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Use of ultra-high pressure liquid chromatography coupled to high resolution mass spectrometry for fast screening in high throughput doping control

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ABSTRACT

We describe a sensitive, comprehensive and fast screening method based on liquid chromatography–high resolution mass spectrometry for the detection of a large number of analytes in sports samples. UHPLC coupled to high resolution mass spectrometry with polarity switching capability is applied for the rapid screening of a large number of analytes in human urine samples. Full scan data are acquired alternating both positive and negative ionisation. Collision-induced dissociation with positive ionisation is also performed to produce fragment ions to improve selectivity for some analytes. Data are reviewed as extracted ion chromatograms based on narrow mass/charge windows (±5 ppm). A simple sample preparation method was developed, using direct enzymatic hydrolysis of glucuronide conjugates, followed by solid phase extraction with mixed mode ion-exchange cartridges. Within a 10 min run time (including re-equilibration) the method presented allows for the analysis of a large number of analytes from most of the classes in the World Anti-Doping Agency (WADA) Prohibited List, including anabolic agents, β2-agonists, hormone antagonists and modulators, diuretics, stimulants, narcotics, glucocorticoids and β-blockers, and does so while meeting the WADA sensitivity requirements. The high throughput of the method and the fast sample pre-treatment reduces analysis cost and increases productivity. The method presented has been used for the analysis of over 5000 samples in about one month and proved to be reliable.

1. Introduction

Anti-doping analysis usually starts with a fast and robust screening (initial testing) method. As every sample has to be screened, methods are designed to be sufficiently sensitive and specific to identify all suspect samples and minimise false suspects that would require targeted additional testing. Aiming to limit the effort required for sample screening, laboratories have invested in the development of a very limited number of comprehensive screening methods which are able to cover the World Anti-Doping Agency (WADA) Prohibited List [1] fully. However, screening methods tend to suffer a short life and need constant updating and modification, because athletes who cheat quickly switch to new compounds in attempts to avoid being caught.

Liquid chromatography coupled to mass spectrometry detection is gaining in popularity, superseding many of the gas chromatographic [2] coupled mass spectrometric methods, especially for polar compounds such as diuretics and stimulants, in part because of the simpler sample preparation needed.

Detection is usually based on low-resolution tandem mass spectrometry (e.g. selective reaction monitoring), and methods are available in the literature which offer the ability to screen for an ever increasing number of targeted analytes [3–7]. Modern triple-quadrupole instruments offer very fast acquisition cycle times and polarity switching, hugely expanding the number of analytes which can be detected in a single run. For example, one method from Thörngren et al. is capable of screening for 130 analytes within a 7.5 min cycle time using UHPLC coupled to a triple-quadrupole mass spectrometer [3]. However, this method (likewise all methods based on triple-quadrupole instruments) suffers from the limitation of being able to perform only targeted analyses, where the analytes to be detected must be established before the analysis is performed.

To overcome this limitation and expand the capability of methods toward screening of unknown substances by low resolution mass spectrometry, Mazzarino et al. [8] suggested the use of a triple-quadrupole instrument in precursor ion scan mode, to detect known and potentially unknown glucocorticoids by screening samples for the presence of a common fragment.

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In recent years, the use of high resolution mass spectrometry has gained in popularity, as a result of the possibility of collecting the full raw data instead of having to pre-select the list of analytes to be detected [2]. A filtering process is then applied *a posteriori* without compromising the integrity of the raw data and with a virtually unlimited potential for the number of analytes to be screened. This also allows for re-processing previously acquired data and investigating samples for new substances (e.g. newly misused compounds) without the need for a new aliquot of the sample.

A few screening methods for doping control based on liquid chromatography coupled to high resolution mass spectrometry (HRMS) are reported in the literature [9–15].

UHPLC coupled to time-of-flight (TOF) instruments is often used [10–13]. This instrument combination, however, does not currently offer the possibility of performing polarity switching and two separate analyses are required to cover positive and negative ionisation modes [10–12]. One UHPLC-TOF method [13] was able to detect a large number of analytes in urine samples in a single run, but as detection was performed in positive mode only, this did not allow for the detection of analytes such as furosemide, dichlorphenamide and ritalinic acid that require negative mode.

An alternative to TOF instruments is provided by orbitrap technology, which offers very high resolution and mass accuracy capability without the need for an internal mass calibrator.

A few methods exploiting orbitrap technology have been published for anti-doping screening [9,14–17]. The first method by Virus et al. [9] mainly focused on anabolic agents, which could be screened by HPLC coupled to an LTQ orbitrap instrument. APCI was used and full scan data were collected with in-source collision-induced dissociation (CID). The instrument used is not capable of performing polarity switching, and was operated in positive ionisation mode. Therefore acidic analytes such as many diuretics could not be included and polar compounds such as stimulants were not considered.

HRMS instruments offering fast polarity switching capability are now available. Currently these use orbitrap technology. When orbitrap technology HRMS with polarity switching was employed, a 30 min run time was used to screen for a large number of analytes [15]. Faster chromatography was achieved by Thomas et al. [14], but only 32 performance enhancing agents were selected as model compounds in human plasma after a sample clean-up consisting of protein precipitation with acetonitrile. This method however does not include hydrolysis nor does it detect intact glucuronide conjugates; thus it is not directly applicable to human urine. A more recent paper from the same group [17] exploits the quadrupole-orbitrap technology Q-Exactive instrument for the analysis of doping agents in dried blood spots. However, this method was only applied to dried blood spots and was tested with 26 representative compounds only.

Similar equipment was used by Moulard et al. [15] for doping control in horses after enzymatic hydrolysis and solid-phase extraction (SPE). Positive and negative ESI was used, but no fragmentation was performed, thus limiting the available data to the protonated (or deprotonated) molecular ion and/or possible adducts. Two hundred and thirty-five compounds could be screened, but the HPLC cycle time is 30 min. A much faster cycle time is desirable when high data throughput and fast sample turnaround are required, in order to reduce the number of instruments, as well as the volume of organic solvents used, thereby minimising costs.

More recently, Giron et al. [16] presented a screening method for more than 120 target analytes using the Exactive mass spectrometer. This method is based on the dilute and inject approach, which is fast but reduces the sensitivity of the method by diluting the sample before analysis. Compounds with very low WADA defined Minimum Requirement Performance Levels (MRPL) [18,19] such as clenbuterol and 3′-hydroxystanozolol were therefore not included. Several compounds are excreted in urine as glucuronide or sulphate conjugates. However, a deconjugation step was not included in the sample preparation, nor were intact glucuronides detected in most cases. This limits the applicability of the method to the free portion of the analytes, which could often represent a minor fraction of the total drug amount, or to analytes which are mainly excreted as non-conjugates. Glucocorticoids and narcotics are often extensively conjugated with glucuronic acid and were not included in the study. Furthermore, in-source generated fragment ions were detected, but no collision cell was used, limiting the availability of additional diagnostic ions. Such screening methods would require samples to be re-analysed with additional liquid chromatographic methods to obtain a comprehensive coverage of the substances prohibited in sport.

Analysis speed is a key factor, especially for fast turnaround events such as Olympic Games, where delivery of the results can be required in periods as short as 24 h. The time required to analyse a sample does not depend only on the instrumental analysis time. Fast sample preparation can be as important. Good selectivity and easy data review are also necessary to avoid false suspect samples and time consuming investigations.

The challenge when developing a suitable sample pre-treatment for a screening method is obtaining good extraction yields for a combination of analytes with very different physico-chemical properties (basic and acidic, lipophilic and hydrophilic). Most Prohibited Substances are basic, yet acidic compounds such as ethacrynic acid and neutrals (e.g. glucocorticoids) need to be detected too. Also compounds such as clenbuterol must be detected at levels less than 2 ng/mL. Besides, the huge number of different endogenous components normally found in urine makes the selective detection of analytes at low concentration very challenging.

The dilute and inject approach [3,12,16] is certainly fast, but it does not allow for the detection of analytes at very low concentrations and does not include deconjugation of glucuronides.

When a clean-up step is used, liquid/liquid extraction at basic pH is commonly adopted [5,6,9,13,20]. However, two consecutive extractions, one at basic and the other at acidic pH, are sometimes required to extract a wide range of analytes [4].

Liquid/liquid extractions require careful separation of the phases, can be time consuming and tend to use large volumes of solvent. Solid-phase extraction can provide good extraction yield, clean samples and high throughput. Reversed-phase SPE has been employed [15] but, because of the ability to extract acidic and basic analytes (such as diuretics) simultaneously, polymeric sorbents are usually preferred [21], sometimes in combination with ion-exchange functionality [11]. We therefore prefer to use SPE whenever suitable.

We present here a UHPLC–HRMS method for the screening of a large number of analytes including anabolic agents, β2-agonists, hormone antagonists and modulators, diuretics, stimulants, narcotics, glucocorticoids and β-blockers. A fast sample clean-up was developed consisting of direct enzymatic hydrolysis of the urine samples, followed by mixed mode polymeric ion-exchange SPE. Analytes with a very wide range of chemistries could be extracted with a single SPE procedure. Instrumental analysis is then performed with a 10 min chromatographic run (including re-equilibration). Full scan data are acquired in positive and negative ESI and using a collision cell to obtain CID fragments in positive mode (nitrogen is used as the collision gas). The instrument operates the fragmentation experiment without selection of the precursor ion, which enables non-targeted fragmentation and no requirement for an *a priori* selection of the analytes to be screened for. The use of CID fragmentation enables the acquisition of all generated fragment ions. Targeted reprocessing of acquired data to monitor for specific fragment ions eliminates false suspects