Detection of 23 Corticosteroids, Anabolic Steroids, and ß2-Agonist in Athens Doping Control Lab, During Athens 2004 Olympic Games Using Agilent 1100 LC/MSD Trap Mass Spectrometers

Application

Forensics

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Abstract
Anabolic steroids, corticosteroids and ß2-agonists are considered performance-enhancing drugs, intentionally regulated by most international sports agencies for the purpose of maintaining fairness in competition. Controlling the use of such drug usage among athletes should also be considered a health benefit. During the 2 weeks of the 2004 Summer Olympics in Athens, Greece, several ion trap instruments were used 24 hours per day for the analysis of extracted urine samples, meeting the World Anti-Doping Agency (WADA) guidelines for minimum required performance limits (MRPLs). In this report Agilent 1100 Series HPLCs are coupled with LC/MSD Trap SLs, each using an electrospray ionization source (ESI) for the analysis of controlled substances. The MRPLs of 10 ng/mL urine for the anabolic steroids, 30 ng/mL urine for the corticosteroids and ß2-agonists are easily met. For example, 1 ng/mL urine for triamcinolone acetonide and less than 0.5 ng/mL for the designer drug tetrahydrogestrinone (THG). Moreover, the instrument sensitivity is shown to be maintained for the entire 2 weeks even though the sample load was extensive.

Introduction
Most international sports agencies introduced drug testing by the 1970’s. The use of anabolic steroids was becoming widespread, especially in strength events, but there was no way of detecting them yet. A reliable test method was finally introduced in 1974 and the International Olympic Committee (IOC) added anabolic steroids to its list of prohibited substances in 1976. This resulted in a marked increase in the number of drug-related disqualifications in the late 1970’s, notably in strength-related sports such as throwing events and weightlifting.

Since then the list of performance enhancing drugs has expanded from anabolic steroids to include corticosteroids and ß2-agonists. Anabolic androgenic steroids are natural or synthetic substances which act in the same way as testosterone. That is, they propose the development of sex characteristics (androgenic effect) and the gradual growth of

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muscle tissue (anabolic effect). Corticosteroids are the most powerful anti-inflammatory drugs available for modern medicine. Also, in large dosages these compounds can help recover an injured muscle or inflamed joint, improving athletic performance. Finally, β2-agonists are used to treat asthma and are allowed only as inhaled medication for those who are otherwise limited by the condition.

Most of the compounds analyzed in this report are polar and thermally labile. Therefore, they are not only amenable to liquid chromatography/mass spectrometry (LC/MS) analysis, but also preferred over the more traditional technique for drug testing in urine of gas chromatography/mass spectrometry (GC/MS). Furthermore, the sensitivity required for measuring ion ratios of the compound-specific product ions, generated by MS/MS (MS2), is unparalleled in an ion trap mass spectrometer. Analyzing compounds using multiple stages of MS also eliminates the need for good chromatographic resolution, as required for ultra-violet (UV) quantitation, because co-eluting compounds are distinguishable. In addition, the specificity of MS/MS/MS (MS3) can further distinguish those compounds, which have identical precursor ion masses and similar product ion distributions. Not only does the sensitivity of the ion trap in product ion generation make it the preferred analytical technique over triple-quadrupole instruments, but their typically lower prices are also an important consideration.

In this work the patented SmartFrag capability of the LC/MSD Trap is implemented to generate reproducible fragment ion spectra at both the MS2 and MS3 stages. Using this technique, it is more likely that each precursor ion will receive exactly the energy it needs to produce an abundant and wider variety of product ions for structural confirmation. By optimizing the generation of certain product ions for each compound, a multiple reaction monitoring (MRM) technique is used for quantitation. It should also be noted that the stringent requirements for confirmation, according to the WADA guidelines, had to be met consistently for at least a 20-day period, analyzing more than 4000 samples, during which the Olympic events took place, so that linearity and reproducibility were critical to not only ensure fairness in competition, but to safeguard the reputation of the Doping Control Lab of Athens. The compounds analyzed are listed below.

Corticosteroids
Triamcinolone, 16α-hydroxyprednisolone (budesonide metabolite), cortisol, prednisolone, fludrocortisone, prednisone, cortisone, methylprednisolone, betamethasone, dexamethasone, flumethasone, beclomethasone, flunisolide, flucortolone, triamcinolone acetonide, desonide, budesonide.

β2-Agonist
Formoterol.

Anabolic Steroids
Trenbolone, epitrenbolone (trenbolone metabolite), gestrinone, THG, hydroxymethylformebolone (formebolone metabolite).

Internal Standard (ISTD)
Methyltestosterone
Note: THG (tetrahydrogestrinone) is a designer steroid derived from another banned steroid, gestrinone. See Figure 1.

Experimental
Sample Preparation
Standard extraction procedure for GC/MS analysis is used except for the final step involving derivatization. Instead, the sample is reconstituted in the LC mobile phase.

To a 2.5-mL urine sample is added the internal standard (methyltestosterone). An enzymatic hydrolysis is then performed with β-glucuronidase (Helix Pomatia) at pH 7, at 50 °C for 1.5 hours. Alkaline extraction at pH 10 with 5.0 mL diethylether, using 1 g of sodium sulphate as the desalting agent. Agitation for 20 min followed by centrifugation. The ether phase is transferred to a conical tube and evaporated with nitrogen flow to dryness. Finally, the urine extract was reconstituted with 100 µL of mobile phase.
**LC/MS/MS Method Details (example)**

<table>
<thead>
<tr>
<th><strong>LC Conditions</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Instrument:</strong></td>
<td>Agilent 1100 HPLC</td>
</tr>
<tr>
<td><strong>Column:</strong></td>
<td>ZORBAX RX-C8, 2.1 mm × 50 mm, 5-µm particle size</td>
</tr>
</tbody>
</table>
| **Mobile phase:** | A = 0.1% acetic acid in water  
                  | B = acetonitrile |
| **Gradient:**     | 15% B at 1 min  
                  | 40% B at 8 min  
                  | 40% B at 12 min  
                  | 90% B at 15 min  
                  | 100% B at 17 min  
                  | Post Run: 4 min |
| **Flow rate:**    | 0.4 mL/min |
| **Injection vol:**| 10 µL |

<table>
<thead>
<tr>
<th><strong>MS Conditions</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Instruments:</strong></td>
<td>Agilent LC/MSD Trap SLs</td>
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<tr>
<td><strong>Ionization mode:</strong></td>
<td>Positive ESI</td>
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<tr>
<td><strong>Drying gas flow:</strong></td>
<td>10 L/min</td>
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<tr>
<td><strong>Nebulizer:</strong></td>
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<tr>
<td><strong>Drying gas temperature:</strong></td>
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<td><strong>V_cap:</strong></td>
<td>3500 V</td>
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<tr>
<td><strong>Skim 1:</strong></td>
<td>15.0 V</td>
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<tr>
<td><strong>Capillary exit:</strong></td>
<td>50.0 V</td>
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<tr>
<td><strong>Scan:</strong></td>
<td>m/z 100–400 amu.</td>
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<tr>
<td><strong>Normal resolution:</strong></td>
<td>13,000 amu/s</td>
</tr>
<tr>
<td><strong>Averages:</strong></td>
<td>2</td>
</tr>
</tbody>
</table>
| **ICC:**          | On  
                  | Maximum accumulation time:  
                  | 20 ms  
                  | Target: 30000  
                  | MRM mode: 10 segments |
| **Qualification:**|  |
| **Fragmentation amplitude:** | 1.0 V |
| **SmartFrag:**    | On, 30%–200% |
| **Quantitation:** |  |
| **Fragmentation amplitude:** | Optimized for each compound |

**Results and Discussion**

One of the limitations of many ion trap MS systems is that fewer product ions exist because of the relatively short time precursor ions are held in the ion trap. The use of the patented SmartFrag feature of the Agilent LC/MSD Trap was central to its successful use for identification of the compounds analyzed during the 2004 Summer Olympic Games in Athens, Greece. An example of the abundant variety of product ions using SmartFrag ramped collisionally induced dissociation (CID) technique is shown in Figure 2. This technique ramps the fragmentation energy from 30% to 200% (user-adjustable) of the user-designated fragmentation voltage, one volt in this work. In this example, we applied the Smart Fragmentation feature of the LC/MSD trap to produce an optimal set of fragment-rich MS/MS spectra for prednisone, as shown in Figure 2C.

Unlike traditional ion traps, the Agilent LC/MSD Trap CID voltage energy is quickly ramped over the range of energies, which results in the optimum energy for each analyte. The SmartFrag feature of the Agilent LC/MSD Trap eliminates the need for time-consuming collision voltage optimization. The analyst efficiently obtains fragment-rich, full-scan product ion spectra for confirmation of identity or structural elucidation.
Figure 2. The usefulness of SmartFrag is demonstrated with the compound prednisone.

Derived mass spectra should produce characteristic ions for the substance's structure. Product ion abundances over 5% relative abundance, as required by WADA, offer a rapid comparison tool for the verification of the presence of a prohibited substance in an athlete sample with respect to standard quality control (QC) samples run with every sequence (blank urine samples spiked with MRPL concentrations of the prohibited substances).
An example of confirmation by identification is shown in the verification of a positive case in which an athlete was given triamcinolone acetonide for therapeutic use. Figure 3 shows the results of a library match for the sample compound mass spectrum, with background subtraction. The Fit, reverse fit (RFit), Purity (NIST algorithm) values are each greater than 950 out of a possible 1000.

Figure 3. Positive case verification procedure - athlete sample. Library match: (P)urity = 967; (F)it = 967; and (R)everse Fit = 968.
Along with confirmation, QC samples run in every sequence were used to monitor system stability throughout the duration of testing at the Olympics. Figure 4 shows the reproducibility of quantitation with the LC/MSD Trap SL for the 20-day period. Each instrument analyzed more than 1100 samples without any routine maintenance beyond the easily accessed source chamber region.

Figure 4. System stability expressed as substance area (triamcinolone acetonide and THG), divided by ISTD area (methyl testosterone).

As a demonstration of linearity, the results of testing three different compounds prior to the start of the Olympic games are shown in Figure 5. Each calibration level is calculated as the average of two identical samples, each run twice (four runs total). The Relative Response is calculated by dividing the substance area by the ISTD area. All calibration curves show a linearity coefficient, \( r^2 \), of at least 0.99, with nearly zero intercepts. Furthermore, the highest calibration point for each plot is the WADA MRPL limits, which means that system sensitivity is far below these limits.

Figure 5. Demonstration of system linearity using the compounds triamcinolone, THG, and desonide.
An example printout of a QC sample is shown in Figure 6, consisting of six compounds, including the ISTD, being analyzed each with three confirmation ions in a vertical descending order of intensity. Relative to this printout, confirmation of drug testing failures can be easily identified just by visual comparison.
Returning to the positive test case verification involving the athlete sample (library match shown in Figure 3), the quantitation of triamcinolone acetonide is confirmed as being below 15 ng/mL urine, but cannot be specified further for the sake of privacy. See Figure 7.
Conclusions

This work demonstrates the robust operation of the Agilent LC/MSD Trap SL for the purpose of testing for the presence of performance-enhancing drugs in Olympic athletes during the 2004 Summer Olympic Games in Athens, Greece. The sensitivity requirements of WADA are easily met using the full scan MS/MS capabilities of the LC/MSD Trap. Moreover, the patented SmartFrag technique involving a ramping of the fragmentation voltage being applied to the precursor ion, produces abundant and specific product ions at both the MS2 and MS3 levels for unique identification and confirmation of the presence of as many as 23 separate controlled substance compounds in urine.

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