Paper spray mass spectrometry – A potential complementary technique for the detection of polar compounds in sports drug testing

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Abstract
In this proof-of-concept study, paper spray mass spectrometry was investigated as a high-throughput and fully automated technique for the initial testing of particularly polar compounds that are prohibited in sports. The technique allows the ionization of analytes from complex sample matrices such as blood and urine when spotted onto a paper strip. By minimizing sample preparation and omitting chromatographic separation, paper spray mass spectrometry benefits from considerable cost- and time-savings compared with conventional high performance liquid chromatography/tandem mass spectrometry, especially in cases where conventional reversed-phase liquid chromatography offers limited applicability. All but one of the investigated model compounds fulfilled the World Anti-Doping Agency’s (WADA’s) requirements regarding the applicable minimum required performance limits for initial testing procedures. In addition, the combination of paper spray mass spectrometry and ion mobility separation results in enhanced selectivity and sensitivity and is a particularly valuable analytical configuration.

KEYWORDS
human urine, ion mobility, paper spray mass spectrometry, sports drug testing, VeriSpray

1 | INTRODUCTION

High performance liquid chromatography coupled to mass spectrometry (HPLC–MS) is used widely in sports drug testing, enabling high-throughput initial testing procedures.1–6 With the development of increasingly powerful instrumentation, a tendency towards simplified assays has been observed in doping controls, where sample pretreatment has been reduced to a minimum, leading eventually to so called “dilute-and-inject” assays.7–11 However, extraordinary hydrophilic compounds still represent a challenging task in HPLC–MS due to their divergent chromatographic behavior and, consequently, limited compatibility with commonly employed reversed-phase HPLC-based test methods.

In this pilot study, paper spray mass spectrometry (PS-MS) was investigated as an alternative technique for the initial testing of highly polar compounds that are considered as prohibited in sports. This technique, first described in 2010,12,13 ionizes analytes directly from complex sample matrices such as blood, dried blood spots, biological tissues, or urine that are spotted onto a paper strip.14,15 Over the years, PS-MS has been explored further in forensic and toxicological applications, particularly in forensic toxicology,16,17 forensic science,18 and clinical drug analysis.19–21 However, to the best of our knowledge, this is the first time that this technique has been applied to sports drug testing.
drug screening. By minimizing sample preparation and omitting chromatographic separation, mass spectrometric results can be obtained in less than 2 minutes.

2 EXPERIMENTAL

2.1 Instrumentation

Experiments were carried out using the Thermo Scientific VeriSpray ion source on a Thermo Scientific TSQ Altis triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA, USA). The paper spray workflow, displayed in Figure 1, typically involves the following steps. In the first step, a defined volume of the urine sample is spotted onto a single use paper strip in a 24 strip VeriSpray sample plate. After drying, the sample plate is placed in the plate loader of the VeriSpray ion source, from where the plate is transported to the inlet of the mass spectrometer for analysis. The application of rewet solvent supports the extraction and transfer of the analytes to the tip of the paper strip. The sample strip then slides out, spray solvent is added, and the application of the spray voltage induces the desired electrospray ionization of the analytes. PS-MS experiments were performed in single-reaction-monitoring (SRM) mode.

The sample spotting volume was 8 μL; the ion transfer tube temperature was set at 400°C, and the spray voltages ranged between 3 kV in negative ionization mode and 4 kV in positive mode. The analytical result is a "chronogram" as depicted in Figure 1, which is typically 0.5 to 2 min wide, depending on the set scan time of the MS. The results are evaluated using Thermo Scientific TraceFinder software version 4.2.

2.2 Model compounds

As model compounds of this proof-of-concept study, meldonium, metformin, p-hydroxyamphetamine, oxilofrine, octopamine and its sulfon conjugate, 5-aminimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), myo-inositol trispyrophosphate (ITPP), ethyl glucuronide (ETG), ethyl sulfate (ETS), tramadol, bemitil, and cyanocobalamin (vitamin B12) were chosen, with corresponding logP values ranging from −5.7 to 3.2 (Table 1). Since the mass spectrometer operated in SRM acquisition mode, compound-specific parameter optimization was required and was conducted by direct infusion of diluted reference standard solutions into the MS. This procedure provided optimized settings for precursor-product ion transitions and corresponding collision energies.

Prior to the analysis of the aforementioned compounds, paper spray experiments to optimize solvents as well as the applied spray voltage were performed and tuned to achieve the highest ionization efficiency by comparison of signal-to-noise (S/N) ratios between blank and spiked urine specimens. In Table 2, the analytical performance of the assay is summarized for selected polar stimulants. For these compounds, the assay provides the highest sensitivity using pure acetonitrile as the rewet and spray solvent. The addition of water or 0.1% aqueous acetic acid as proton donor negatively affected the S/N ratio. Moreover, to further investigate the sensitivity of the assay for all model compounds, calibration curves were determined in spiked urine specimens, and the limit of quantification (LOQ) was estimated at a S/N ratio of 4, %CV < 15% and ± 20% accuracy.

3 RESULTS

3.1 Polar compounds

All of the aforementioned hydrophilic model compounds except for ITPP were successfully determined in human urine using paper spray mass spectrometry at relevant concentrations. The coefficient of determination for linear regression (R²) for each calibration curve was greater than 0.98, indicating good linearity with detection limits that meet the criteria of sports drug testing (Figure S1).19 Table 1 provides the results of all tested compounds and specifies the selected analytical parameters, e.g. spray voltage, ionization mode, paper spray solvents, quantifier precursor-product ion transition, and internal standard.
| Compound      | Structural formula | logP<sup>a</sup> | Spray voltage (ionization mode) | Rewet/spray solvent | Ion transition (m/z) | ISTD            | WADA MRPL | LOQ          |
|---------------|--------------------|------------------|---------------------------------|---------------------|----------------------|------------------|-----------|--------------|
| ITPP          | ![ITPP structure](https://via.placeholder.com/150) | −5.7             | −3.0 kV MeOH + 0.01% NH₄OH | 301.9 → 524.9       | ITPP-D₆              | -                | 10 ng/mL  |
| AICAR         | ![AICAR structure](https://via.placeholder.com/150) | −4.3             | 3.8 kV 50:50 MeOH: H₂O         | 259.1 → 82.1        | AICAR-¹³C₂-¹⁵N     | -                | 2.5 μg/mL |
| Meldonium     | ![Meldonium structure](https://via.placeholder.com/150) | −2.2             | 3.8 kV 90:10 ACN: H₂O + 0.1% AA | 147.1 → 58.3        | Meldonium-D₃       | 200 ng/mL       | 100 ng/mL |
| Metformin     | ![Metformin structure](https://via.placeholder.com/150) | −1.4             | 4.0 kV ACN + 0.1% AA           | 129.9 → 60.4        | -                    | -                | 25 ng/mL  |
| Octopamine    | ![Octopamine structure](https://via.placeholder.com/150) | −0.4 (−4.2)      | 3.8 kV ACN                      | 154.0 → 65.0 (234.1 → 83.9) | Isosuprine-D₅   | 1000 ng/mL       | 300 (5) ng/mL |

(Continues)
**Table 1** (Continued)

| Compound          | Structural formula | logP a | Spray voltage (ionization mode) | Rewet/spray solvent | Ion transition (m/z) | ISTD      | WADA MRPL | LOQ          |
|-------------------|--------------------|--------|--------------------------------|---------------------|----------------------|-----------|-----------|--------------|
| ETG               |                    | −0.3   | −3.0 kV 90:10 MeOH: iPrOH      | 221.1 → 75.1        | ETG-D₅               | 5 µg/mL   | 1000 ng/mL| FAIMS: 100 ng/mL |
| ETS               |                    | −0.3   | −3.0 kV 90:10 MeOH: iPrOH      | 125.0 → 96.5        | ETG-D₅               | -         | 250 ng/mL | FAIMS: 50 ng/mL  |
| Oxilofrine        |                    | 0.5    | 3.8 kV ACN                      | 182.0 → 91.4        | Isoxsuprine-D₅       | 100 ng/mL | 15 ng/mL |
| p-OH-amphetamine  |                    | 1.0    | 3.8 kV ACN                      | 152.0 → 73.0        | Isoxsuprine-D₅       | 100 ng/mL | 25 ng/mL |
| Cyanocobalamin    | (Na-adduct)        | 1.9    | 4.0 kV 60:40:0.1 MeOH: Water:NaOAc | 700.3 → 657.7       | Isoxsuprine-D₅       | -         | 100 ng/mL |

(Continues)
Myo-inositol trispyrophosphate (ITPP) is the most difficult analyte from the set of compounds tested due to its chromatographic and mass spectrometric behavior. Routine analytical applications targeting ITPP utilize hydrophilic-interaction liquid chromatography mass spectrometry (HILIC-MS) combined with dilute-and-inject or solid-phase extraction protocols using weak-anion-exchange cartridges. In this study using PS-MS, the negative ionization mode was used to determine ion transitions derived from the 2-fold deprotonated precursor ion. In addition, the Na-adduct of ITPP was investigated. As quantifier precursor/product ion pairs, m/z 301.9 → 524.9 (ITPP) and m/z 312.9 → 546.9 (ITPP Na-adduct) were chosen.

To verify the ability of the method effectively to ionize ITPP from the paper strip, the first step was to analyze the compound in pure methanol. Both ITPP itself and its Na-adduct provided an LOQ of 10 ng/mL in neat solution and a coefficient of determination for linear regression of 0.99 in the range of 10 to 1000 ng/mL. However, it was not possible to detect the analyte in spiked urine samples, even at concentrations of 5 μg/mL. According to the first results in pure methanol, interfering adsorption effects caused by the cellulose-based paper strip can be excluded. More likely it appears to be the presence of substantial ion suppression effects resulting from the urinary matrix. Alternatively, extensive protein-binding might contribute to the observed phenomenon. Further studies where the urine or the paper substrate is pretreated to reduce matrix effects are required to understand how paper spray can best be used to analyze ITPP in urine.

### 3.2 Compounds of moderate polarity

Besides the highly polar compounds with logP values ≤ 1.0, three examples of compounds with moderate polarity (cyanocobalamin (vitamin B₁₂), tramadol, and bemitil), which are not prohibited by WADA but have been the subject of complementary studies, were investigated using PS-MS.

As a source of organically bound cobalt, vitamin B₁₂ was investigated in a recent study aimed at determining the contribution of organically bound cobalt (in the form of vitamin B₁₂) to the total amount of cobalt in human urine, in order to account for the fact that the use of inorganic cobalt preparations are not permitted in sports.

With an LOQ of 100 ng/mL and a linear range of quantitation between 100 and 2500 ng/mL, PS-MS proved suitable to detect cyanocobalamin and therefore might be helpful for adequate result interpretation and management.

In an ongoing study in our laboratory, dried blood spots are investigated as a potential novel matrix for the detection of tramadol misuse in sports. PS-MS was investigated as an alternative analytical technique since it has been extensively used to detect drugs in blood. Here, analytical results for tramadol demonstrated a linear response with R² > 0.99 in the range 1.0 to 10.0 ng/mL. The estimated LOQ of 1.0 ng/mL meets the required level of quantitation.

According to the manufacturer, bemitil is known to improve both physical performance and resistance to stress. Therefore, WADA
 decided to include the compound in its 2018 monitoring program.\textsuperscript{26} To verify the analytical performance of the assay a calibration curve in the range 0.5 to 250 ng/mL was prepared in urine. The coefficient of determination for linear regression (R^2) was greater than 0.99 indicating good linearity with a detection limit of 10 ng/mL with a S/N > 5.

### 3.3 Internal standards

The use of appropriate internal standards is of utmost importance in paper spray mass spectrometry, and optimal results are typically achieved when an isotopically labeled internal standard is used as it has the same extraction efficiency from the paper and ionization efficiency as the matching analyte. The importance of choosing an appropriate internal standard was exemplified when studying metformin, a metabolic modulator. As a first attempt, meldonium-D\textsubscript{3} was chosen as the internal standard for metformin because isotopically labeled metformin was not available. However, using meldonium-D\textsubscript{3} as an ISTD resulted in moderate linearity (R^2 = 0.94) and excluding the ISTD resulted in improved results (R^2 > 0.99). Due to the absence of any chromatographic separation, omitting ISTDs is not recommended to allow for compensating unpredictable matrix effects.

### 3.4 Ion mobility/paper spray MS

Ion mobility spectrometry offers separation based on a combination of compound-specific factors, such as molecular shape, conformation, charge state, and size of gas phase ions. Although the use of internal standard in paper spray can lead to successful quantitation, the high background from the paper, minimal sample cleanup, and lack of chromatographic separation could limit its detection capability. The combination of ion mobility with paper spray mass spectrometry can help to overcome the above-mentioned limitations and makes it possible to have short turnaround times with increased levels of quantitation.

FAIMS refers to (high) field asymmetric ion mobility spectrometry and is an atmospheric pressure separation technique based on differences in ion mobility by applying alternating low and high electric

### Table 2: Optimization of the S/N for polar stimulants by adjusting the rewet and spray solvent

| Solvent       | S/N p-OH amphetamine (50 ng/mL) | S/N Octopamine (500 ng/mL) | S/N Octopamine sulfo-conjugate (500 ng/mL) | S/N Oxilofrine (50 ng/mL) |
|---------------|---------------------------------|-----------------------------|--------------------------------------------|---------------------------|
| 95:5:0.01 MeOH:H\textsubscript{2}O:AA | 1.1                             | 2.0                         | 97                                         | 3.9                       |
| 90:10:0.1 ACN:H\textsubscript{2}O:AA | 1.6                             | 1.9                         | 337                                        | 5.9                       |
| ACN + 0.1% AA | 2.5                             | 2.4                         | 557                                        | 5.4                       |
| MeOH + 0.1% AA| 2.5                             | 2.1                         | 192                                        | 6.6                       |
| ACN           | 8.4                             | 6.5                         | 857                                        | 15.5                      |

**Figure 2** Meldonium calibration curve in the range of 0.1–10 μg/mL in urine without FAIMS Pro (A) and in the range of 0.01–10 μg/mL in urine with the use of FAIMS Pro (B).
fields (asymmetric waveform) between two cylindric electrodes. Desolvated ions are introduced into the system by a carrier gas (N₂) and move radially between the aforementioned cylindric electrodes as the result of the applied asymmetric waveform. While high electric fields have a stronger impact on an ion’s mobility, ions begin to drift towards one of the electrodes during the low-voltage portion of the waveform. Ions introduced into the FAIMS Pro™ interface can only pass the pair of electrodes by applying a compound-specific compensation voltage (CV). All non-optimized species that are reaching an electrode surface are discharged and filtered out. For PS-MS experiments, a Thermo Scientific™ FAIMS Pro™ Interface was used.

Table 3 demonstrates a comparison of area intensities and corresponding S/N ratios for 10 blank urine specimens spiked with 10 and 100 ng/mL meldonium. Noise reduction: ~99%
S/N: 2.1 → 67.6
S/N: 1.3 → 5.8

4 | CONCLUSION

This proof-of-concept-study demonstrates that paper spray mass spectrometry is a novel and complementary technique for polar compounds in sports drug testing. In comparison with conventional LC-MS approaches, the key benefits of this technique are the cost- and time-savings due to minimal sample pretreatment and omission of chromatographic separation. These aspects suggest a significant added value to routine doping controls, especially target analytes that have proved to be challenging for detection by conventional reversed-phase chromatography-based analytical approaches due to their physico-chemical properties. All investigated model compounds in appropriate human sample matrices, except ITPP, fulfilled WADA requirements regarding the applicable minimum required performance limits for initial testing procedures. The combination of the VeriSpray™ ion source and the FAIMS Pro™ interface adds additional selectivity and sensitivity to the assay.

However, due to omission of chromatographic separation the use of appropriate internal standards is of utmost importance to compensate for matrix effects, which was exemplified when studying metformin. Another currently existing limitation of the technique has been the substantial influence of rewet and spray solvents on the individual analytical performance of the system, necessitating optimization for each compound. Consequently, multi-target assays require a well-balanced compromise to meet the desired analytical goals.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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