Received: 5 December 2017 | Revised: 9 February 2018 | Accepted: 13 March 2018

DOI: 10.1002/rcm.8119

RESEARCH ARTICLE

WILEY Rapid Communications in Mass Spectrometry

Development of a multi-functional concurrent assay using weak cation-exchange solid-phase extraction (WCX-SPE) and reconstitution with a diluted sample aliquot for anti-doping analysis

Yongseok Kim[1,2] | Mijin Jeon[1] | Hophil Min[1] | Junghyun Son[1] | Jaeick Lee[1] | Oh-Seung Kwon[1] | Myeong Hee Moon[2] | Ki Hun Kim[1]

[1] Doping Control Center, Korea Institute of Science and Technology, Hwarang-ro 14-gil 5, Seongbuk-gu, Seoul 02792, Republic of Korea

[2] Department of Chemistry, Yonsei University, Yonsei-ro 50, Seodaemun-gu, Seoul 03722, Republic of Korea

Correspondence
K.H. Kim, Doping Control Center, Korea Institute of Science and Technology, Hwarang-ro 14-gil 5, Seongbuk-gu, Seoul 02792, Republic of Korea.
Email: kihun.kim@kist.re.kr

Funding information
Korea Institute of Science and Technology

Rationale: In addition to the development of adequate screening methods for multiple compounds, the World Anti-Doping Agency (WADA) requires anti-doping laboratories to analyze prohibited substances and their metabolites from various classes. This task presents a difficult challenge for all agencies and interests involved in the field of doping control.

Methods: A screening method is reported in which hybrid sample preparation was performed using a combination of weak cation-exchange solid-phase extraction (WCX-SPE) and the 'Dilute and Shoot' strategy in order to take advantage of both the methodologies. Target substances were extracted using a WCX cartridge and reconstituted with a diluted sample aliquot that included 20% of an untreated urine sample. The target substances were further analyzed by high-performance liquid chromatography/triple quadrupole mass spectrometry (LC/MS).

Results: The SPE procedure was optimized using a cartridge-washing step, elution conditions, and elution volume. The cartridge-washing step, which was performed using 10% methanol, improved the overall recovery of target substances. Since the recovery was observed to vary according to the pH of the eluting solution, we applied an elution step using both an acid and a basic organic solvent to achieve complementary recovery. Reconstitution of the diluted aliquot sample was performed to recover the polar substances.

Conclusions: The method was validated and applied to real samples in accordance with the external quality assessment scheme of WADA and to the previously reported samples that had provided positive test results. This novel method using hybrid sample preparation and LC/MS could be useful to screen multiple classes of the 264 targeted substances in anti-doping analysis.

1 | INTRODUCTION

The World Anti-Doping Agency (WADA) annually publishes a list in which prohibited drugs are organized into twelve classes and the three forbidden methods of chemical and physical manipulation, gene doping, and the manipulation of blood and blood components.[1] The number of drugs deemed as "prohibited" has dramatically increased over the decades, which has led to increased demands for stricter anti-doping policies and control methods.[2] In light of these demands for more control, class-dependent or multi-class approaches for analysis have evolved over the years. Liquid chromatography/tandem mass spectrometry (LC/MS/MS) is now extensively utilized due to its

high selectivity and sensitivity. In addition, many doping analyses are conducted using various extraction methodologies, such as liquid–liquid extraction (LLE), protein precipitation (PP), or solid-phase extraction (SPE).[3-11] SPE presents several advantages over LLE or PP because it includes a relatively simple pre-treatment procedure and is less dependent on the proficiency of the researcher. Moreover, it involves less sample manipulation, which should provide good reproducibility and thus higher sensitivity and selectivity. Various SPE sorbents have also been developed and can be selected according to the target compounds or the sample matrix.[5,12-15] Among the various types of SPE, mixed-mode weak cation-exchange solid-phase extraction (WCX-SPE) offers both cation-exchange and reversed-phase interactions, which are properties that facilitate the extraction of various analytes across an extensive range of physicochemical characteristics. In the anti-doping field, WADA-prohibited peptides that stimulate the secretion of growth hormones, such as growth hormone releasing peptides (GHRPs), have been successfully analyzed recently, and the analytical method was validated using WCX-SPE.[16,17]

However, an extraction method based on WCX-SPE has its drawbacks; it is difficult to extract highly polar analytes as SPE requires the use of an organic solvent as an eluent. Meldonium is a characteristic example of a highly polar prohibited compound that is difficult to extract. Since 2016, meldonium (mildronate; 3-(2,2,2-trimethylhydrazinium)propionate) has been prohibited by WADA because of its effect on the production of lactic acid. Meldonium is known to reduce lactic acid levels, thus enhancing both the energy storage and the endurance of elite athletes.[18] Furthermore, to evaluate the exact concentration of drugs in urine, the conjugated metabolites, such as glucuronides, sulfates, or cysteinyl conjugates, have to be de-conjugated in order to use the multiclass doping analysis method.[19-24]

Ethyl glucuronide (EtG) is considered to be a biomarker for ethanol consumption since high levels of EtG are often correlated with abnormal steroid profiles. In particular, consumption of ethanol is assumed to cause an elevation of the testosterone/epitestosterone ratio and a decrease in the androsterone/testosterone ratio. To correlate these abnormal steroid profile ratios, it is necessary for doping control to determine the concentration of EtG in urine.[21,25] Several analytical methods have been reported for the analysis of these compounds; however, most of these approaches require a variety of sample preparation methods or instrumental conditions, which in turn leads to more complicated assays.[26-29] Recently, an approach was developed that is highly compatible with the analysis of hydrophilic compounds.[30,31] Known as the "Dilute and Shoot" (DnS) strategy, this method does not require any additional sample extraction step but, rather, a simple dilution of the sample itself. The absence of an extraction step allows for the detection of hydrophilic compounds and facilitates the development of multi-class analyses. The DnS strategy also has its drawbacks, however; it is difficult to analyze a sample containing low concentrations of analytes because of the high matrix effect.

In this study, an initial testing method was developed based on hybrid sample preparation by utilizing a combination of WCX-SPE and the "Dilute and Shoot" strategy for 264 prohibited drugs and their analogs. The extraction step was optimized in order to encompass multiple classes of compounds, and a dual elution step for both acidic and basic conditions was developed to ensure higher recovery of specific compounds. The DnS strategy was also introduced via the addition of urine samples in reconstituted solutions to simultaneously analyze the levels of meldonium and ethyl glucuronide. The optimized initial screening method was validated for routine analysis and was applied to real samples. A triple quadrupole mass spectrometer was used because it offered some advantages over other types of mass spectrometers. Mass spectrometers based on Orbitrap technology tend to have a much higher resolution and are extensively used for assays. Musenga and Cowan developed a fast screening method using these high-resolution mass spectrometers; however, the method was mostly performed in full scan mode without product ion scanning, which does not provide product ions that can confirm the presence of the target analytes.[32] To minimize the occurrence of false negative or false positive results, we employed selected reaction monitoring (SRM) analysis on a triple quadrupole mass spectrometer, which provides the benefit of monitoring both the precursor and the product ions within a short transition time. Although SRM analysis has the limitation that it loses information other than that of the selected precursor-product ion transition, monitoring a single ion transition is enough for a screening method. By applying SRM analysis on the triple quadrupole mass spectrometer, we are able to analyze a total of 264 prohibited compounds, including anabolic agents, peptide hormones and growth factors, beta-2 agonists, hormone and metabolic modulators, diuretics and masking agents, stimulants, narcotics, cannabinoids, glucocorticoids, and beta-blockers. This enabled us to monitor these compounds and to detect the manipulation of blood and blood components.

Thus, the optimization, validation, and real application of a new SPE method based on dual elution are reported in this study. In addition, the use of this method for increasing the recovery of specific substances after analysis is described.

2 | EXPERIMENTAL

2.1 | Chemicals and reagents

All the standard substances were purchased in pharmaceutical grade from various suppliers. Supplier information is provided in supporting information 1. Methanol was obtained from J.T. Baker (Phillipsburg, NJ, USA). Citric acid, sodium citrate, and ammonium hydroxide were obtained from Sigma-Aldrich (St Louis, MO, USA). β-Glucuronidase/sulfatase was purchased from Roche Diagnostics (Mannheim, Germany). Phosphoric acid and formic acid were supplied by Wako Pure Chemical Industries (Osaka, Japan). The water used in the experiments was purified using a Milli-Q system (Millipore, Bedford, MA, USA). All the reagents and solvents were of analytical grade.

2.2 | LC/MS analysis

The liquid chromatography and mass spectrometry conditions employed were similar to those in our previously reported study.[9] Briefly, chromatographic separation was conducted using a Thermo Scientific™ Vanquish™ UHPLC system (ThermoFinnigan, San Jose, CA, USA) with a Kinetex® C18 column (100 mm × 2.1 mm I.D.;

2.6-μm particle size; Phenomenex, Torrance, CA, USA) connected to a guard column (2.1 mm I.D.). The mobile phase consisted of water (A) and methanol (B), both of which contained 0.1% formic acid. The initial gradient composition (98% A/2% B) was maintained for 0.5 min and was linearly increased up to 95% B over 8 min and maintained for 0.5 min before being decreased to 2% B over 0.01 min. Afterwards, equilibration was performed for 1 min. The flow rate was set to 0.5 mL/min. The column temperature was fixed at 35°C. The mass spectrometer was a TSQ Quantiva™ triple quadrupole mass spectrometer (ThermoFinnigan) equipped with an electrospray ionization (ESI) source. The ion spray voltages were set to 4.5 kV (positive mode) and 3.5 kV (negative mode); the capillary temperature was 320°C and the vaporizer temperature was 340°C in both modes. Nitrogen gas was used for both the sheath gas and the auxiliary gas at flow rates of 60 arbitrary units (arb) and 15 arb, respectively. The experiments were performed using SRM with collision-induced dissociation. An optimized SRM method was obtained via continuous infusion of the analytes into the ESI source in both positive and negative mode at a flow rate of 5 μL/min. The optimized SRM method and the minimum required performance level (MRPL) results are reported in supporting information 2.[33]

2.3 | Preparation of standard solutions and samples for WCX-SPE

All the stock solutions were prepared by dissolving each standard compound in methanol or dimethyl sulfoxide (DMSO) at a concentration of 1000 μg/mL. The dissolved standard compounds were further diluted, along with compounds in the same groups, to specific concentrations before being stored at −20°C. The internal standard mixture consisted of methaqualone (25 ng/mL), ethyltheophiline (100 ng/mL), heavy-isotope-labeled [(^{13}C,^{15}N)Lys] GHRP-2 (H-D-Ala-D-2-Nal-Ala-Trp-D-Phe-Lys-NH_2) and GHRP-6 (His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2) (2 ng/mL) to evaluate the assay performance, and potassium 4-nitrophenyl sulfate (100 ng/mL) and 4-nitrophenyl β-D-glucuronide (100 ng/mL) to evaluate the enzymatic hydrolysis. Formic acid (5%) in methanol was prepared by diluting 500 μL of 99% formic acid in 9.5 mL of methanol. To obtain 1% ammonium hydroxide in methanol, 400 μL of 25% ammonium hydroxide was diluted using 9.6 mL of methanol. 21 g of citric acid monohydrate was dissolved in 1000 mL of water. The potassium carbonate solution was produced by dissolving 50 g of potassium carbonate in 1000 mL of water. 95 mL of 0.1 M citric acid and 415 mL of 0.1 M sodium citrate were diluted with water to obtain a final volume of 1000 mL, and 2 mL of 5 N potassium hydroxide was added to adjust the pH to 6.0.[9,34]

An aliquot of 40 μL was taken from the 2 mL sample and was transferred to an Eppendorf tube for reconstitution. An internal standard mixture (10 μL, ISTD), 700 μL of 0.1 M citrate buffer, and 50 μL of β-glucuronidase/arylsulfatase were added to the remaining sample volume of 1960 μL. Hydrolysis was performed according to the previously established in-house methodologies.[9,34] The SPE cartridge was washed using 2 mL of methanol and was conditioned with 2 mL of water. Before the sample was loaded onto the SPE cartridge, 100 μL of 4% phosphoric acid was added to the hydrolyzed sample. In order to remove interferences, 2 mL of 10% methanol in water was used to load the sample cartridge. 2 mL of 5% formic acid in methanol and 1% ammonium hydroxide in methanol were added to the cartridge, consecutively. The eluted sample was dried using an N_2 evaporator for 25 min at 50°C. The resultant residue was reconstituted using 200 μL of the diluted sample aliquot, which consisted of 40 μL of the previously transferred sample and 160 μL of the mobile phase mixture (95% A/5% B, *v*/v). The aliquot (150 μL) was further transferred to a 1.5-mL vial after being centrifuged for 10 min at 10,000 *g*. A sample (10 μL) was then injected into the LC/MS instrument for analysis.

2.4 | Complementary effect of the SPE and DnS methods

To evaluate the complementary effect of the two different sample preparation methods, the sample with the fortified standard compounds at 50% of the MRPL level (required LOD level from WADA guideline) was prepared using the Dilute and Shoot method that was previously developed in our laboratory:[35] 300 μL of the sample was centrifuged for 10 min, and 90 μL of the supernatant was combined with 10 μL of ISTD. Further analysis using LC/MS was performed with the method previously described.

3 | RESULTS AND DISCUSSION

3.1 | Sample preparation for WCX-SPE

The effectiveness of the de-conjugation step was evaluated by monitoring the amount of 4-nitrophenol that was released as a product of the potassium 4-nitrophenyl sulfate and the 4-nitrophenyl β-D-glucuronide that were present in the internal standard mixture. Sample loading was performed under acidic conditions in order to protonate the analytes, which readily promoted the interaction with the cation exchanger via the addition of 100 μL of 4% phosphoric acid. To obtain sufficient overall sensitivity to the target compounds with increased specificity for the analytical procedure, each SPE step was optimized after loading the sample. Different elution conditions, wash procedures, and reconstitution steps were considered. The measure for optimization was based on the overall recovery of the 264 target compounds and was not based on the highest recovery of only a few compounds. To eliminate interferences from the matrix after sample loading, an optimized wash protocol was required. Four different concentrations of methanol in water (0%, 10%, 20%, and 50%) were compared. Of the 264 compounds that were examined, 187 showed the highest intensities when washed on the WCX-SPE cartridge using 50% methanol in water. However, since 25 of these compounds provided less than 10% intensity using this wash protocol rather than the other wash protocols, it was concluded that a protocol using 50% methanol in water was not suitable for the analysis of various classes of compounds. In order to obtain satisfactory intensities for all the 264 target compounds, 10% methanol in water was chosen as the wash protocol. (Figure 1).

At low pH levels, organic solvents, such as methanol or acetonitrile, are extensively employed for elution on WCX-SPE. Elution of the analytes was performed by collapsing the ion-exchange interaction

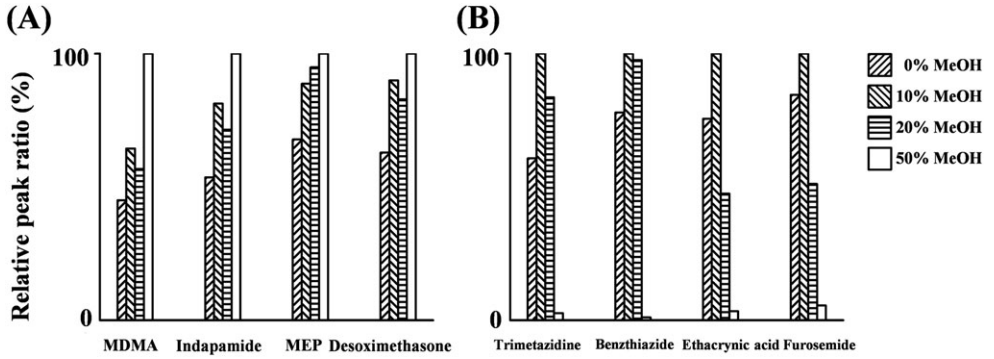

FIGURE 1 Optimization of the wash protocol using concentrations of methanol in water ranging from 0% to 50%. Under the protocol, the highest intensities were noted for 187 compounds while using 50% methanol in water (A), whereas 25 compounds were completely lost (B)

via the introduction of a high concentration of counterions (hydrogen ions in this case).[36-39] Although 5% formic acid in methanol was initially introduced for elution, some compounds (especially prohibited peptides such as alexamorelin (2-7), GHRP-1 (2-7), and ipamorelin) were not detected at all or showed poor recovery. Alternative approaches that were aimed at overcoming these flaws were investigated. When 1% ammonium hydroxide in methanol was introduced,[5] the GHRPs that were not previously extracted under low pH conditions were successfully detected. Furthermore, 99 of the 264 compounds showed enhanced recovery levels of more than 50%. It is suggested that this method utilizes a mechanism for cation-exchange chromatography, which is similar to that used for the separation of peptides or proteins by strategically increasing the pH levels.[40,41] High pH levels of the buffer induce disruption of the electrostatic interaction between the analytes and the SPE cartridge stationary phase by modifying the positively charged analytes into neutral or negatively charged ones.[42] However, 34 of the compounds were observed to have drastically reduced intensities under high pH conditions compared with when using elution with 5% formic acid in methanol. Thus, in order to take advantage of both sets of elution conditions, elution with 5% formic acid in methanol was followed by elution with 1% ammonium hydroxide in methanol. Both eluates were collected in the same glass tube. The reason for the significant difference in the recovery of the target substances under the various elution conditions is not clear. However, consecutive elution steps facilitated the elution of all the target compounds. Figure 2 depicts the trends observed under different elution conditions. For comparative purposes, 4 mL of 5% formic acid in methanol (top), 4 mL of 1% ammonium hydroxide in methanol (middle), and a 2-mL aliquot of each elution solvent (bottom) were applied, consecutively.

The volume of the elution solvent is also an important factor for achieving higher recovery levels. Small amounts of the elution solvent do not seem to provide enough capacity for analyte elution. However, large amounts of the elution solvent increase the sample preparation time and can also decrease the peak area of analytes; this could be because the impurities that are not eliminated during the washing step could be easily extracted.[12] In order to achieve the optimum elution volume, analyses were conducted using the elution volumes of 1, 2, 4, and 6 mL. It was observed that an elution volume of 4 mL provided sufficient capacity for elution and minimized both the sample preparation time and the possibility of eluting unexpected interferences. Therefore, this volume was chosen for our optimized protocol. It is worth noting that elution volumes greater than 4 mL did not show any significant variations in the amounts of target compound recovered (data not shown).

A diluted sample aliquot comprising 40 μL of the previously transferred sample and 160 μL of the reconstituted solvent (95%/5%/0.1%, water/methanol/formic acid, *v*/*v*/*v*) was applied to reconstitute the dry residues. Figure 3 illustrates two conditions under which reconstitution was conducted. It was revealed that using a dilute sample aliquot for reconstitution caused the detection of both meldonium and ethyl glucuronide. Although those two compounds were lost during the sample extraction procedure, we were able to detect them using the sample aliquot. Small amounts of interference in the sample aliquot did not affect the recovery of other compounds. Chromatograms for all the target substances are depicted in supporting information 3.

3.2 | Method validation

The developed procedure was validated by considering parameters, such as selectivity, matrix effect, intra- and inter-day precision values, the limit of detection (LOD), and the recovery amount, as required by the ISO/IEC 17025 and WADA guidelines.

Compounds which were both spiked and not spiked with five different urine samples were prepared and analyzed in accordance with the established protocol in order to investigate the selectivity of our method. As the presence of interference peaks may cause false negative or false positive results, chromatograms for the prepared spiked samples were overlapped with those of the non-spiked samples to check for the appearance of any interference peaks at the same retention time (RT). No interference peaks were observed.

The intra- and inter-day precisions were investigated by analyzing the six replicated urine samples that had been fortified with the target substances at three different concentrations (low, mid, and high) in both single and 3-day periods (n = 6/6/6 and 18/18/18). These were evaluated in relation to the relative standard deviation (% RSD) of the chromatogram area. For precision data, each concentration range for the mixed standard solution was prepared using half of the differences in serial dilution. The values obtained for the mid-range concentration

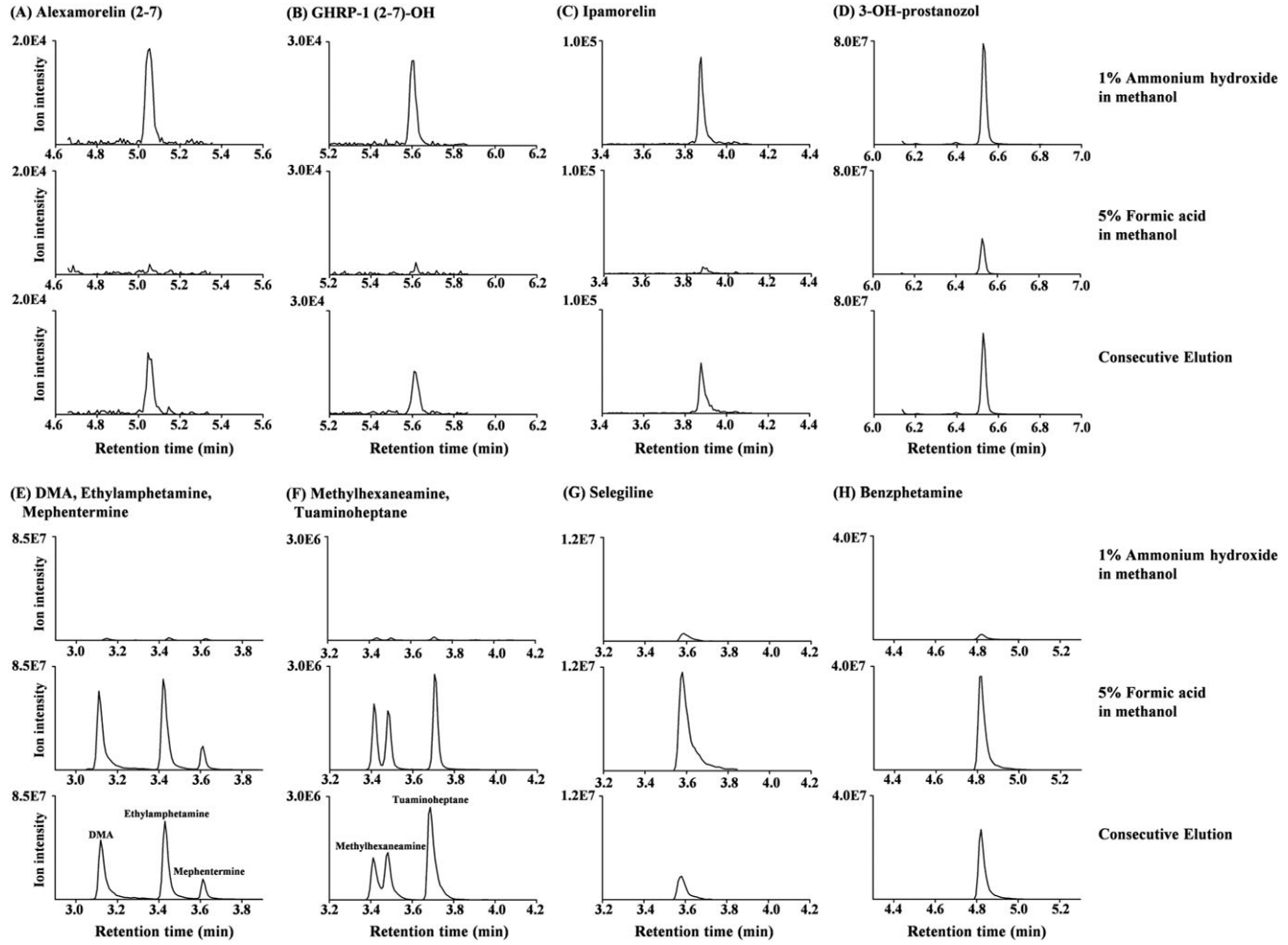

FIGURE 2 Chromatograms of the representative target compounds under three different elution conditions. The compounds that depicted higher recovery in conditions of 1% ammonium hydroxide in methanol are illustrated by the initial four figures: (A) Alexamorelin (2-7) (RT: 5.04 min); (B) GHRP-1 (2–7)-OH (RT: 5.61 min); (C) Ipamorelin (RT: 3.87 min); and (D) 3-OH-prostanozol (RT: 6.52 min). However, the exemplified compounds that illustrated higher recovery by the elution of 5% formic acid in methanol are represented in the last four chromatograms: (E) DMA (RT: 3.13 min); Ethylamphetamine (RT: 3.43 min) and Mephentermine (RT: 3.61 min); (F) Methylhexaneamine (RT: 3.42 min) and Tuaminoheptane (RT: 3.71 min); (G) Selegiline (RT: 3.58 min); and (H) Benzphetamine (RT: 4.81 min)

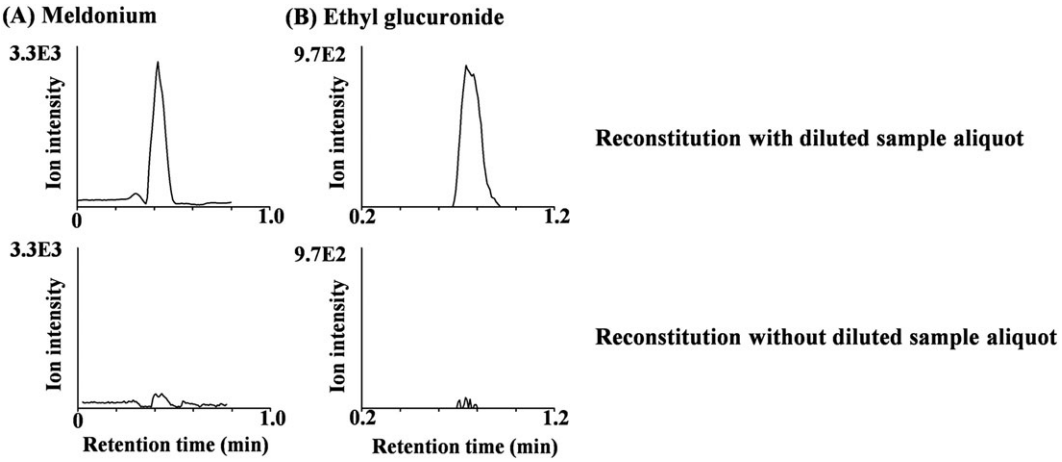

FIGURE 3 Chromatograms of meldonium (A) and ethyl glucuronide (B) obtained via either reconstitution with dilute sample aliquot [40 μL of sample aliquot and 160 μL of the reconstitution solvent (95%/5%/0.1%, water/methanol/formic acid, v/v/v)] (top) or reconstitution without the diluted sample aliquot (bottom). The concentration of meldonium was 200 ng/mL, whereas the concentration of ethyl glucuronide was 1000 ng/mL

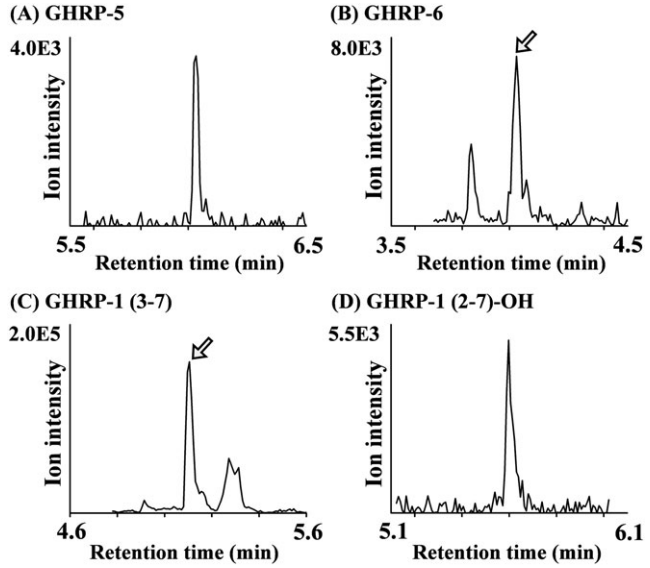

FIGURE 4 Compounds of GHRP depicted equal or higher LOD than that depicted by the previously developed method. The 1 ng/mL of LOD that was obtained using the developed method and (A) GHRP-5; (B) GHRP-6; (C) GHRP-1 (3-7); (D) GHRP-1 (2–7)-OH) are illustrated

of the target substances are illustrated in supporting information 4. Intra- and inter-day precision limits help to evaluate the robustness of the retention times and the abundance of the diagnostic ion transitions for the target substances. The RSD values for intra-day precision ranged from 1.30% to 22.73%, whereas those for the inter-day precision ranged from 5.30% to 24.25%. In all cases, the coefficient of variance (CV) was less than 25%. Only the precision data for the mid-range concentration is depicted.

The matrix effect of the method was assessed by conducting three replicate analyses of urine samples that were spiked with either the target compounds (QC_pos) or water (QC_water). The matrix effect was determined by comparing the peak area of QC_pos with that of the QC_water samples. The matrix effect was calculated using the following equation, with the results ranging from 7.55% to 164.98%:

$$\text{Matrix effect (ME)} = \text{Peak area of target compound of sample in urine} / \text{Peak area of target compound of sample in water} \times 100\%$$

To evaluate the analyte recovery, we analyzed three replicates of the QC_pos samples and three replicates of the recovery sample in which the target substances were spiked after extracting the pooled urine. The peak areas were determined in order to obtain the recovery values. The peak areas of the QC_pos samples were divided into those of the recovery sample. The recovery values ranged from 2.06% to 124.99%. Meldonium and ethyl glucuronide, which were lost through WCX-SPE, showed a recovery range of 2.52% and 2.06%. These values were estimated since these two compounds only existed in the 40-μL sample aliquot that had been introduced in the reconstitution step.

Five replicates of the urine samples that had been spiked with the target compounds at different concentrations were prepared and analyzed in order to define the LOD. The concentrations were 1-, 0.5-, 0.2-, 0.1-, 0.05-, 0.02-, 0.01-, 0.005-, 0.002-, and 0.001-fold of the low-range concentrations for precision calculations. The LOD was defined as the lowest concentration at which all five samples can be detected with a signal-to-noise ratio of ≥3. Using the established method, the LODs of the analytes ranged from 0.005 to

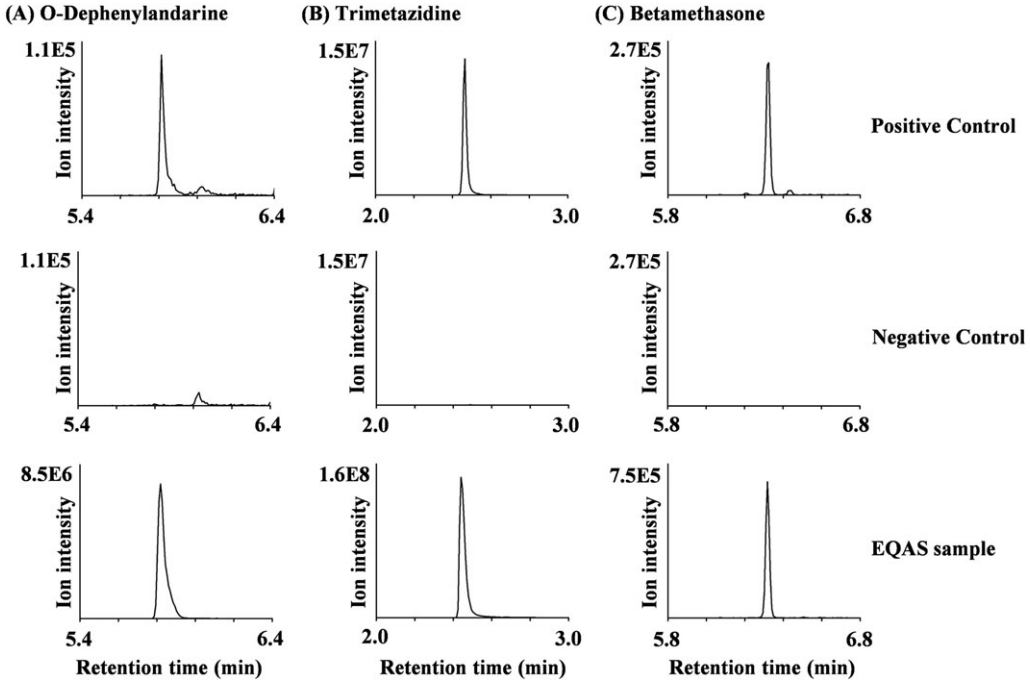

FIGURE 5 Chromatograms obtained after applying the developed method. The left column shows the positive control urine samples at specific concentrations: (A) O-dephenylandarine, 20 ng/mL; (B) trimetazidine, 20 ng/mL; and (C) betamethasone, 30 ng/mL). The middle column represents negative control urine samples, whereas the right column shows the results for the WADA EQAS sample

250 ng/mL and were equal to or less than 50% of the MRPL. The validation results are depicted in supporting information 4.

3.3 | Complementary effect of the SPE and DnS methods

In the developed method, the solid-phase extraction and dilute and shoot methods were associated. The combination of the two sample preparation steps provided better sensitivity than when simply using dilute and shoot because this method does not possess any extraction or enrichment step. Furthermore, eight compounds (norclostebol, hexarelin, GHRP-1 (2-7), GHRP-1 (2–7)-OH, GHRP-1 (3-7), GHRP-5, GHRP-6, and zearalenone) were not detected at 50% of the MRPL level using the DnS method. They were only detected after the extraction step using WCX-SPE.

3.4 | Comparison with previous methods

To evaluate the sensitivity of the developed method, we compared it with the previous in-house methodologies.[9,43] Even if the assays were performed under different chromatographic and mass spectrometric conditions, we were still able to appraise the sensitivity of our novel method with some degree of certainty. Of the 264 target compounds, 181 were investigated. Of the target substances, 70.2% (127/138) yielded lower LOD values than in the previous assays. In the case of benzoylecgonine the sensitivity was 200-fold higher than that by the LOD comparison. For GHRP-6 (2–5)-OH and GHRP-1 (3-7), the developed method was observed to be 10- and 5-fold better than the LOD, respectively; however, these values still satisfied WADA's guideline requirements for the LOD, which states that the results for an initial screening method should be equal to or less than that of the MRPL. The value obtained for GHRP was 1 ng/mL (Figure 4).

3.5 | Applications to real samples

The developed method was employed to analyze 37 blind urine samples, including those from WADA EQAS for 2017. Three results of the WADA EQAS samples (O-dephenylandarine, trimetazidine, and betamethasone) are depicted in Figure 5.

4 | CONCLUSIONS

In this study, a new screening method for the evaluation of 264 prohibited drugs and their metabolites was developed and validated for use in anti-doping analyses. This novel method is based on WCX-SPE sample preparation and LC/MS. To cover the diverse chemical properties of the numerous target analytes, various parts of the sample preparation step were optimized, including the wash protocol, the elution conditions and volume, and the reconstitution steps. Recovery of the compounds depends on the elution conditions being utilized. Some compounds, including the GHRPs, were efficiently extracted under basic conditions using an ammonium hydroxide/methanol solution instead of formic acid. In contrast, an acidic elution solvent provided higher extraction efficiency for 34 compounds, some of which contained amino groups. In order to establish the ability of the assay to examine different classes of compounds, acidic–basic double extraction of WCX-SPE was employed. The optimized elution volume was determined to be 4 mL of the total volume of the eluent and was simultaneously shown to have high extraction efficiencies although it did require adequate extraction times. The "Dilute and Shoot" strategy was employed for the reconstitution step of meldonium because extraction was challenging using WCX-SPE and of ethyl glucuronide that was degraded by enzyme hydrolysis. All the validation results satisfied the requirements of ISO/IEC 17025 and WADA guidelines. To evaluate the applicability of the developed method, we analyzed 37 samples, including those of WADA EQAS. In addition, the presented method was used to analyze more than 800 samples with no observed false negative or positive results, which implied that it is a reliable tool in the field of anti-doping analysis.

ACKNOWLEDGEMENT

This study was supported by an intramural grant from the Korea Institute of Science and Technology.

ORCID

Yongseok Kim http://orcid.org/0000-0001-7861-7375
Junghyun Son http://orcid.org/0000-0002-0000-0591
Jaeick Lee http://orcid.org/0000-0001-7548-0297
Myeong Hee Moon http://orcid.org/0000-0002-5454-2601
Ki Hun Kim http://orcid.org/0000-0002-0962-1837

REFERENCES

1. The 2017 Prohibited List. World Anti-Doping Agencys. Available: http://www.wada-ama.org/en/World-Anti-Doping-Program/Sports-and-Anitidoping-Organizations/International-Standards/Prohibited-List.
2. Aguilar M, Munoz-Guerra J, Plata MDM, Del Coso J. Thirteen years of the fight against doping in figures. *Drug Test Anal*. 2017;9(6):866-869.
3. Dominguez-Romero JC, Garcia-Reyes JF, Lara-Ortega FJ, Molina-Diaz A. Screening and confirmation capabilities of liquid chromatography-time-of-flight mass spectrometry for the determination of 200 multiclass sport drugs in urine. *Talanta*. 2015;134:74-88.
4. Santos MG, Tavares IM, Boralli VB, Figueiredo EC. Direct doping analysis of beta-blocker drugs from urinary samples by on-line molecularly imprinted solid-phase extraction coupled to liquid chromatography/mass spectrometry. *Analyst*. 2015;140(8):2696-2703.
5. Mazzarino M, Calvaresi V, de la Torre X, et al. Development and validation of a liquid chromatography–mass spectrometry procedure after solid-phase extraction for detection of 19 doping peptides in human urine. *Forensic Toxicol*. 2015;33(2):321-337.
6. Matabosch X, Pozo OJ, Papaseit E, et al. Detection and characterization of triamcinolone acetonide metabolites in human urine by liquid chromatography/tandem mass spectrometry after intramuscular administration. *Rapid Commun Mass Spectrom*. 2014;28(16):1829-1839.
7. Kioussi MK, Lyris EM, Angelis YS, et al. A generic screening methodology for horse doping control by LC-TOF-MS, GC-HRMS and GC-MS. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2013;941:69-80.
8. Mazzarino M, Fiacco I, de la Torre X, Botre F. Screening and confirmation analysis of stimulants, narcotics and beta-adrenergic agents in human urine by hydrophilic interaction liquid chromatography coupled to mass spectrometry. *J Chromatogr A*. 2011;1218(45):8156-8167.

9. Jeong ES, Kim SH, Cha EJ, et al. Simultaneous analysis of 210 prohibited substances in human urine by ultrafast liquid chromatography/tandem mass spectrometry in doping control. *Rapid Commun Mass Spectrom*. 2015;29(4):367-384.
10. Liu Y, Uboh CE, Soma LR, et al. Efficient use of retention time for the analysis of 302 drugs in equine plasma by liquid chromatography-MS/MS with scheduled multiple reaction monitoring and instant library searching for doping control. *Anal Chem*. 2011;83(17):6834-6841.
11. Moller I, Thomas A, Delahaut P, et al. Mass spectrometric detection of peginesatide in human urine in doping control analysis. *J Pharm Biomed Anal*. 2012;70:512-517.
12. Wang J, Fan X, Liu Y, et al. Extraction optimization of sixteen cephalosporins in milk by filtered solid phase extraction and ultra high pressure liquid chromatography coupled to tandem mass spectrometry. *Anal Methods*. 2017;9(8):1282-1289.
13. Lalovic B, Durkic T, Vukcevic M, et al. Solid-phase extraction of multiclass pharmaceuticals from environmental water samples onto modified multi-walled carbon nanotubes followed by LC-MS/MS. *Environ Sci Pollut Res Int*. 2017;24(25):20784-20793.
14. Ji W, Sun R, Duan W, et al. Selective solid phase extraction of chloroacetamide herbicides from environmental water samples by amphiphilic magnetic molecularly imprinted polymers. *Talanta*. 2017;170:111-118.
15. Pichon V, Combes A. Selective tools for the solid-phase extraction of Ochratoxin A from various complex samples: immunosorbents, oligosorbents, and molecularly imprinted polymers. *Anal Bioanal Chem*. 2016;408(25):6983-6999.
16. Arvat E, di Vito L, Maccagno B, et al. Effects of GHRP-2 and hexarelin, two synthetic GH-releasing peptides, on GH, prolactin, ACTH and cortisol levels in man. Comparison with the effects of GHRH, TRH and hCRH. *Peptides*. 1997;18(6):885-891.
17. Semenistaya E, Zvereva I, Thomas A, et al. Determination of growth hormone releasing peptides metabolites in human urine after nasal administration of GHRP-1, GHRP-2, GHRP-6, Hexarelin, and Ipamorelin. *Drug Test Anal*. 2015;7(10):919-925.
18. Lippi G, Mattiuzzi C. Misuse of the metabolic modulator meldonium in sports. *J Sport Health Sci*. 2017;6(1):49-51.
19. Gomez C, Pozo OJ, Fabregat A, et al. Detection and characterization of urinary metabolites of boldione by LC-MS/MS. Part I: Phase I metabolites excreted free, as glucuronide and sulfate conjugates, and released after alkaline treatment of the urine. *Drug Test Anal*. 2012;4(10):775-785.
20. Gomez C, Pozo OJ, Marcos J, Segura J, Ventura R. Alternative long-term markers for the detection of methyltestosterone misuse. *Steroids*. 2013;78(1):44-52.
21. Grosse J, Anielski P, Sachs H, Thieme D. Ethylglucuronide as a potential marker for alcohol-induced elevation of urinary testosterone/epitestosterone ratios. *Drug Test Anal*. 2009;1(11–12):526-530.
22. Lu J, Fernandez-Alvarez M, Yang S, et al. New potential biomarkers for mesterolone misuse in human urine by liquid chromatography quadrupole time-of-flight mass spectrometry. *J Mass Spectrom*. 2015;50(1):153-159.
23. Mazzarino M, de la Torre X, Fiacco I, et al. A simplified procedure for the analysis of formoterol in human urine by liquid chromatography-electrospray tandem mass spectrometry: application to the characterization of the metabolic profile and stability of formoterol in urine. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2013;931:75-83.
24. Desrosiers NA, Lee D, Concheiro-Guisan M, et al. Urinary cannabinoid disposition in occasional and frequent smokers: is THC-glucuronide in sequential urine samples a marker of recent use in frequent smokers? *Clin Chem*. 2014;60(2):361-372.
25. Thieme D, Grosse J, Keller L, Graw M. Urinary concentrations of ethyl glucuronide and ethyl sulfate as thresholds to determine potential ethanol-induced alteration of steroid profiles. *Drug Test Anal*. 2011;3(11–12):851-856.
26. Slampova A, Kuban P. Rapid determination of meldonium in urine samples by capillary electrophoresis with capacitively coupled contactless conductivity detection. *J Chromatogr A*. 2016;1468:236-240.
27. Weinmann W, Schaefer P, Thierauf A, Schreiber A, Wurst FM. Confirmatory analysis of ethylglucuronide in urine by liquid-chromatography/electrospray ionization/tandem mass spectrometry according to forensic guidelines. *J Am Soc Mass Spectrom*. 2004;15(2):188-193.
28. Moller I, Thomas A, Geyer H, Schanzer W, Thevis M. Development and validation of a mass spectrometric detection method of peginesatide in dried blood spots for sports drug testing. *Anal Bioanal Chem*. 2012;403(9):2715-2724.
29. Bicker W, Lammerhofer M, Keller T, et al. Validated method for the determination of the ethanol consumption markers ethyl glucuronide, ethyl phosphate, and ethyl sulfate in human urine by reversed-phase/weak anion exchange liquid chromatography-tandem mass spectrometry. *Anal Chem*. 2006;78(16):5884-5892.
30. Gorgens C, Guddat S, Thomas A, et al. Simplifying and expanding analytical capabilities for various classes of doping agents by means of direct urine injection high performance liquid chromatography high resolution/high accuracy mass spectrometry. *J Pharm Biomed Anal*. 2016;131:482-496.
31. Gorgens C, Guddat S, Orlovius AK, et al. "Dilute-and-inject" multi-target screening assay for highly polar doping agents using hydrophilic interaction liquid chromatography high resolution/high accuracy mass spectrometry for sports drug testing. *Anal Bioanal Chem*. 2015;407(18):5365-5379.
32. Musenga A, Cowan DA. Use of ultra-high pressure liquid chromatography coupled to high resolution mass spectrometry for fast screening in high throughput doping control. *J Chromatogr A*. 2013;1288:82-95.
33. WADA Technical Document – TD2017MRPL. World Anti-Doping Agency, 2017. Available: https://www.wada-ama.org/sites/default/files/resources/files/wada-td2017mrpl-en_0.pdf, 2017.
34. Lee KM, Kim HJ, Jeong ES, et al. Simple and accurate quantitative analysis of seven prohibited threshold substances in human urine by liquid chromatography/tandem mass spectrometry in doping control. *Rapid Commun Mass Spectrom*. 2011;25(16):2261-2267.
35. Kim Y, Jeong D, Min H, et al. Method for screening and confirming meldonium in human urine by high-resoluton mass spectrometry and identification of endogenous interferences for anti-doping testing. *MSL*. 2017;8(2):39-43.
36. Ho EN, Kwok WH, Wong AS, Wan TS. Detection of singly- and doubly-charged quaternary ammonium drugs in equine urine by liquid chromatography/tandem mass spectrometry. *Anal Chim Acta*. 2012;710:94-101.
37. Boles TH, Wells MJ. Analysis of amphetamine and methamphetamine in municipal wastewater influent and effluent using weak cation-exchange SPE and LC-MS/MS. *Electrophoresis*. 2016;37(23–24):3101-3108.
38. Zhao M, Wu XJ, Fan YX, Guo BN, Zhang J. Development and validation of a UHPLC-MS/MS assay for colistin methanesulphonate (CMS) and colistin in human plasma and urine using weak-cation exchange solid-phase extraction. *J Pharm Biomed Anal*. 2016;124:303-308.
39. Thomas A, Solymos E, Schanzer W, et al. Determination of Vasopressin and Desmopressin in urine by means of liquid chromatography coupled to quadrupole time-of-flight mass spectrometry for doping control purposes. *Anal Chim Acta*. 2011;707(1–2):107-113.
40. Dai J, Shieh CH, Sheng QH, Zhou H, Zeng R. Proteomic analysis with integrated multiple dimensional liquid chromatography/mass spectrometry based on elution of ion exchange column using pH steps. *Anal Chem*. 2005;77(18):5793-5799.
41. Cai X, Guo Z, Xue X, et al. Two-dimensional liquid chromatography separation of peptides using reversed-phase/weak cation-exchange mixed-mode column in first dimension. *J Chromatogr A*. 2012;1228:242-249.
42. Karlsson D, Jakobsson N, Brink KJ, Axelsson A, Nilsson B. Methodologies for model calibration to assist the design of a preparative ion-exchange step for antibody purification. *J Chromatogr A*. 2004;1033(1):71-82.

43. Min H, Han B, Sung C, et al. LC-MS/MS method for simultaneous analysis of growth hormone-releasing peptides and secretagogues in human urine. *MSL*. 2016;7(3):55-63.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Kim Y, Jeon M, Min H, et al. Development of a multi-functional concurrent assay using weak cation-exchange solid-phase extraction (WCX-SPE) and reconstitution with a diluted sample aliquot for anti-doping analysis. *Rapid Commun Mass Spectrom*. 2018;32:897-905. https://doi.org/10.1002/rcm.8119