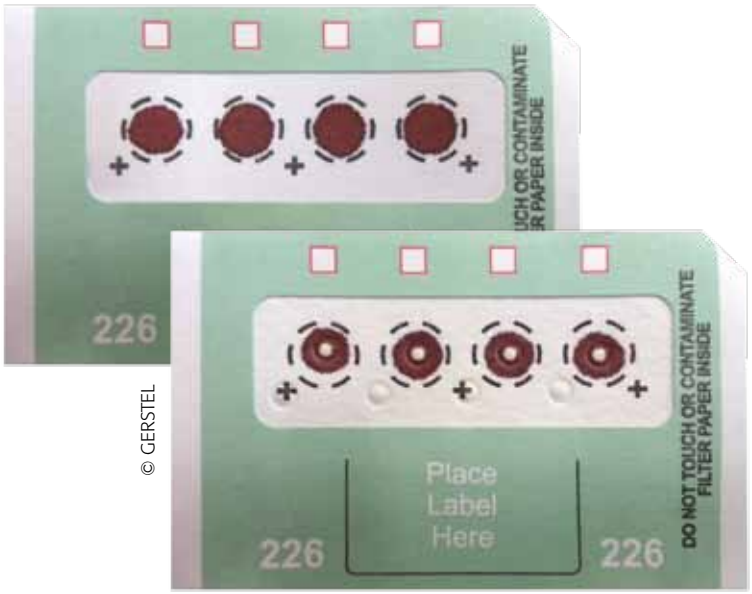
Fully automated DBS system based on the MultiPurpose Sampler (MPS).



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DBS desorption is performed with high accuracy in the user-defined section of the blood spot. Quantitative recovery is achieved resulting in high reproducibility.

est. In addition, blood sampled in this way exhibits good long term stability at room temperature. Samples dry very quickly and the absence of humidity means that enzymes are deactivated, as the scientists point out in their article in the Journal of Pharmaceutical and Biomedical Analysis [1].

To perform DBS analysis, several sample preparation steps are required: "Currently, the pre-analysis workflow includes punching out and dissolving each dried blood spot. Then an extraction is performed with a suitable solvent, occasionally including ultra-sonication. Further clean-up steps involve: Protein precipitation, filtration and transfer of the resulting extract into a sample vial followed by LC-MS/MS analysis", according to Thevis et al.. Automation is an absolute necessity in order to minimize the significant manual workload and to qualify DBS sampling for high throughput analysis in a routine laboratory setting.

Commercially available automation

The scientists set out to determine nicotine, the main metabolites nornicotine, cotinine and trans-3'-hydrocotinine (trans-3'-HCOT), as well as the alkaloids Anabasine and Anatabine using a fully automated system: A MultiPurpose Sampler (MPS) and a Dried Blood Spot Autosampler (DBSA) coupled to an online SPE system (SPE*xos*) all from GERSTEL. This setup was coupled with a high-resolution LC-MS/MS system.

Prof. Thevis explains why this setup is different from previous systems used in the laboratory: "When using an online SPE system, we can extract the DBS sample, clean up the extract, and proceed directly with the analysis". Automation is only one important aspect of the system, according to Prof. Thevis: "Not only does the automated DBS sample preparation reduce the workload, it also improves extraction efficiency and limits of detection". The patented Flow Through Desorption (FTD™) technology enables good concentration factors to be achieved while minimizing the risk of sample to sample carry over. The Online DBS SPE-LC-MS/MS method was developed using standard solutions at different concentration levels, which were spiked into samples from volunteers who had neither smoked nor consumed snuff. The validation was performed following the recommendations of WADA and the European Bioanalysis Forum (EBF). Deuterated analogues were used for quantification of target compounds. In order to investigate potential differences in pharmacokinetics, Thevis et al. used their method on authentic samples, that is, blood samples taken from cigarette- and e-cigarette smokers, as well as snuff users. The project was set up with permission from the local ethics commission, in some countries known as the Institutional Review Board, whose task it is to protect human subjects from harm by overseeing research performed on humans or animals. Written permission was obtained from the volunteers.

Differentiating between normal consumption and doping

When they developed the method, the authors focused on optimizing it for reproducibility and workflow efficiency, according to Thevis and his colleagues [1]. The process steps that held the most promise for improvement was DBS elution, SPE cleanup, as well as Mass Spectrometric Detection. Using the DBS method they developed, the authors succeeded in determining all target analytes with excellent precision and accuracy. The limit of detection for all analytes was 5 ng/mL. The successful analysis of blood samples taken from real smokers as well as e-cigarette and snuff users demonstrated that the method could be implemented for routine doping controls as well.

"All target compounds were found in the real samples", Thevis et al. wrote. Additionally, the statistical evaluation had shown a significant difference in the ratio between nicotine and nornicotine concentrations in the blood depending on whether nicotine was administered via the lungs (inhalational) or via mucous membranes (buccal uptake). This means that based on pharmacokinetic properties, conclusions can be drawn as to the athlete's method of consumption and maybe the longer term pattern of use.

Literature

[1] Laura Tretzel, Andreas Thomas, Thomas Piper, Mikael Hedeland, Hans Geyer, Wilhelm Schänzer, Mario Thevis, Fully automated determination of nicotine and its major metabolites in whole blood by means of a DBS online-SPE LC-HR-MS/MS approach for sports drug testing, Journal of Pharmaceutical and Biomedical Analysis 123 (2016) 132–140