Development of Targeted and Non-targeted Analysis for Illegal Doping Drugs by Liquid Chromatography-Mass Spectrometry

Boyoung Han

Department of Medical Science

The Graduate School, Yonsei University

Development of Targeted and Non-targeted Analysis for Illegal Doping Drugs by Liquid Chromatography-Mass Spectrometry

Directed by Professor Je-Wook Yu and Junghyun Son

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Boyoung Han

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연세대학교
YONSEI UNIVERSITY

This certifies that the Master's Thesis
of Boyoung Han is approved.

Thesis Supervisor: Je-Wook Yu

Thesis Committee Member#1: Eun Jig Lee

Thesis Committee Member#2: Junghyun Son

The Graduate School

Yonsei University

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한보영 올림

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ABSTRACT

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Boyoung Han

Department of Medical Science
The Graduate School, Yonsei University

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In 2016, growth hormone-releasing peptides, growth hormone secretagogues, and insulin-like growth factor-1 are listed by the World Anti-Doping Agency as illegal doping drugs based on increasing of its usage in athletes to promote the secretion of growth hormone. This study developed and validated the targeted and non-targeted methods for the simultaneous analysis of illegal doping drugs. First section of this study was focused on qualification analysis (PART 1), targeted method using HPLC-MS/MS for doping control was developed for growth hormone-releasing peptides and growth hormone secretagogues, and validated to selectivity (no interference), linearity (R^2 >0.9986), matrix effects (50.0–141.2%), recovery (10.4–100.8%), and intra- (2.8-16.5%) and inter-day (7.0-22.6%) precisions with the limit of detection (0.05 to 0.5 ng/mL). Furthermore, new non-targeted qualitative method that has a traceability for unknown compounds was developed with growth hormone-related drugs include small chemicals. The feature of non-targeted method is direct application for unknown drugs without additional optimization, and is expected to contribute to next generation of doping test. The method was

validated in terms of selectivity (no interferences), recovery (29-131%), matrix effect (35-237%) and intra- and inter-day precision (%CV lower than 25%) with limit of detection (0.0002 to 100 ng/mL). In second part (PART 2), endogenous insulin-like growth factor-1 that is an endogenous biomarker and increases when an athlete injects growth hormone, was quantitatively analyzed using a calibration curve in human serum. This method was validated with selectivity (no interferences), matrix effect (90-94%), carry-over (lower than 5%) and intra- and inter-day precision (%CV lower than 15%) to target substances (T1 and T2) with limit of detection (10 ng/mL). Newly developed targeted and non-targeted methods were accredited by KOLAS and are playing important role of anti-doping tests in Korea.

Key words: growth hormone, growth hormone-releasing peptides, growth hormone secretagogues, insulin-like growth factor-1, bio-mimetics, targeted, non-targeted, doping, validation

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I. INTRODUCTION

There are an increasing number of cases in which athletes' misuse and abuse drugs for the purpose of improving their performance in all sorts of sports. Thus, the International Olympic Committee has ordered that top athletes be tested for drugs from the 1968 Winter Olympics. Since the establishment of the World Anti-Doping Agency (WADA) in 1999, drug testing has been strengthened and mandatory drug testing is required for all competitions organized by the Sports Federation (https://www.wada-ama.org/en/resources /science-medicine/td2015-mrpl).

The initial stage of doping drugs was mainly composed of stimulants such as Amphetamine and Methamphetamine.[1] Stimulants have the effect of improving athletic ability, such as reduced tiredness, increased agility, and

aggression.[2] As a result, stimulants became the first group of prohibited drugs.[1]

Since the 1950s, anabolic androgenic steroid (AAS) which has the effect of increasing muscle size and strength without training is a dominant group in doping events.[1,3,4] If accompanied by drug intake and training, there is a great effect on muscle fiber hypertrophy. Currently, AAS is detected by the method of identifying the Testosterone/Epitestosterone (T/E) ratio using gas chromatography mass spectrometry (GC-MS) and confirming the $^{13}C/^{12}C$ ratio using gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) to distinguish between endogenous and synthetic agents.[1,4,5]

Besides this, various kinds of small chemical drugs such as beta-2 agonists, hormone and metabolic modulators, diuretics and masking agents, narcotics, cannabinoids, and glucocorticoids etc. are designated as doping prohibited drugs. As for this substance, analysis method using liquid chromatography-coupled mass spectrometry (LC-MS/MS) or GC-MS is used for doping test.

In recent doping events, the use of bio-doping methods such as erythropoiesis stimulating hormone (EPO), growth hormone (GH), growth hormone-releasing hormone (GHRH), growth hormone-releasing peptides (GHRPs), non-peptidyl GH secretagogues (GHSs), insulin, insulin like growth factor 1 (IGF-1), and other proteins or peptides are increasing. As the interest in analytical techniques for detecting bio-doping has increased so much, a variety of analytical techniques have been reported.[6,7] Most of proteins or peptides contained in the International Standard Prohibited List published by WADA are naturally produced in the body. These hormones are known to work in various parts such as cell metabolism, muscle or organ growth, erythropoiesis, and insulin secretion. However, it has been reported to be accompanied by serious side effects such as muscle, joint and bone pain, cardiovascular disease, hypertension, and abnormal cell division.

Human growth hormone (hGH) is a powerful anabolic hormone that reduces body fat, increases muscle mass, and accelerates recovery from sports injuries.[8] For these reasons, hGH is listed on the Prohibited List for competitors "in" and "out" of athletic competitions by the WADA.[9] Despite its prohibition, athletes, particularly in power and endurance sports, often use hGH. However, since 2010, hGH has become detectable using two distinct tests, the hGH isoform immunoassay and hGH biomarker test.[10]

Since Bowers *et al.* reported a GHRP that stimulates hGH secretion,[11] similar GHRPs (GHRP-1, GHRP-2, and Hexarelin) have been developed.[12] In particular, GHRP-2 has been reported as a stimulant in many studies and is used in diagnostic tests for hGH deficiency and is also used to hide the signs of hGH abuse by rapidly reducing the rate of recombinant/pituitary hGH after injection.[13] In addition, nonpeptidyl GHSs, such as Anamorelin[14] and MK-677 (Ibutamoren),[15] have been developed to stimulate hGH release. Thus, these hGH-releasing substances are on the WADA Prohibited List and must be assessed by antidoping laboratories. To date, several methods have been developed for doping control analysis of GHRPs and GHSs using LC-MS/MS,[16] and metabolites of certain GHRPs have been identified by an excretion study.[17] Interestingly, the use of GHRP-1 and Alexamorelin can only be detected by their metabolites excreted in urine.[18] During the 2014 Sochi Winter Olympic Games, GHRPs and GHSs, except Anamorelin, were analyzed in more than 1000 official samples by LC-MS/MS.[19] However, each antidoping laboratory needs to have their evaluation method accredited by International Organization for Standardization (ISO)/International Electrotechnical Commission (IEC) 17025. The aim of the current study was to develop and validate a method for a simultaneous analysis of hGH-releasing substances and their metabolites in human urine samples for doping control testing.

Furthermore, in this study, GHRPs, GHSs, and small chemical drugs were

integrated into one preparation process analyzed by non-target qualitative method with full scan and vDIA mode. This method has advantage of open method that can identify MS/MS information of all the substances present in the sample. Therefore, this method can discover unknown compounds and trace new drugs from the past sample data whereas targeted method cannot discover or trace new drugs. And this method was set up with fixed *m/z* ranges and collision energy (CE), it is a permanent method that does not need to be modified for new drugs. In anti-doping field, the athlete biological passport (ABP) recently progressed that based on individual character by longitudinal monitoring of markers in blood or urine.[20-22] However, despite all these efforts, these analytical methods have limitation that is impossible of retrospective evaluation of the newly known black-market product[23] contrary to non-targeted qualitative method. By the reason, this non-targeted qualitative method overcome limitation described above and it can be effectively used for conducting official new generation doping tests (Fig.1).

IGF-1 is a downstream of hGH that is similar in structure and function to insulin, and is involved in muscle, bone, and tissue growth.[24] In addition, when taken with hGH and insulin, it can have a synergetic effect on protein metabolism.[25] Secretion of hGH from the pituitary promotes the secretion of IGF-1 in the liver and its levels can regulate by hGH intake, IGF-1 is used as biomarkers to detect hGH misuse. However, the level of IGF-1 in blood is different according to age and sex, it is necessary to analyze by quantitative analysis, and using IGF-1 as a biomarker can be a weak point because it is also high in people with diseases such as acremegaly.[26] Nevertheless, quantitative method using mass spectrometry has become a powerful method than conventional immunoassay method[27] because immunoassay has disadvantage in that there is a large deviation among laboratories from instruments, reagents, and method.[28]

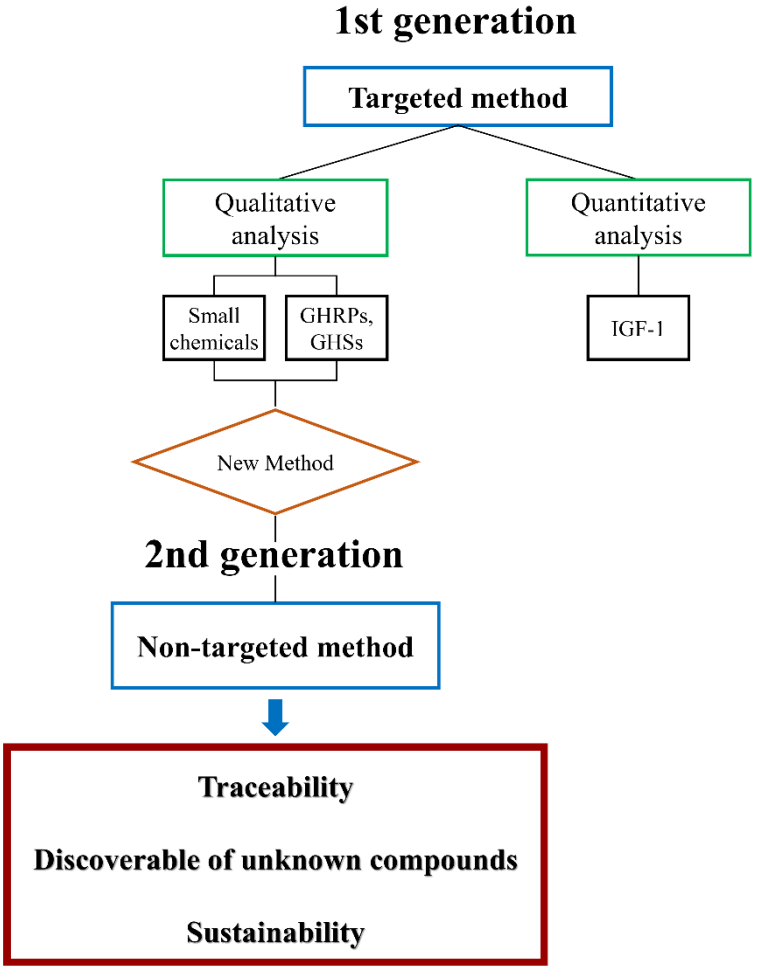


Figure 1. Overall scheme of this study. In this study, targeted methods that composed of qualitative analysis for GHRPs and GHSs and quantitative analysis for IGF-1 were validated. Also, non-targeted method for GHRPs, GHSs, and small chemicals was developed and validated to propose a new method of doping analysis.

In this study, the appropriate preparation and analytical methods for each analyte are explained by each chapter. Figure 1 describes overall strategy of this study. The analytical equipment was detected by applying high performance liquid chromatography-mass spectrometry (HPLC-MS/MS), high performance liquid chromatography-high resolution mass spectrometry (HPLC-HRMS) and nano flow liquid chromatography-high resolution mass spectrometry (nanoLC-HRMS). Development of analytical method using HRMS is expected to accurately distinguish and quantify the *m/z* difference between endogenous protein and synthetic protein to the fifth decimal point which could not be done in the previous analysis method.

The purpose of this study is to develop a targeted and non-targeted method for the analysis of major illegal drugs. Each antidoping laboratory needs to have their evaluation method accredited by ISO/IEC 17025. So, all analytical methods were evaluated for effectiveness according to Korea Laboratory Accreditation Scheme (KOLAS), so that it could be used for actual doping test in the future.

II. MATERIALS AND METHODS

1. Materials

GHRP-2 and GHRP-6 were purchased from ProSpec (East Brunswick, NJ, USA). GHRP-1, -4, -5, hexarelin and ipamorelin were purchased from Abbiotec (San Diego, CA, USA). Ibutamoren was purchased from Axon Medchem (Groningen, Netherlands). Alexamorelin and metabolites of all GHRPs were synthesized by Peptron (Daejeon, South Korea). Anamorelin was purchased from MedChem Express (Princeton, NJ, USA). Heavy isotope-labeled [(^{13}C, ^{15}N) Lys] GHRP-2 and -6 used as internal standards (ISTD) were synthesized by JPT Peptide Technologies (Berlin, Germany). All of small chemical drugs standard substances were purchased from various pharmaceutical suppliers in pharmaceutical purity. Ethyltheophilline used as ISTD for small chemicals was purchased from Sigma-Aldrich (St. Louis, MO, USA). IGF-1 was purchased from NIBSC (Potters Bar, UK), IGF-2 was purchased from Sigma-Aldrich (St. Louis, MO, USA), heavy isotope-labeled (^{15}N) IGF-1 was purchased from ProSpec (East Brunswick, NJ, USA). Rat serum, ammonium bicarbonate (NH_4HCO_3), iodoacetamide (IAA), glacial acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). The enzyme trypsin was purchased from Promega (Madison, WI, USA), DL-Dithiothreitol (DTT) purchased from VWR (West Chester, PA, USA). The enzyme β-glucuronidase was purchased from Roche Diagnostics (Mannheim, Germany). HPLC grade acetonitrile and methanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). Formic acid was purchased from Wako Pure Chemicals (Osaka, Japan), and phosphoric acid was purchased from Yakuri

Pure Chemicals (Kyoto, Japan). Ammonium hydroxide, citric acid monohydrate and sodium citrate tribasic dehydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was produced by Milli-Q water purification system (Millipore, Bedford, MA, USA).

2. Instrumentations

A. PART 1: Targeted qualitative method by HPLC-MS/MS

The Shimadzu Prominence LC-20A series reverse-phase LC system comprised LC-20AD XR binary pump system, SIL-20AC XR autosampler, CTO-20AC column oven and DUG-20AS degasser (Shimadzu, Kyoto, Japan). Chromatographic separation was performed on a Kinetex C18 column (100 mm × 2.1 mm, 2.6 μm; Phenomenex, Torrance, CA, USA). Mobile phase-A comprised 0.1% formic acid in water, whereas mobile phase-B contained 0.1% formic acid in acetonitrile. The column oven was set at 35 °C, and the flow rate was 500 μL/min. Gradient elution was completed as follows: isocratic flow in 5% mobile phase-B for 0.5 min, 5% mobile phase-B linear to 95% mobile phase-B for 5 min, isocratic flow in 95% mobile phase-B for 1 min, followed by isocratic flow in 5% mobile phase-B for 0.1 min and equilibration for 1 min (total run time, 8 min).

The HPLC system was coupled to a TSQ ultra triple stage quadrupole mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with a heated electrospray ionization source. Ion spray voltages were set at 4.5 kV in positive mode. The capillary and vaporizer temperatures were set at 320 °C. The sheath gas flow rate was 60 arbitrary units, and the auxiliary gas flow rate was 20

arbitrary units. The tube lens voltage was automatically optimized by the instrument calibration procedure with a solution of polytyrosine-1, 3, 6 (CS Bio, Menlo Park, CA, USA) according to the manufacturer's instructions. Selected reaction monitoring (SRM) mode was applied for analysis using precursor/product ion information (Fig. 2). All the data were acquired using Xcalibur™ software (Thermo Scientific, San Jose, CA, USA), and data processing was performed using Skyline software (University of Washington).

B. PART 1: Non-targeted qualitative method by HPLC-HRMS

The Shimadzu Prominence LC-20A series reverse-phase LC system comprised an LC-20AD XR binary pump system, SIL-20AC XR autosampler, CTO-20AC column oven and a DUG-20AS degasser (Shimadzu, Kyoto, Japan). Chromatographic separation was performed on a Kinetex C18 column (100 mm × 2.1 mm, 2.6 μm; Phenomenex, Torrance, CA, USA). Mobile phase-A comprised 0.1% formic acid in water, whereas mobile phase-B contained 0.1% formic acid in methanol. The column oven was set at 35 °C, and the flow rate was 500 μL/min. Gradient elution was completed as follows: isocratic flow in 2% mobile phase-B for 0.5 min, 2% mobile phase-B linear to 95% mobile phase-B for 8 min, isocratic flow in 95% mobile phase-B for 0.5 min, followed by isocratic flow in 2% mobile phase-B in 0.01 min, and equilibration for 1 min (total run time, 10 min).

The HPLC system was coupled to a Q-Exactive plus mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with a heated electrospray ionization source. Ion spray voltages were set at 4.5 kV in positive mode. The capillary and vaporizer temperatures were set at 320 °C and 300 °C, respectively. The sheath gas flow rate was 50 arbitrary units, and the auxiliary gas flow rate was 30 arbitrary units. The S-lens RF level was 60. Full scan and

variable data-independent acquisition (vDIA) mode was applied for analysis (Fig. 2). All the data were acquired using Xcalibur™ software (Thermo Scientific, San Jose, CA, USA), and data processing was performed using TraceFinder™ software (Thermo Scientific, San Jose, CA, USA).

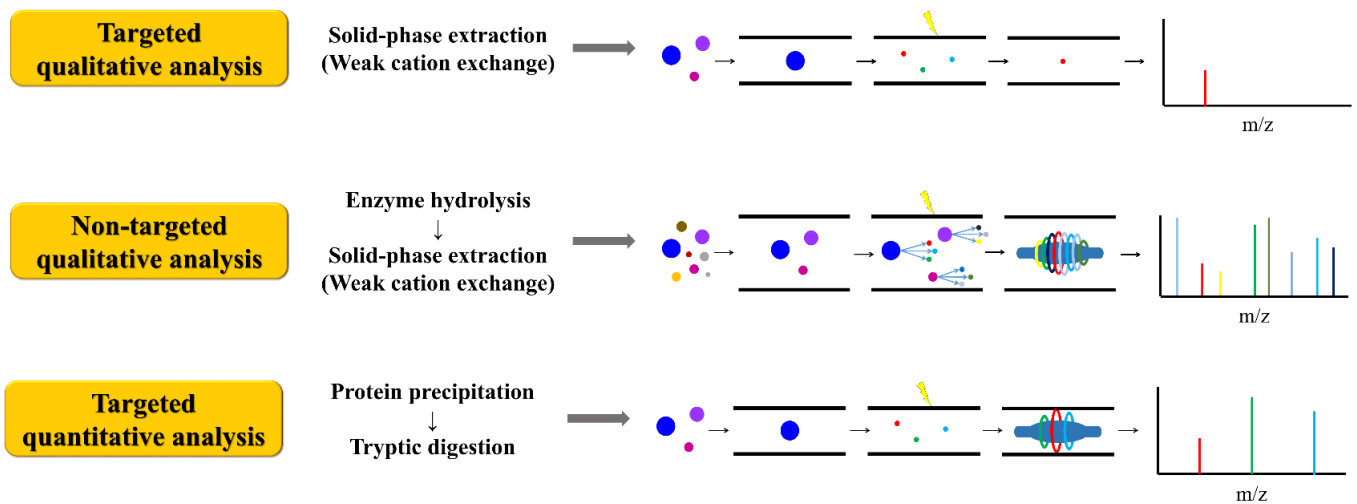


Figure 2. Overall strategy of each method. Each method was pretreated with solid-phase extraction, enzyme hydrolysis followed by solid-phase extraction, and protein precipitation followed by tryptic digestion. The concentrated and purified samples were analyzed using selected reaction monitoring (SRM), variable data independent acquisition (vDIA), and parallel reaction monitoring (PRM) mode respectively. In these three analysis methods, SRM and PRM mode must know the information of the substances to be filtered, but vDIA mode can be performed without knowing the information of each substance. Also, it is easy to analyze at low concentration in vDIA mode because this method analyze all ranges of samples.

C. PART 2: Quantitative method of IGF-1 by nanoLC-HRMS

Thermo Scientific™ Dionex™ Ultimate™ 3000 RSLCnano system (Thermo Scientific, San Jose, CA, USA) coupled to a Thermo Scientific™ Acclaim™ PepMap™ RSLC C18 column (75 μm × 15 cm, 3 μm; Thermo Scientific, San Jose, CA, USA) and a Thermo Scientific™ Acclaim™ PepMap™ 100 trap column (75 μm × 2 cm, 3 μm; Thermo Scientific, San Jose, CA, USA). Mobile phase-A comprised 0.1% formic acid in water, whereas mobile phase-B contained 0.1% formic acid in acetonitrile. Buffer-A comprised 0.1% formic acid/3% acetonitrile in water. The targets were loaded on the trapping column with 99% of buffer-A at a flow of 5 μL/min for 5 min. The gradient started at 1% of B, raised to 40% of B in 20 min, raised to 95% of B within 5 min, isocratic flow in 95% mobile phase-B for 5 min, followed by 0.1 min isocratic flow in 1% mobile phase-B and was followed by re-equilibration for 20 min. The flow rate was set to 0.3 μL/min.

The nanoLC system was coupled to a Q-Exactive mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with a nano-heated electrospray ionization source. Ion spray voltages were set at 2.2 kV in positive mode. The capillary temperatures were set at 275 °C. The sheath gas, auxiliary gas, sweep gas flow rate was 0 arbitrary units. The S-lens RF level was 65. Parallel reaction monitoring (PRM) mode was applied for analysis (Fig. 2). All the data were acquired using Xcalibur™ software (Thermo Scientific, San Jose, CA, USA), and data processing was performed using Skyline software (University of Washington).

3. Sample preparations

A. PART 1: GHRPs, GHSs and small chemical drugs

Urine samples were prepared as a modified method previously described.[29] Briefly, each urine sample (2 mL) was spiked with 10 μL of ISTD solution. Adjust pH 6.0 adding 1 mL of citrate buffer (0.1 mol/L, pH 6.0). Then add 50 μL of β-glucuronidase for enzyme hydrolysis. And sample was incubated at 55 ℃ for 1 h on water bath. After incubation step, sample was spiked with 100 μL of 4% phosphoric acid and then centrifuged for 5 min at 3,000 g. Mixed-mode weak cation exchange (WCX) cartridges (60 mg; Waters, Milford, MA, USA) were conditioned with 2 mL of methanol and 2 mL of water before applying samples. After apply samples, wash with 2 mL of water and 2 mL of methanol. And then, target substances were eluted with 1 mL of elution buffer comprising 2% ammonia and 1.2% formic acid in methanol. Extracts were evaporated to dryness under an N_2 stream at 50 ℃, reconstituted in 200 μL of the reconstitution buffer mixture (1% formic acid in 95% A/5% B), and then injected (10 μL aliquot) into the HPLC-MS/MS and HPLC-HRMS system.

B. PART 2: IGF-1

The IGF-1 calibration standards were prepared in a matrix of rat serum purchased from Sigma-Aldrich. IGF-1 stock solution (10 μg/mL) was spiked in rat serum to concentrations ranging from 0 to 1000 μg/mL. The low quality control sample (QC_{low}) was 200 μg/mL and high quality control sample (QC_{high}) was 600 μg/mL.

Transfer calibration standards, QCs, and samples 100 μL in Eppendorf® Protein LoBind™ tubes (Stevenage, UK), respectively. 2 μL of IGF-2 (200 ng) and 5 μL of N15-IGF-1 (20 ng) were spiked and then 200 μL of 1% acetic acid was added to each tube followed by vortexing. The next, 400 μL of acetonitrile was added for protein precipitation followed by gentle shaking (900 rpm) with Thermomixer® (Eppendorf®, Stevenage, UK) for 10 min at room temperature followed by further vortexing (30 s). The protein precipitate was pelleted by centrifugation at 13,000 g for 10 min at 4 °C in a fixed rotormicro-centrifuge (Eppendorf®). The supernatant (600 μL) was transferred to a fresh Protein LoBind™ tube and evaporated to dryness in a vacuum centrifuge (Speed Vac Thermo Scientific, San Jose, CA, USA) with heating at 40 °C. Reconstitute each sample with 50 μL of 100 mM NH_4HCO_3 (ammonium bicarbonate). Add 5 μL of 100 mM DTT and mixing (900 rpm) in the Thermomixer for 10 min at 60 °C followed by incubated for 50 min more for reduction. And then, add 5 μL of 250 mM IAA and incubate in the dark for 30 min at room temperature for alkylation. After that, samples were incubated in the Thermomixer at 37 °C, 14,000 rpm for overnight with trypsin (400 ng). Finally, add 10 μL of 1% formic acid to stop digestion and centrifugation at 13,000 g for 10 min at 4 °C. Transfer 60 μL of supernatants to vials and injected (2 μL aliquot) into nanoLC-HRMS system.

4. Validation

In PART 1, analysis method of GHRPs, GHSs, and small chemical drugs was validated for urine under consideration of the following parameters: Selectivity, recovery, matrix effect, limit of detection (LOD), and precision. The selectivity of the method was estimated by analysis of five positive control

urine (PCU) and five negative control urine (NCU) samples. PCU samples were spiked with the working reference and internal standard (ISTD) solutions, whereas NCU samples were spiked only with ISTD solution. Interference signals from endogenous substances were considered on the chromatograms at the expected retention times. For determining recovery, urine samples were prepared in two ways. Five samples were spiked before WCX extraction with the working reference solution in blank urine, whereas another five samples were spiked after extraction with the working reference solution. ISTD solution was added to both sample groups before extraction. For determining matrix effect, samples were prepared in two ways. Five samples were spiked before WCX extraction with the working reference solution in blank urine, whereas another five samples were spiked in blank buffer with the working reference solution. ISTD solution was added to both sample groups before extraction. All samples with working reference solution were spiked with minimum required performance level (MRPL) concentration. Intra- and inter-day precisions were determined by analyzing five replicates of each PCU sample per day on three independent days. PCU samples were prepared for low (50% of MRPL), middle (MRPL), high (200% of MRPL) concentration. The results were expressed as the coefficient of variation (%CV) of the peak areas. LOD were defined as the lowest concentration with a signal-to-noise ratio of >3 and %CV of <25%. For determination of the LOD, five PCU samples were analyzed at each of six concentration points (MRPL, 50% of MRPL, 20% of MRPL 10% of MRPL, 2% of MRPL, 1% of MRPL and 0.1% of MRPL for each group of substances).

In PART 2, analysis of IGF-1 calibration standards, the method was validated for rat serum under consideration of the following parameters: Selectivity, matrix effect, linearity, limit of detection, precision and carry over. The specificity of the method was estimated by analysis of five positive control serum (PCS) and five negative control serum (NCS) samples. PCS samples

were spiked with the working reference and ISTD solutions, whereas NCS samples were spiked only with ISTD solution. Interference signals from endogenous substances were considered on the chromatograms at the expected retention times. If the highest signal obtained had interference, the second signal was considered as the qualifier ion. For determining matrix effect, samples were prepared in two ways. Five samples were spiked with the working reference solution in blank rat serum, whereas another five samples were spiked in blank buffer with the working reference solution. ISTD solution was added to both sample groups before extraction. Calibration curves for linearity were analyzed at eleven concentration points (5, 10, 25, 50, 100, 200, 400, 600, 1000, 1500 and 2000 ng/mL of substances); three urine samples were analyzed at each concentration point. Intra- and inter-day precisions were determined by analyzing six replicates of each PCS sample per day on three independent days. The results were expressed as the coefficient of variation (%CV) of the peak areas. LOD were defined as the lowest concentration with a signal-to-noise ratio of >3 and %CV of <25%. For determination of the LOD, five PCS samples were analyzed at each of six concentration points (0.5, 1, 2, 5, 10 and 50 ng/mL of substances).

III. RESULTS

In this study, GHRPs, GHSs, and small chemicals were analyzed by targeted qualitative method and non-targeted qualitative method. IGF-1 was analyzed by targeted quantitative method. Each method was optimized and validated. These results were explained PART 1 and PART 2 separately.

1. PART 1: Analysis of GHRPs, GHSs and small chemical drugs

A. Optimization targeted qualitative method

Prior to SRM assay, this study evaluated possible product ions by direct infusion with each reference stock (Fig. 3 (A)), and three product ions per target were selected (Table 1). Then, for higher sensitivity, the collision energy (CE) was optimized by Skyline software. An additional optimization process through the Skyline is based on direct infusion raw data. Skyline set the predicted optimal CE that matches each product ion and apply four steps from the predicted optimal CE to obtain the second optimal CE (Fig. 3 (B)). Finally, apply one steps from second optimal CE, and precisely dispose the CE showing the highest peak area (Fig. 3 (C)). SRM assay using optimized CE, peak area of all target substances was increased by 10-30% (Fig. 4). The selected charge state of the precursor ion and three product ions with optimized CE are shown in Table 1. The representative SRM chromatograms at the MRPL (2 ng/mL)[25] for the qualitative of 10 substances and their metabolites are shown in Fig. 5. All target analytes can be detected with sufficient peak intensities at the 50% of MRPL concentrations and achieve complete chromatographic separation to

exclude false-positives/false-negatives or misinterpretation. These results indicated that the present method is acceptable for routine drug testing in doping control.

Table 1. HPLC-MS/MS mass spectrometry parameters for 27 substances

Name	Precursor charge state	Precursor ion (m/z)	Product ion (m/z)	Collision energy (V)	Retention time (min)
			84	34	
GHRP-1	2	478	129	21	3.0
			209	20	
			110	35	
GHRP-1-M1	2	443	129	21	3.0
			335	29	
			153	35	
GHRP-1-M2	2	374	170	18	3.2
			479	10	
			110	20	
GHRP-1-M3	2	443	147	16	3.2
			307	23	
			159	33	
GHRP-1-M4	1	620	335	27	3.8
			352	17	
			170	28	
GHRP-2	2	410	269	12	3.3
			550	8	
			153	37	
GHRP-2-M1	2	358	170	22	3.1
			269	10	
			153	32	
GHRP-2-M2	2	410	170	20	3.4
			551	13	
			159	35	
GHRP-4	1	608	351	19	3.5
			444	19	
			159	31	
GHRP-4-M	1	462	230	18	3.2
			258	10	
			258	33	
GHRP-5	1	771	350	34	3.6
			421	27	
			191	22	
GHRP-5-M1	1	439	322	18	3.0
			350	14	
			322	28	
GHRP-5-M2	1	625	350	23	3.4
			421	17	
			129	21	
GHRP-6	2	437	296	25	2.8
			324	25	

			84	34	
GHRP-6-M1	2	369	129	20	3.1
			346	10	
			159	39	
GHRP-6-M2	1	609	335	27	3.8
			352	19	
			110	29	
Hexarelin	2	444	129	21	2.9
			338	25	
			110	32	
Hexarelin-M	2	427	273	20	2.4
			310	20	
			84	35	
Ipamorelin	2	357	129	16	2.6
			223	16	
			166	30	
Ipamoreli-M	1	585	223	22	3.1
			420	22	
			129	22	
Alexamorelin	2	480	209	22	2.9
			813	14	
			110	27	
Alexamorelin-M1	2	445	130	27	3.0
			147	21	
			144	38	
Alexamorelin-M2	1	623	335	24	3.7
			352	16	
			174	37	
Anamorelin	1	547	202	33	3.9
			276	22	
			235	24	
Ibutamoren	1	529	263	17	3.7
			267	21	
			137	25	
ISTD 1	2	414	153	28	3.3
			170	24	
			110	28	
ISTD 2	2	441	159	26	2.8
			248	31	

GHRP, growth hormone-releasing peptide; M1/M2, metabolite 1/2, ISTD, internal standards

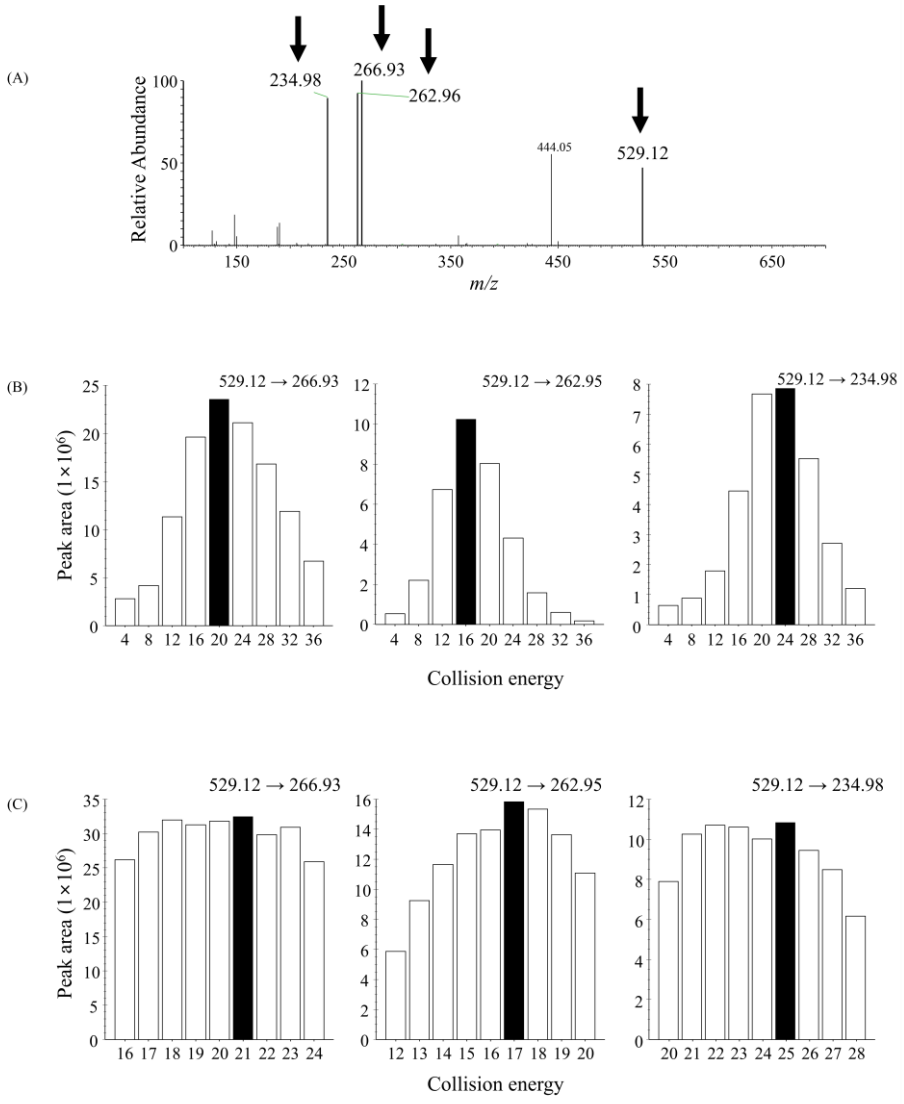


Figure 3. CE optimization of Ibutamoren. Collision energy (CE) decided through direct infusion and additional optimization process through Skyline program. (A) is a direct infusion result and precursor ions and product ions pair (*m/z* 529.12 → 266.93, 262.95, and 234.98) of Ibutamoren. (B) is results of process for obtain second optimal CE of each product ion. For first product ion (*m/z* 266.93), CE was decided to 20, for second product ion (*m/z* 262.95), CE was decided to 16, and for third product ion (*m/z* 234.98), CE was decided to 24. (C) is result of final CE value of each product ion. For first product ion (*m/z* 266.93), CE was decided to 21, for second product ion (*m/z* 262.95), CE was decided to 17, and for third product ion (*m/z* 234.98), CE was decided to 25.

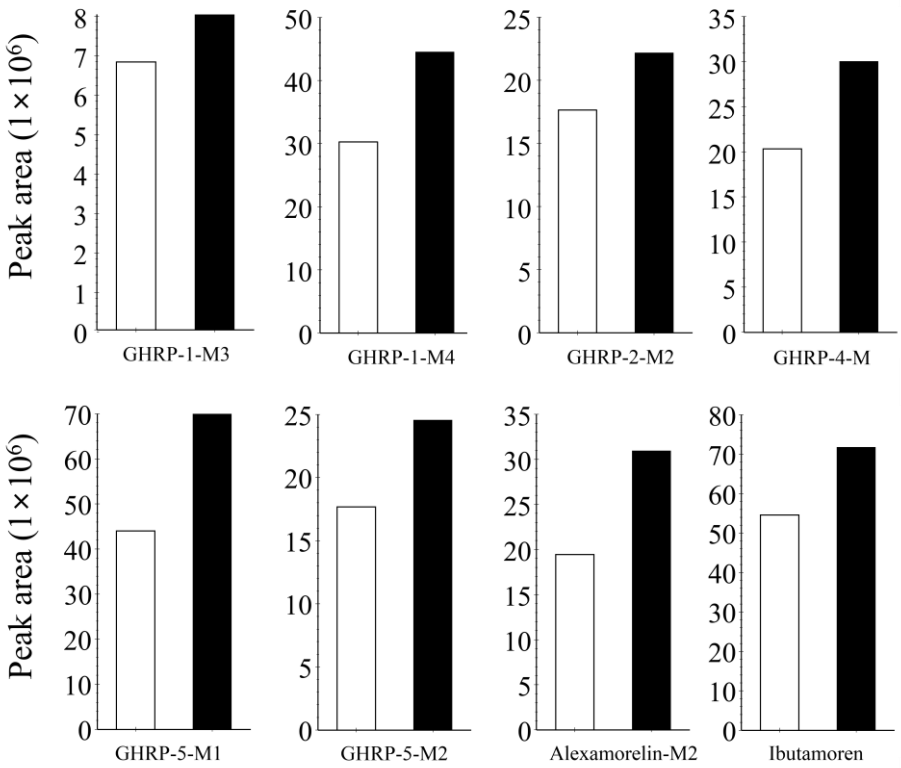


Figure 4. The result of optimized CE values of targets. The white bar is the result before optimization and the black bar is the result after optimization. Selected reaction monitoring (SRM) assay using optimized collision energy (CE), peak area of all target substances was increased by 10-30%.

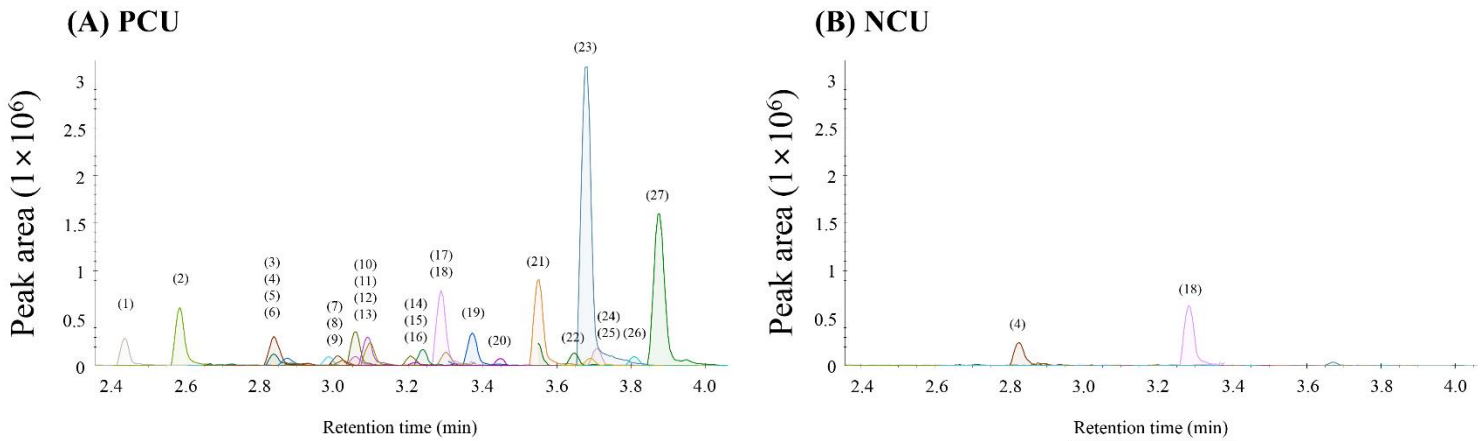


Figure 5. Extract ion chromatograms of 25 substances and 2 internal standards (ISTDs) in positive control urine (PCU) and negative control urine (NCU). (1) Hexarelin-M, (2) Ipamorelin, (3) GHRP-6, (4) ISTD 2, (5) Alexamorelin, (6) Hexarelin, (7) GHRP-5-M1, (8) GHRP-1-M1, (9) GHRP-1, (10) GHRP-2-M1, (11) Alexamorelin-M1, (12) Ipamorelin-M, (13) GHRP-6-M1, (14) GHRP-1-M2, (15) GHRP-1-M3 (16) GHRP-4-M, (17) ISTD 1, (18) GHRP-2, (19) GHRP-2-M2, (20) GHRP-5-M2, (21) GHRP-4, (22) GHRP-6 M2, (23) Ibutamoren, (24) Alexaremolin-M2, (25) GHRP-5, (26) GHRP-1-M4, and (27) Anamorelin.

B. Optimization non-targeted qualitative method

In overall strategy of non-targeted qualitative method (Fig. 2), there are explained about preparation step and analysis step. The process of enzyme hydrolysis that removes glucuronide is necessary because drug enters the body, it goes through metabolism such as glucuronidation. After enzyme hydrolysis step, samples were concentrated by extraction such as liquid-liquid extraction (LLE) or solid phase extraction (SPE). Small chemical drugs currently uses a method of extracting by applying LLE twice at acidic pH and basic pH in order to pretreat as many substances as possible at the same time. This method is poor detection limits for some of compounds and requires frequent modification as additional substances acquire "prohibited" status. This is time consuming and requires a lot of human resources. Therefore, in this study, small chemical drugs, GHRPs, and GHSs were integrated with SPE method to reduce preparation time. Finally, about 111 target substances extracted by SPE method were optimized (Table 2), and samples are analyzed via HPLC-HRMS instrument. Some substances were not fragmented with CE 40, so these substances were analyzed with only a precursor ion *m/z* value.

Prior to non-target qualitative method, this study preceded the process to prove whether the target substances were detected correctly by the non-target qualitative method. 111 target substances were individually analyzed using data-dependent MS/MS (ddMS/MS) method, and *m/z* of precursor ion and product ion were confirmed for each target substance. And then, this study developed and validated a non-target qualitative method that combines full scan and vDIA mode using HRMS. Full scan was optimized with 35,000 resolution (automatic gain control (AGC) target: 5.00E+06, maximum injection time (IT): 50 ms) without fragmentation and this event covered mass range from m/z 100 to 800.

In vDIA mode, fixed m/z range (*m/z* 100-800) was divided into five events (*m/z* 100-200, 200-300, 300-400, 400-500, and 500-800). Four vDIA events isolation window were 100 Da and fifth vDIA event isolation window was 300 Da. These five vDIA events were performed at a resolution of 17,500 (AGC target: 1.00E+05, maximum IT: 30 ms). Analysis with these optimized methods showed that the data points of the non-targeted qualitative method was sufficient compared to the targeted qualitative method (Fig. 6). So, this six scan events were constructed in the analysis of 111 substances. This non-targeted qualitative method with full scan and vDIA mode has MS/MS information of all the substances present in the sample, so no later modification is required. The mixture of all target substances without direct infusion process was analyzed by the vDIA mode and data was confirmed through comparison with ddMS/MS mode results.

Table 2. HPLC-HRMS parameters for 111 substances

N	Name	Precursor ion (*m/z*)	Product ion (*m/z*)	Retention time (min)
1	Anamorelin	547.33957	174.12810	6.8
2	GHRP-1	478.25072	129.10257	5.2
3	GHRP-1-M	443.22394	110.07169	5.8
4	GHRP-2	409.72128	170.09676	5.8
5	GHRP-2-M	410.21300	170.09662	6.2
6	GHRP-4	608.29833	159.09192	6.1
7	Ibutamoren	529.24823	91.05492	6.3
8	Ipamorelin	356.70029	110.07179	4.0
9	5'-Hydroxyphenyl Carvedilol	423.19064	240.12245	5.4
10	Acebutolol	337.21145	116.10738	4.4
11	Alprenolol	250.17986	173.09618	5.5
12	Atenolol	267.16993	190.08652	2.6
13	Befunolol	292.15387	215.06987	4.5
14	Betaxolol	308.22149	116.10733	5.6
15	Bisoprolol	326.23197	116.10731	5.2
16	Bufuralol	262.17993	188.10672	5.8
17	Bupranolol	272.14090	216.07828	5.7
18	Carteolol	293.18543	237.12354	3.6
19	Carvedilol	407.19524	100.07624	6.0
20	Celiprolol	380.25327	251.10257	4.9
21	Desmethylcarvedilol	393.17925	210.11250	5.6
22	Esmolol	296.18515	219.10162	4.7
23	Labetalol	329.18534	162.05510	5.1
24	Levobunolol	292.19018	236.12828	4.5
25	Metipranolol	310.20067	191.10675	5.4
26	Metoprolol	268.19032	191.10672	4.4
27	Nadolol	310.20065	201.09111	3.8
28	Nebivolol	406.18170	151.05522	6.5
29	Oxprenolol	266.17473	116.10736	4.9
30	Penbutolol	292.22655	236.16451	6.6

31	Pindolol	249.15937	116.10738	3.4
32	Propranolol	260.16415	183.08069	5.4
33	Sotalol	273.12622	213.06941	2.3
34	Timolol	317.16379	74.06075	4.3
35	Clenbuterol	277.08658	203.01396	4.7
36	Methyltrienolone	285.18524	-	7.2
37	Bambuterol	368.21727	294.14474	5.0
38	Brombuterol	366.98239	292.91003	4.6
39	Cimaterol	220.14438	160.08678	2.3
40	Cimbuterol	234.15994	160.08672	2.9
41	Clenpenterol	291.10208	203.01331	4.7
42	Clenproperol	265.06784	205.01051	3.7
43	Fenoterol	304.15367	135.08073	3.2
44	Indaterol	393.21667	173.13240	6.3
45	Mabuterol	311.11270	237.03952	4.6
46	Mapenterol	325.12848	237.03966	5.2
47	Olodaterol	387.19037	163.11148	5.0
48	Pirbuterol	241.15451	167.08128	2.3
49	Procaterol	291.17001	231.11246	3.1
50	Reproterol	402.17640	221.10298	3.2
51	Ritodrine	288.15912	121.06490	3.3
52	Salmeterol	416.27828	91.05480	6.7
53	Terbutaline	226.14346	152.07072	2.4
54	Tulobuterol	228.11482	154.04153	4.5
55	Vilanterol	486.18039	158.97617	6.4
56	Raloxifene	474.17286	112.11253	5.8
57	Trimetrazidine	267.16987	-	2.6
58	Amiloride	230.05481	60.05646	3.0
59	Triamterene	254.11445	237.08861	4.1
60	Phenylephrine	168.10165	150.09163	0.9
61	2, 5-dimethoxy-4-bromophenethylamine	260.02792	227.97768	4.7
62	3, 4-Mthylenedioxyethylamfetamine	208.13323	163.07513	3.7
63	Benzylpiperazine	177.13850	85.07672	1.0
64	Benzphetamine	240.17436	91.05482	5.1
65	Dimethylamphetamine	164.14331	91.05487	3.2

66	Ethylamphetamine	164.14331	91.05487	3.6
67	Etilefrine	182.11738	164.10715	1.4
68	Fencamfamine	216.17468	67.05498	5.1
69	Fencamine	385.23329	236.11439	4.3
70	Fenetylline	342.19180	91.05486	4.6
71	Fenfluramine	232.13057	159.04184	5.0
72	Heptaminol	146.15376	128.14374	2.3
73	Methylenedioxyamphetamine	180.10159	133.06509	3.3
74	Methylenedioxymethamphetamine	194.11744	135.04427	3.4
75	Mefenorex	212.11990	91.05480	4.5
76	Mephedrone	178.12239	160.11166	3.1
77	Mephentermine	164.14334	133.10149	3.8
78	Methoxyphenamine	180.13812	149.09639	3.9
79	Methylephedrine	180.13815	162.12796	2.7
80	Methylphenidate	234.14861	84.08141	4.4
81	Norephedrine	152.10681	117.07035	2.2
82	Norfenefrine	154.08600	136.07544	0.8
83	Norfenfluramine	204.09932	159.04179	4.8
84	Octopamine	154.08606	136.07549	0.6
85	Ortetamine	150.12758	105.07043	3.9
86	Oxilofrine	182.11734	164.10733	1.0
87	Phenmetrazine	178.12256	-	3.1
88	Pholedrine	166.12245	135.08072	2.1
89	p-Methylamphetamine	150.12762	133.10143	4.1
90	p-OH-amphetamine	152.10676	107.04932	2.0
91	Prenylamine	330.22163	91.05479	6.8
92	Prenylamine-M	212.14323	117.07033	5.6
93	Prolintane	218.19019	91.05482	4.8
94	Sibutramine-M	252.15107	125.01569	6.8
95	Strychnine	335.17465	184.07542	3.7
96	Hydromorphone	286.14335	-	2.3
97	Methadone	310.21609	105.03397	6.3
98	Norfentanyl	233.16470	177.13882	4.1
99	Oxycodone	316.15400	298.14383	3.0
100	Oxymorphone	302.13825	284.12833	2.1

101	Pentazocine	286.21628	218.15410	5.0
102	Pethidine	248.16436	174.12782	4.6
103	Flumethasone	411.19753	-	6.8
104	Cathine	152.10681	134.09671	2.4
105	Ephedrine	166.12247	148.11230	2.5
106	Morphine	286.14322	201.09058	1.7
107	Pseudoephedrine	166.12246	148.11234	2.7
108	Codeine	300.15881	215.10623	2.8
109	Hydrocodone	300.15880	199.07478	3.2
110	Pipradol	268.16910	-	5.0
111	Tramadol	264.19532	58.06596	4.3

GHRP, growth hormone-releasing peptide; M1/M2, metabolite 1/2

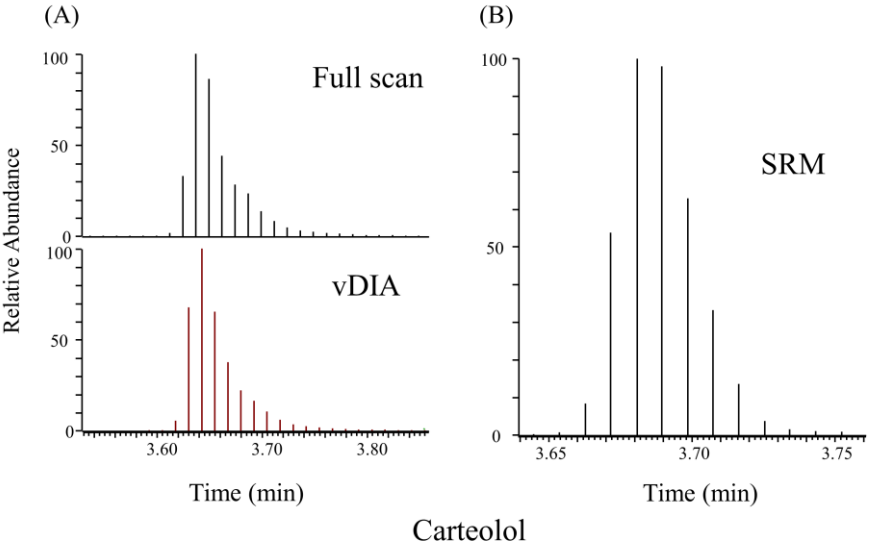


Figure 6. Data peaks of non-targeted qualitative and targeted qualitative method. (A) is non-target screening method data of Carteolol. The upper row is full scan data and the bottom row is variable data independent acquisition (vDIA) mode data. (B) is target screening method using selected reaction monitoring (SRM) mode data of Carteolol. Compared to (A) and (B), data points were sufficient to analyze 111 target substances.

C. Validation

In case of targeted qualitative method, GHRPs and GHSs validated with parameters such as selectivity, recovery, matrix effect, intra- and inter-day precision, and LOD (Table 3). The results of selectivity showed no observable interference signal from endogenous substances at the expected retention time. The results of recovery, almost all target substances were extracted with 50-100% recovery. GHRP-4 and GHRP-5 were detected without loss of signal by extraction but six substances showed low recovery for preparation step. Specially, GHRP-2 M1 and GHRP-6 M2 showed only 20.5% and 10.4% of recovery, but these 2 substances were adequately detected at MRPL concentrations despite their low recovery. Thus, the recovery of the method is adequate for all analytes. The results of matrix effect, five substances, GHRP-2 M1, GHRP-6 M1, GHRP-6 M2, hexarelin M, and ipamorelin M, showed ion suppression signal with less than <70% in matrix compared with the signal in buffer, whereas 3 substances such as GHRP-1 M3, anamorelin, and ibutamoren were significantly increased by >10%. The results of intra- and inter-day precision, the relative retention times per day exhibited a %CV of <0.1% for each group and the %CV for the peak area of qualifier ions was almost <15% (Table 3). For inter-day precision, relative retention time and peak area of 18 PCU samples from 3 days were calculated. The %CV of all target substances were <25% (Table 3). Finally, for determination of LOD, a series of diluted reference substances ranging from 0.05 to 2 ng/mL in PCU samples were prepared and then performed SRM analysis of five samples per concentration point. The LOD of all substances was <50% of the MRPL (1 ng/mL). In particular, seven substances could be detected at the 0.05 ng/mL concentration point with a %CV of <25% and signal-to-noise ratio of >3.

Table 3. Method validation results of targeted qualitative method: matrix effect, recovery, LOD, and precision

Name	Matrix effect (%)	Recovery (%)	LOD (ng/mL)	Intra-day precision %CV	Inter-day precision %CV
GHRP-1	77.3	57.3	0.50	6.9	22.6
GHRP-1-M1	102.7	48.0	0.50	11.0	16.9
GHRP-1-M2	84.8	62.1	0.10	11.3	18.9
GHRP-1-M3	110.7	82.4	0.20	6.2	18.2
GHRP-1-M4	71.3	38.8	0.10	7.8	9.8
GHRP-2	98.9	75.1	0.20	7.9	13.8
GHRP-2-M1	50.0	20.5	0.10	9.4	18.6
GHRP-2-M2	79.6	93.8	0.05	6.2	7.0
GHRP-4	71.7	100.8	0.05	4.9	13.0
GHRP-4-M	73.6	60.6	0.10	10.1	14.6
GHRP-5	101.1	100.3	0.20	14.1	19.1
GHRP-5-M1	85.0	54.1	0.10	10.0	18.6
GHRP-5-M2	77.0	21.0	0.50	12.1	14.0
GHRP-6	103.0	57.7	0.10	5.6	18.0
GHRP-6-M1	59.3	61.0	0.20	5.0	13.2
GHRP-6-M2	64.9	10.4	0.50	16.5	18.8
Hexarelin	86.7	65.5	0.10	7.1	15.7
Hexarelin-M	65.9	89.1	0.05	6.2	20.5
Ipamorelin	76.7	90.6	0.05	4.4	16.7
Ipamorelin-M	68.4	95.8	0.05	7.2	14.8
Alexamorelin	87.8	54.3	0.50	8.1	18.9
Alexamorelin-M1	89.1	93.2	0.20	6.0	16.5
Alexamorelin-M2	84.9	23.0	0.10	11.3	12.2
Anamorelin	141.2	99.7	0.05	7.9	9.3
Ibutamoren	112.5	97.6	0.05	2.8	8.0

GHRP, growth hormone-releasing protein; CV, coefficient of variation; M1/M2, metabolite 1/2; LOD, limit of detection

In case of non-targeted qualitative method, 111 substances of GHRPs, GHSs, and small chemical drugs validated with parameters such as selectivity, recovery, matrix effect, intra- and inter-day precision, and LOD (Table 4). There are no observable interference signal from endogenous substances at the expected retention time in the results of selectivity showed. The results of recovery, almost target substances were extracted without loss. However, 18 substances showed low recovery (29-49%) for preparation step. The results of matrix effect, 20 substances showed ion suppression signal with less than <70% in matrix. Conversely, the signals for 6 substances are significantly enhanced by >30% owing to coelution of endogenous substances within the matrix. The results of intra- and inter-day precision, the %CV for the peak area of PCU at three different concentration (low, middle, and high) was <25% (Table 4). For inter-day precision, relative retention time and peak area of all PCU samples at low, middle, and high concentration from 3 different days were calculated. The %CV of almost target substances were <25% (Table 4). For determination of LOD, a series of diluted reference substances ranging from 100%, 50%, 20%, 10%, 2%, 1%, and 0.1% of MRPL in PCU samples were prepared and then analysis of five samples per concentration point. The LOD for almost all the analytes are <50% of the MRPL, whereas some substances are detected at <0.1% of the MRPL. Collected resulting data were according to ISO/IEC 17025 and WADA guidelines.

Table 4. Method validation results of non-targeted qualitative method: matrix effect, recovery, LOD, and precision

N	Name	Matrix effect	Recovery	LOD	Intra-day precision (%CV)			Inter-day precision (%CV)		
		(%)	(%)	(ng/mL)	Low	Mid	High	Low	Mid	High
1	Anamorelin	122	30	0.02	9.6	6.1	5.2	7.5	12.4	7.2
2	GHRP-1	158	66	0.40	11.3	13.2	4.5	11.9	9.7	4.6
3	GHRP-1-M	70	57	1.00	11.1	10.6	8.8	10.1	9.8	9.3
4	GHRP-2	128	64	<0.002	12.8	10.3	6.0	9.0	10.1	5.6
5	GHRP-2-M	171	78	0.20	5.3	3.7	3.5	5.3	9.5	4.1
6	GHRP-4	153	66	<0.002	6.1	3.7	5.4	6.8	7.1	5.9
7	Ibutamoren	124	32	0.40	7.0	7.4	6.5	24.6	9.8	12.8
8	Ipamorelin	178	121	<0.002	3.8	1.6	1.2	3.6	1.9	3.8
9	5'-Hydroxyphenyl Carvedilol	91	67	1.00	2.5	2.9	4.5	6.4	4.2	4.0
10	Acebutolol	99	108	<0.1	4.0	2.5	1.4	4.3	2.3	1.8
11	Alprenolol	93	98	<0.1	3.4	1.4	2.4	4.1	3.2	2.2
12	Atenolol	64	118	<0.1	3.1	1.4	3.4	7.9	1.5	2.8
13	Befunolol	90	113	<0.1	3.2	1.2	2.3	4.4	2.5	5.9
14	Betaxolol	85	113	1.00	3.3	2.8	2.6	3.8	2.7	3.9
15	Bisoprolol	88	104	<0.1	4.0	2.2	1.7	5.3	2.6	4.6
16	Bufuralol	85	93	<0.1	5.7	1.7	2.4	4.3	4.0	3.4
17	Bupranolol	87	100	<0.1	2.4	1.0	1.5	3.5	3.5	1.8
18	Carteolol	87	116	<0.1	6.8	2.2	2.8	5.4	2.4	3.3
19	Carvedilol	82	85	<0.1	1.9	7.4	4.9	4.7	4.8	4.2
20	Celiprolol	87	105	<0.1	1.9	1.7	2.5	2.9	1.9	2.0
21	Desmethylcarvedilol	109	92	1.00	2.9	5.6	5.9	6.0	4.9	5.5
22	Esmolol	92	103	<0.1	4.5	1.1	3.2	5.8	2.1	5.3
23	Labetalol	103	107	<0.1	3.2	2.1	1.7	4.9	4.0	1.9
24	Levobunolol	105	130	<0.1	4.8	2.1	1.8	5.0	2.7	4.3
25	Metipranolol	76	109	1.00	3.9	1.3	1.7	4.0	1.9	2.2
26	Metoprolol	113	118	<0.1	2.4	2.3	2.0	4.9	2.0	4.2

27	Nadolol	99	115	<0.1	3.2	1.9	1.3	3.9	1.9	2.5
28	Nebivolol	90	89	<0.1	4.3	4.1	4.0	3.8	3.6	4.1
29	Oxprenolol	80	107	<0.1	3.8	4.7	1.4	4.0	3.8	3.3
30	Penbutolol	107	99	<0.1	4.1	1.5	2.2	4.1	1.8	4.8
31	Pindolol	59	92	<0.1	7.3	7.2	5.1	17.1	7.3	11.3
32	Propranolol	84	96	<0.1	4.2	1.5	1.1	5.1	1.5	1.9
33	Sotalol	71	114	<0.1	4.3	1.9	2.2	4.4	2.3	1.8
34	Timolol	102	114	<0.1	3.2	1.7	3.9	3.4	2.2	3.0
35	Clenbuterol	63	115	<0.0002	0.9	2.3	2.6	5.0	4.5	2.7
36	Methyltrienolone	100	35	2.50	23.0	24.6	8.1	19.6	19.9	14.4
37	Bambuterol	78	101	<0.02	0.9	1.6	0.9	3.8	2.7	2.9
38	Brombuterol	98	131	<0.02	2.1	4.4	2.5	3.0	3.8	3.4
39	Cimaterol	65	107	<0.02	3.3	4.5	2.7	3.2	3.7	3.7
40	Cimbuterol	76	103	0.20	3.4	3.5	2.9	3.1	5.4	4.3
41	Clenpenterol	68	115	0.20	1.5	5.3	2.6	5.5	4.9	2.7
42	Clenproperol	83	111	0.20	2.7	3.4	2.1	3.7	4.2	2.5
43	Fenoterol	61	91	0.20	5.5	11.0	8.4	8.2	9.2	7.6
44	Indaterol	118	73	0.20	5.3	5.8	6.6	5.5	10.1	6.6
45	Mabuterol	99	99	<0.02	1.0	1.2	1.8	2.9	3.2	3.2
46	Mapenterol	92	91	<0.02	2.0	2.2	1.1	4.5	3.1	3.1
47	Olodaterol	90	95	0.20	2.0	2.8	3.4	5.7	8.3	4.8
48	Pirbuterol	62	104	0.02	0.6	3.0	2.4	2.0	3.5	2.9
49	Procaterol	121	99	0.02	3.4	8.5	10.4	4.2	10.6	6.5
50	Reproterol	99	56	0.20	5.2	4.4	7.8	7.4	6.3	6.2
51	Ritodrine	60	111	<0.02	1.4	2.0	1.9	2.2	5.7	2.3
52	Salmeterol	103	89	0.40	2.7	5.0	4.3	7.4	6.0	3.5
53	Terbutaline	56	99	0.20	2.0	3.5	1.8	4.5	2.8	4.0
54	Tulobuterol	100	92	<0.02	2.2	2.2	2.9	5.1	11.0	4.1
55	Vilanterol	89	97	<0.02	1.6	3.8	2.1	4.1	8.2	2.9
56	Raloxifene	53	82	0.20	5.5	12.8	9.4	6.3	10.8	10.0
57	Trimetrazidine	68	117	<0.02	4.3	1.4	3.8	6.1	2.6	3.0
58	Amiloride	64	105	0.20	3.5	2.5	2.4	13.2	14.3	10.0
59	Triamterene	77	78	4.00	3.3	1.9	8.8	3.9	3.6	9.3

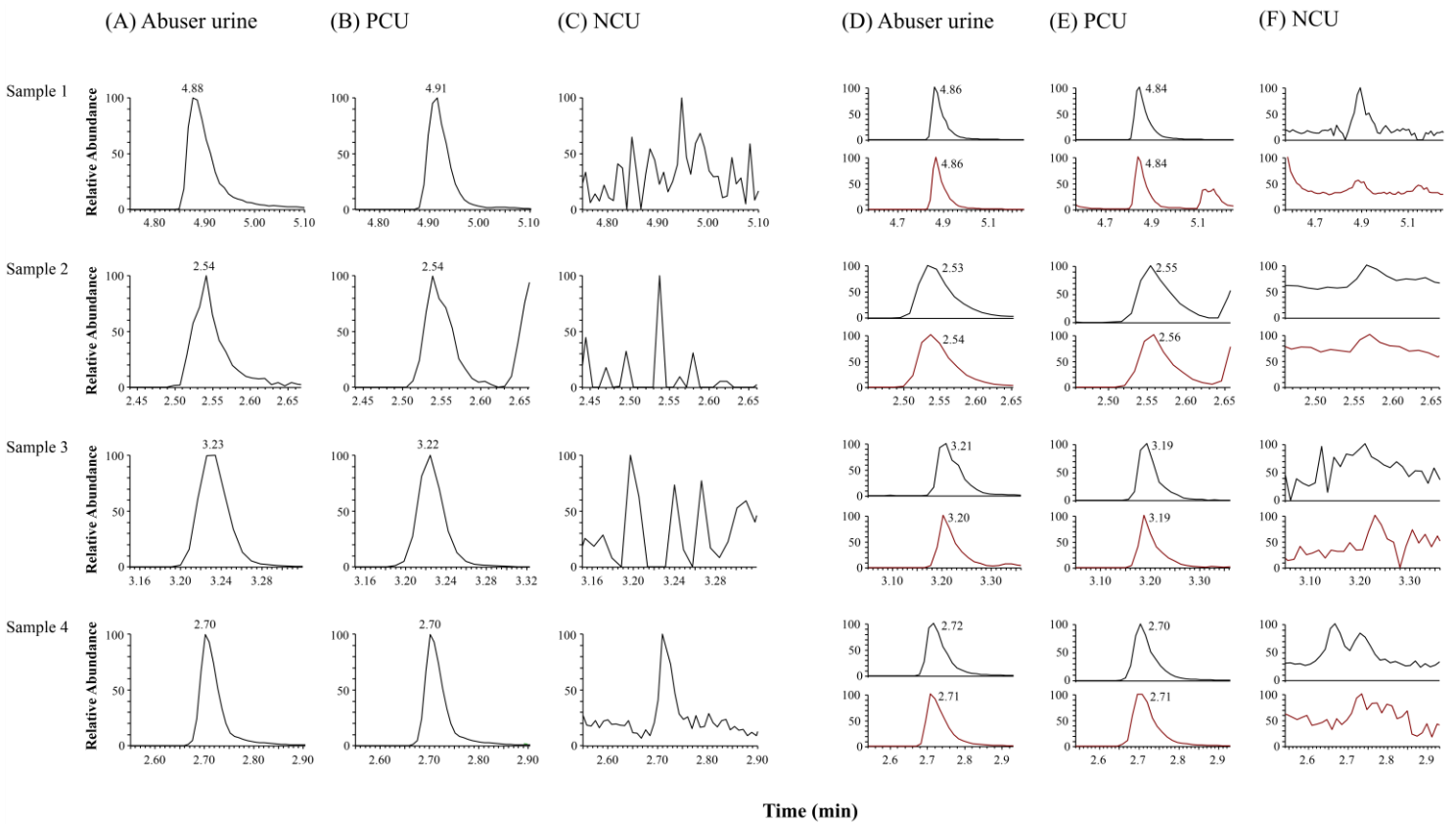
60	Phenylephrine	76	97	0.10	2.9	3.8	2.5	6.0	10.7	4.9
61	2, 5-dimethoxy-4-bromophenethylamine	116	90	<0.1	1.6	2.1	1.9	4.5	6.0	4.0
62	3, 4-Mthylenedioxyethylamfetamine	86	90	<0.1	3.8	5.8	6.6	5.4	11.7	4.6
63	Benzylpiperazine	59	58	1.00	16.2	3.6	10.5	20.9	10.3	9.8
64	Benzphetamine	87	29	1.00	8.8	6.9	6.9	7.3	11.2	7.6
65	Dimethylamphetamine	124	40	0.10	17.7	5.6	15.6	17.5	14.0	20.5
66	Ethylamphetamine	111	55	<0.1	18.8	4.7	13.1	20.4	14.7	13.4
67	Etilefrine	78	119	<0.1	2.5	3.8	1.6	4.7	3.3	2.6
68	Fencamfamine	86	67	<0.1	3.1	7.7	2.8	6.4	12.3	3.2
69	Fencamine	88	91	<0.1	3.3	1.6	5.2	3.9	4.4	4.5
70	Fenetylline	97	32	2.00	5.1	4.6	6.2	6.9	9.6	7.5
71	Fenfluramine	127	45	<0.1	23.6	7.1	18.4	21.7	18.7	16.3
72	Heptaminol	115	92	<0.1	2.9	5.5	2.9	9.7	5.6	5.3
73	Methylenedioxyamphetamine	101	97	1.00	4.2	4.3	1.9	5.1	4.5	3.3
74	Methylenedioxymethamphetamine	89	81	<0.1	3.5	7.0	2.6	4.7	9.4	5.1
75	Mefenorex	102	59	<0.1	3.9	6.6	4.0	8.1	14.7	6.4
76	Mephedrone	98	34	0.10	4.2	5.4	5.4	6.4	13.3	11.8
77	Mephentermine	103	61	1.00	9.7	6.8	8.6	14.1	16.3	9.2
78	Methoxyphenamine	101	82	<0.1	3.4	6.3	4.5	4.9	10.2	4.1
79	Methylephedrine	85	76	<0.1	3.1	8.5	3.7	4.4	11.9	4.3
80	Methylphenidate	101	53	<0.1	7.2	5.3	4.7	7.5	13.1	6.0
81	Norephedrine	167	91	<0.1	1.2	3.1	1.9	4.6	4.7	4.6
82	Norfenefrine	107	98	<0.1	7.5	9.5	5.4	8.5	14.8	6.5
83	Norfenfluramine	100	69	<0.1	9.7	6.9	5.6	8.6	12.4	8.1
84	Octopamine	107	98	100.00	7.4	10.2	5.4	7.3	14.9	6.8
85	Ortetamine	96	83	<0.1	8.5	6.5	3.8	7.9	16.0	5.8
86	Oxilofrine	44	115	<0.1	4.1	2.0	1.5	5.4	3.4	1.8
87	Phenmetrazine	104	37	1.00	7.0	5.0	6.6	6.8	16.0	11.1
88	Pholedrine	81	108	<0.1	2.5	3.4	1.7	3.1	5.0	1.4
89	p-Methylamphetamine	110	76	1.00	7.4	6.1	7.2	8.9	15.7	6.6
90	p-OH-amphetamine	80	105	1.00	4.3	2.5	24.5	6.4	1.6	14.3
91	Prenylamine	104	92	0.10	4.1	6.6	5.1	4.8	5.2	4.7
92	Prenylamine-M	107	94	0.10	3.6	1.9	2.7	3.9	2.0	6.9

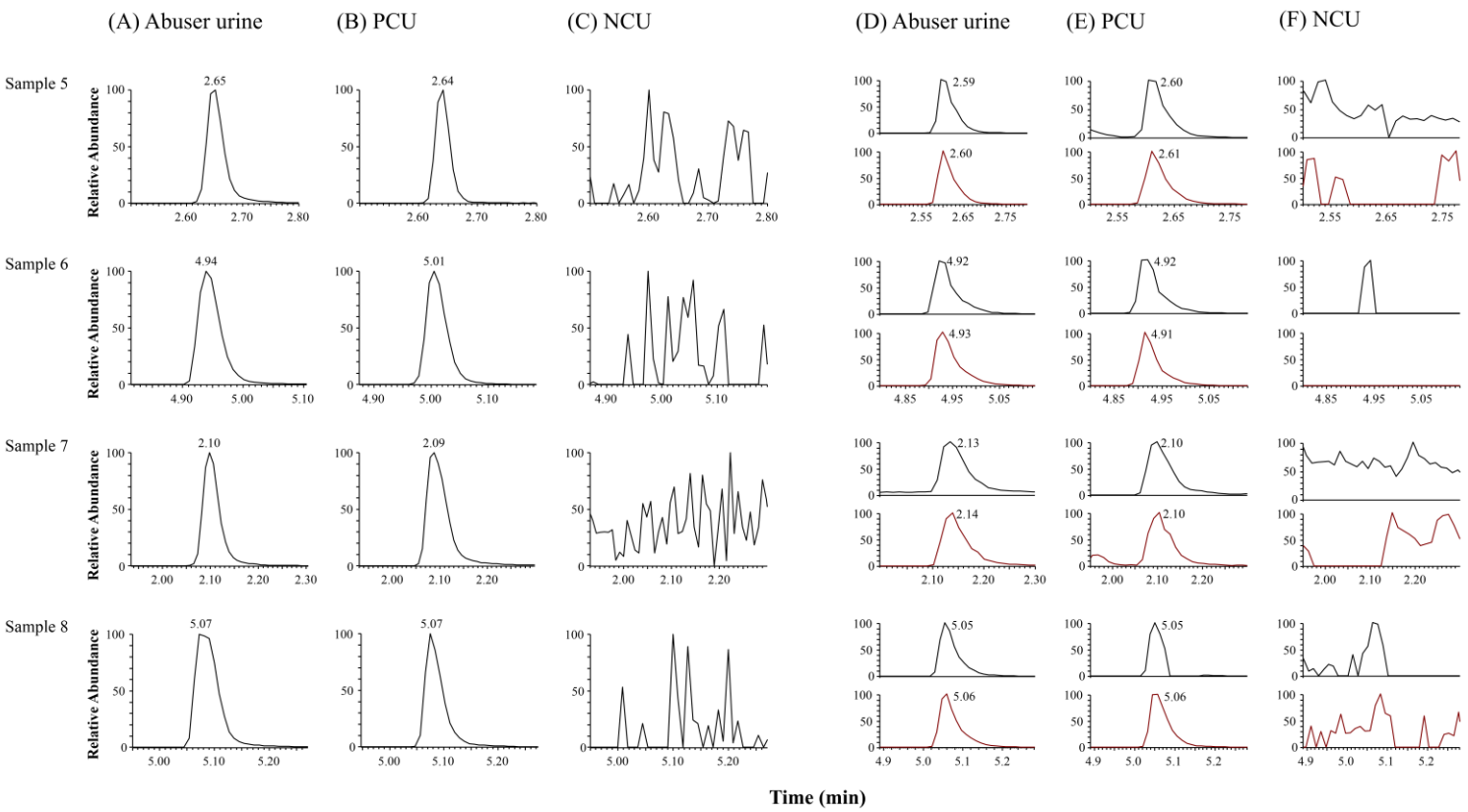
93	Prolintane	99	59	<0.1	13.4	6.5	9.6	13.0	15.3	9.3
94	Sibutramine-M	117	60	<0.1	4.2	10.1	4.0	6.7	18.6	3.4
95	Strychnine	98	107	<0.1	5.3	1.9	4.5	7.9	3.4	4.3
96	Hydromorphone	57	77	<0.05	7.2	7.5	9.8	10.9	13.2	8.4
97	Methadone	97	87	<0.05	3.9	8.7	3.1	3.5	6.6	2.6
98	Norfentanyl	237	88	2.00	9.1	9.9	4.1	9.0	9.7	6.2
99	Oxycodone	69	49	0.50	15.7	9.8	19.7	15.2	18.1	18.6
100	Oxymorphone	35	66	0.50	11.5	7.3	16.2	11.1	10.3	17.4
101	Pentazocine	88	102	<0.05	3.5	3.5	2.6	6.7	2.7	3.9
102	Pethidine	100	29	0.05	5.8	8.4	6.2	7.3	21.3	5.8
103	Flumethasone	102	79	0.30	4.5	3.5	4.0	4.5	9.9	3.8
104	Cathine	122	85	<0.1	2.5	2.0	0.6	5.7	3.2	2.3
105	Ephedrine	109	89	<0.1	1.7	3.5	3.9	3.6	3.8	4.2
106	Morphine	44	124	100.00	4.6	2.6	3.1	6.1	5.3	7.9
107	Pseudoephedrine	69	91	<0.1	1.7	2.3	3.9	3.6	3.9	5.3
108	Codeine	62	86	1.00	6.5	4.9	6.8	6.1	6.3	10.1
109	Hydrocodone	74	61	0.05	18.4	6.6	10.0	15.4	15.0	11.2
110	Pipradol	93	96	1.00	1.6	3.5	1.2	3.0	5.5	1.6
111	Tramadol	92	77	0.05	6.0	3.2	4.2	5.5	8.9	4.5

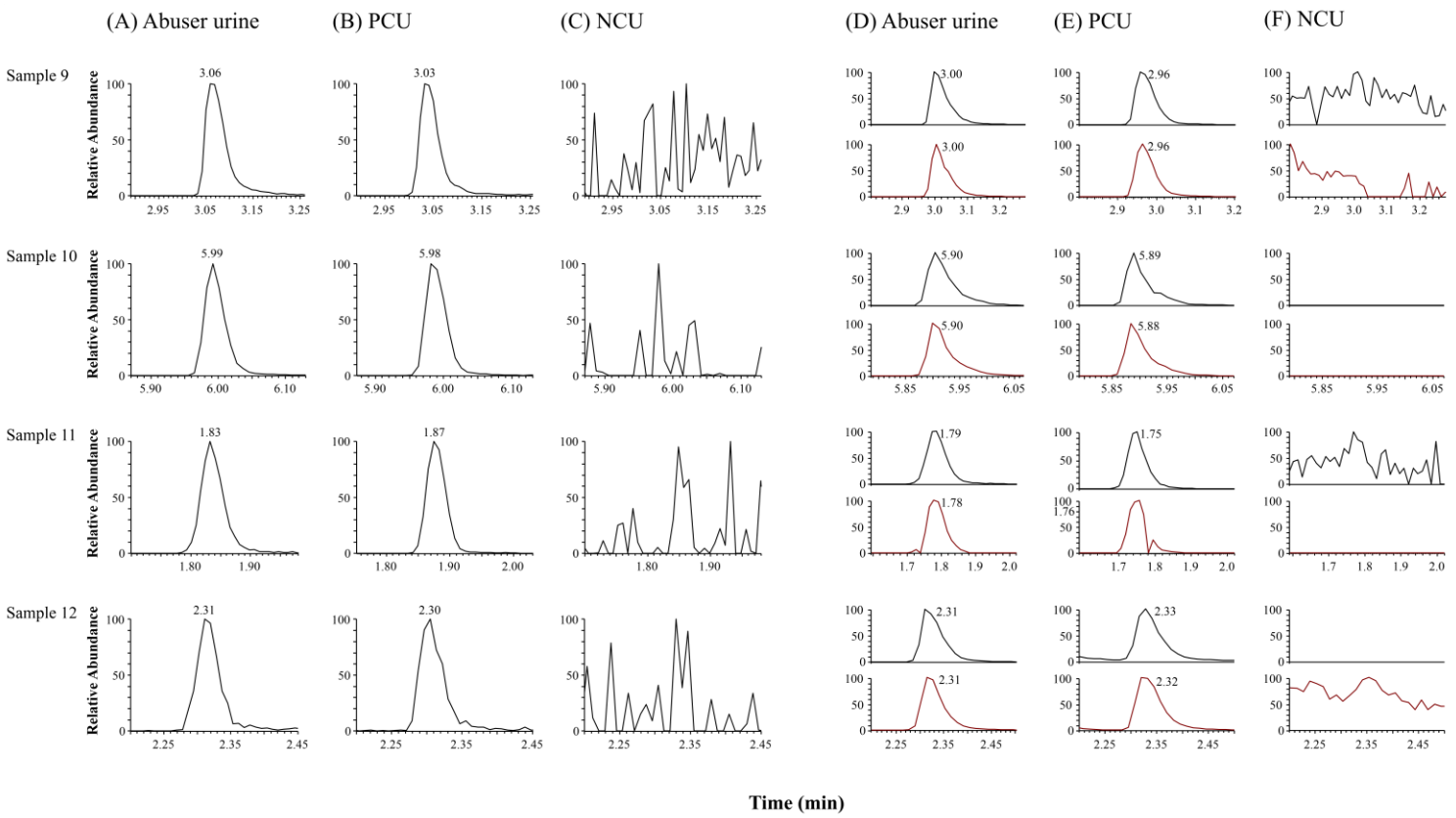
GHRP, growth hormone-releasing peptide; CV, coefficient of variation; M1/M2, metabolite 1/2; LOD, limit of detection

D. Application of External Quality Assurance Scheme (EQAS) sample with targeted and non-targeted qualitative method.

In order to confirm the applicability of the non-targeted qualitative method optimized in this research, 14 actual urine samples from WADA-EQAS for 2014-2017 were analyzed. The results were compared with those of the conventional targeted method. The 14 prohibited substances were successfully detected using non-targeted qualitative method (Fig. 7). Figure 7 shows the chromatograms of the actual urine, PCU, and NCU samples analyzed using both the traditional targeted method and non-targeted qualitative method. Methylephedrine, Atenolol, Celiprolol, Pholedrine, Pentazocine, Amiloride, Raloxifene, Morphine, Cathine, Ephedrine, GHRP-2, Prolintane, and Fenoterol are detected at the expected retention times, demonstrating that non-targeted qualitative method is suitable for doping control. Furthermore, we can confirm that a prohibited drug featured on the WADA prohibited list but not specifically targeted in this study was detected and correctly identified by non-targeted qualitative method. Figure 8 shows that Salbutamol (*m/z* 240.15900 → 148.07594) was detected in one of the 14 actual urine samples. Salbutamol is a prohibited drug but is not included in the 111 target substances in this study. This result demonstrates that all prohibited drugs present in the sample can be detected, unless they are lost in the preparation process.







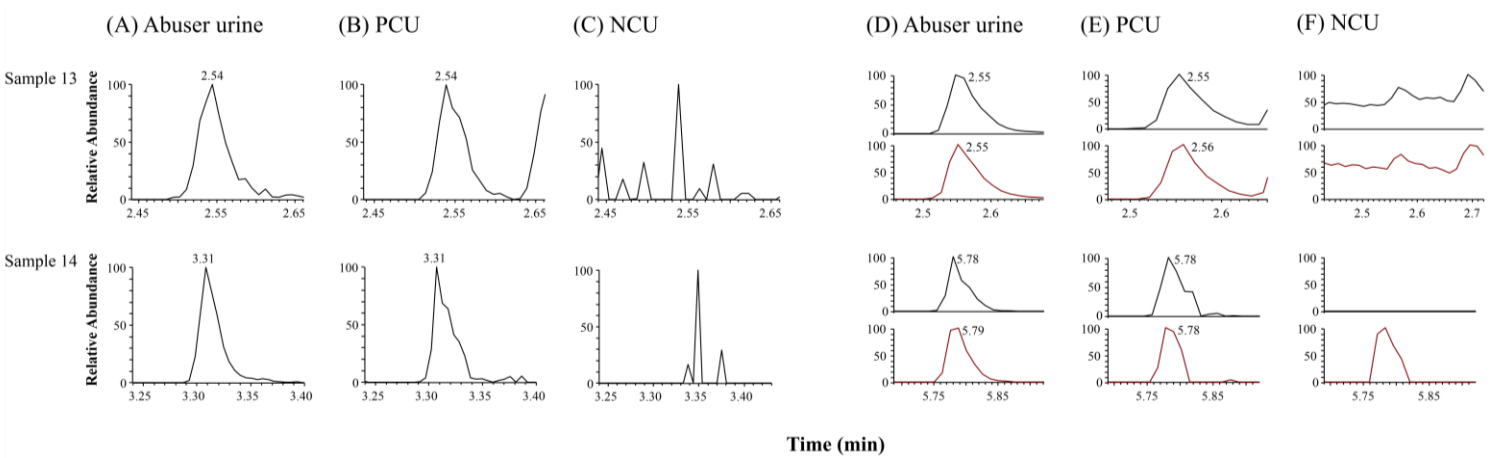


Figure 7. EQAS samples data. The applicability of this method for conducting doping tests was evaluated by analyzing 14 actual urine samples. (A), (B), and (C) show the results for the target screening method ((A): drug abuser urine, (B): positive control urine (PCU) and (C): negative control urine (NCU)), whereas (D), (E), and (F) show the results for the non-target screening method ((D): drug abuser urine, (E): positive control urine (PCU), and (F): negative control urine (NCU)). Sample 1: Prolintane, Sample 2: Ephedrine, Sample 3, Fenoterol, and Sample 4: Methylephedrine, Sample 5: Atenolol, Sample 6: Celiprolol, Sample 7: Pholedrine, Sample 8: Pentazocine, Sample 9: Amiloride, Sample 10: Raloxifene, Sample 11: Morphine, Sample 12: Cathine, Sample 13: Ephedrine, and Sample 14: GHRP-2.

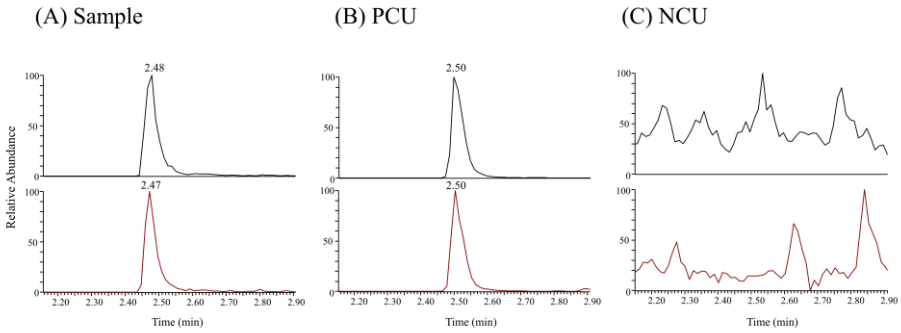


Figure 8. Analysis results for Salbutamol (*m/z* 240.15900 → 148.07594). Salbutamol features in the WADA prohibited drug list. (A) shows the result from an actual urine sample, (B) shows the result for positive control urine (PCU), and (C) shows the result for negative control urine (NCU). The upper trace is the full scan data and the bottom trace is the variable data independent acquisition (vDIA) mode data. Salbutamol is not included in the 111 target substances for this study, but it was detected in one of the 14 samples (Sample 5) by non-target qualitative method

2. Part 2: Analysis of IGF-1

A. Optimization of sample preparation

First, this study try to optimizing protein precipitation (PPT) step. PPT is a process to remove unnecessary proteins in rat serum for the analysis of IGF-1. This process is experimented with a combination of various acidic reagents and organic solvents. This study optimized PPT process using acetic acid and acetonitrile (ACN). The ratio of sample and ACN at 1:1, 1:2, 1:4, and 1:8 were tested, and 1% acetic acid was used together to establish the combination with the best PPT efficiency. As a result of this study, a method of PPT was performed by adding 200 μL of 1% acetic acid and 400 μL of ACN to 100 μL of sample.

Second, tryptic digestion step was optimized for best enzymatic efficiency. This study targeted peptide fragments (named T1 and T2) obtained by trypsin enzyme (Table 5) which is difficult to analyze in intact state (21,841 Da). Before using trypsin, the reduction process was applied in various ways such as using DTT with sodium dodecylsulfate (SDS) and urea and using only DTT. The reduction method using DTT with SDS and urea is not friendly to mass spectrometry, a further cleanup procedure was needed and it has problems like time consuming and additional loss of IGF-1. Finally, applying reduction method using only 100 mM DTT was optimized. Since this method uses as mass spectrometry friendly reagents, it was able to shorten the preparation time which does not require an additional cleanup process.

Table 5. Amino acid sequence of IGF-1

	Amino acid sequence
IGF-1	GPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRA PQTGIVDECCFRSCDLRRLEMYCAPLKPAKSA
T1 (*m/z* 769.6963)	GPETLCGAELVDALQFVCGDR
T2 (*m/z* 556.5986)	GFYFNKPTGYGSSSR

IGF1 size is 7649 Da. T1 and T2 are fragments obtained via tryptic digestion. In the first fragment T1, the *m/z* value is 769.6963 and the charge state is 3. The second fragment T2 has an *m/z* value of 556.5986 and a charge state is 2.

B. Optimization of nanoLC-HRMS conditions

In this study, IGF-1 was quantitatively analyzed using the targeted method with parallel reaction monitoring (PRM) mode by nanoLC-HRMS. The resolution and sensitivity were increased by using LC with nano flow (300 nL/min). PRM mode was optimized with 35,000 resolution (AGC target: 2.00E+05, maximum IT: 100 ms), CE 27 in positive mode for 55 min. Using this method, data was obtained by designating five or more product ions of T1 (*m/z* 769.6963) and T2 (*m/z* 556.5986) (Table 6).

Table 6. nanoLC-HRMS mass spectrometry parameters for fragment of T1 and T2

Precursor	Precursor charge state	Precursor ion (*m/z*)	Product ion (*m/z*)	Ion types
T1	3	769.6963	881.3934	y7
			753.3348	y6
			606.2664	y5
			507.1980	y4
			347.1674	y3
			915.3877	b9
T2	2	556.5986	911.4217	y9
			814.3690	y8
			713.3213	y7
			493.2365	y5
			349.1830	y3

C. Validation

IGF-1 validated with parameters such as selectivity, matrix effect, linearity, LOD, precision and carry-over. The precursor ion and product ion of IGF-1 used for validation are summarized in a Table 7. The results of selectivity showed no observable interference signal from endogenous substances at the expected retention time and results are shown in the Fig. 9. The results of matrix effect, ion suppression/enhancement by the matrix evaluated, and it was confirmed that there was no false negative of the positive sample and false positive of the negative sample due to the matrix effect. The results of linearity, a calibration curve (n=3) ranging from 0 to 2000 ng/mL of concentrations (0, 5, 10, 25, 50, 100, 200, 400, 600, 1000, 1500, and 2000) was analyzed. Calculate the calibration curve using the average value of three calibration point ratios (peak area ratio to heavy) of the each concentration at T1 and T2, and check the value of R^2. The results are shown in the Table 8 and Fig. 10. The results of LOD, 10 ng/mL was the minimum concentration at which s/n ratio >3 and the %CV value >25. For determination intra-, inter-day precision, in order to fulfill the criteria of the reproducibility test and reproducibility test, the %CV value of each day's data must be within 25%. As a result of the test, it satisfied all the criteria presented above, and the results are shown in Table 7. Finally, for determination carry-over, the IGF-1 spiked sample at the concentration of 1000 ng/mL prepared and the blank sample without adding IGF-1 were prepared and alternately analyzed. It was evaluated whether the ratio between the area value of the IGF-1 spiked sample and blank sample was less than 5%. As a result of the carry-over test, it was confirmed that the ratio between IGF-1 spiked sample and blank sample was less than 5% (Table 9).

Table 7. Method validation results of targeted quantitative method of IGF-1: matrix effect, linearity, LOD, carry-over and precision

Parameter		T1 (m/z 769.6963)	T2 (m/z 556.5986)
Matrix effect (%)		94	91
LOD (ng/mL)		10	10
Linearity (R^2)		0.999	0.996
Carry-over (%)		0.7	0.2
Accuracy (%)	QC_High	106	103
	QC_Low	98	99
Intra-day precision CV (%)	QC_High	2.9	2.9
	QC_Low	4.3	3.6
Inter-day precision CV (%)	QC_High	7.6	7.3
	QC_Low	6.4	5.4

LOD, limit of detection; CV, coefficient of variation

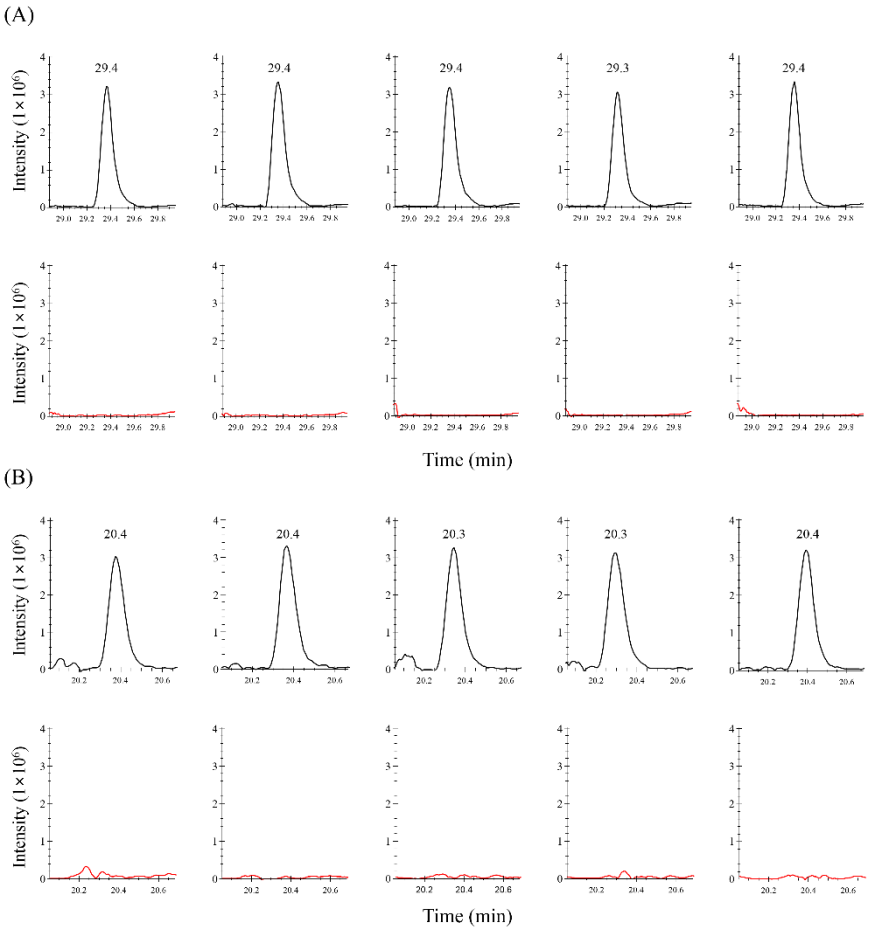


Figure 9. Extract ion chromatograms of positive control serum (PCS) and negative control serum (NCS). (A) is selectivity result of T1, the upper line is PCS and the lower line is NCS. (B) is result of T2, the upper line is PCS and the lower line is NCS.

Table 8. Linearity of T1 and T2. Each fragment was calculated as peak area ratio to heavy

Conc.	T1 (Peak area ratio to heavy)						T2 (Peak area ratio to heavy)						**Average of T1+T2**
	1	2	3	Average	SD	%CV	1	2	3	Average	SD	%CV	
5	0.026	0.025	0.023	0.025	0.002	6.356	0.029	0.029	0.024	0.027	0.003	10.188	**0.026**
10	0.055	0.050	0.055	0.053	0.003	5.260	0.046	0.044	0.049	0.046	0.003	5.805	**0.050**
25	0.138	0.144	0.140	0.141	0.003	2.270	0.128	0.137	0.114	0.126	0.012	9.139	**0.134**
50	0.307	0.291	0.294	0.297	0.008	2.837	0.280	0.263	0.266	0.270	0.009	3.234	**0.284**
100	0.612	0.638	0.535	0.595	0.054	9.010	0.558	0.579	0.537	0.558	0.021	3.814	**0.576**
200	1.158	1.261	1.349	1.256	0.096	7.623	1.032	1.156	1.160	1.116	0.073	6.522	**1.186**
400	2.243	2.203	2.425	2.290	0.118	5.166	2.150	2.160	2.338	2.216	0.105	4.758	**2.253**
600	3.763	3.336	3.517	3.539	0.214	6.051	3.688	3.238	3.413	3.446	0.227	6.589	**3.492**
1000	5.815	5.888	6.712	6.139	0.498	8.115	5.704	5.565	6.788	6.019	0.670	11.126	**6.079**
1500	9.724	9.145	9.444	9.437	0.290	3.068	9.211	9.038	9.085	9.111	0.089	0.981	**9.274**
2000	13.130	13.600	14.484	13.738	0.687	5.002	12.937	13.372	13.650	13.320	0.359	2.698	**13.529**

SD, standard deviation; CV, coefficient of variation

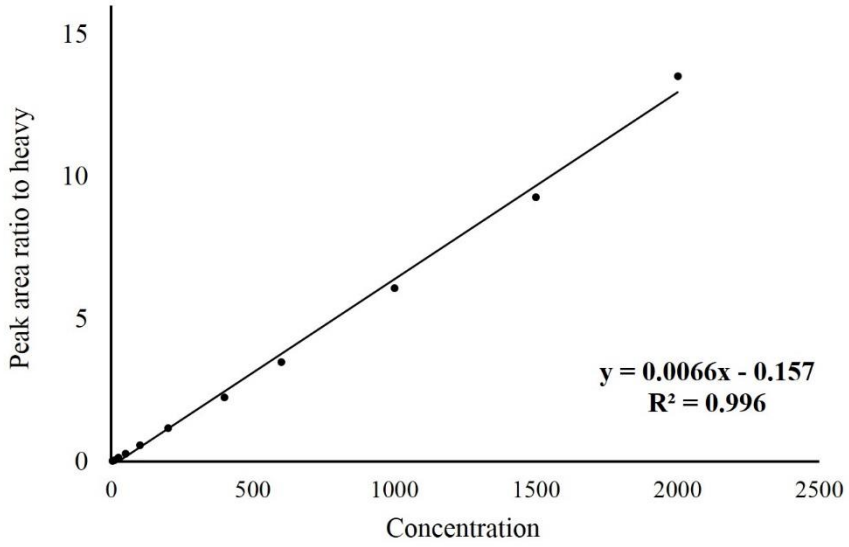


Figure 10. Linearity of T1 and T2. For the calibration curve, the average value of T1 and T2 was used. The value of R^2 was 0.996.

Table 9. The result of carry-over test

Peak Area (T1)		
PCS sample (IGF-1 at 1000 ng/mL)	Rat serum sample (Blank sample)	**Carry-over (%)**
734490624	4918943	**0.67**
672347328	5732398	**0.85**
Peak Area (T2)		
PCS sample (IGF-1 at 1000 ng/mL)	Rat serum sample (Blank sample)	**Carry-over (%)**
354242944	661844	**0.19**
347867488	976854	**0.28**

Carry-over test evaluates whether analyte remains and influences the next sample. The ratio between IGF-1 spiked PCS (positive control serum) sample and blank sample was less than 5%.

D. Application of EQAS samples with targeted quantitative method

The method optimized in this study was confirmed that it can actually be applied to doping test. Therefore, 3 samples of 2015 WADA-EQAS, quality control high (QC_{high}), and quality control low (QC_{low}) were analyzed. The result was as follows that concentration of QC_{low} were 178 ng/mL but the analyzed value was 225.7 ng/mL and there was deviation between ideal value and experimental value (33.7 ng/mL). According to what is known, the IGF-1 quantitative analysis method has variations depending on the equipment and preparation method, and sometimes a large error occurs among interlaboratory. Therefore, WADA has distributed 1 point calibrator to doping laboratory of each country since 2017 in an effort to reduce errors in IGF-1 quantitative analysis for doping test. The deviation of the QC_{high} sample before calibration was 125.7 ng/mL, but the deviation was reduced to 4.3 ng/mL after calibration. Therefore, if appropriate 1 point calibration is followed, this quantitative analysis method can be used for doping test.

IV. DISCUSSION

Recently bio-doping has been exploited many different ways and athletes would not stop using wrong drugs for reaching higher performances even with the risk to health. Especially athletes are abusing protein drugs because the doping test method about protein drugs is not universally establish yet in doping laboratories around the world. Most of these bio-doping drugs are mainly related to GH that improves muscle mass, strength and has the effect of helping recovery from injury. There are various doping ways using GH such as an injection of small peptide drugs that increases GH and a direct injection of GH. Therefore, this study developed analysis method of growth hormone releasing substances such as GHRPs and GHSs. In addition, GHRPs, GHSs, and small chemical drugs are unified by one preparation method, and applied non-targeted qualitative method that will be useful for next generation doping test. And I developed a quantitative analytical method for IGF-1 as a bio marker which is indirect evidence of GH injection.

PART 1 introduced the research about the development and validation procedure of the targeted qualitative method for GHRPs and GHSs. In order to fulfil the analytical condition of each substance, various mass parameters were optimized and used SPE method for enriching all substances in preparation step. This analysis method was applied to the actual doping after validated process that contains the selectivity, linearity, matrix effect, recovery, LOD, and precision. Most substances satisfied the reference value but some substances showed low recovery and matrix effect. Since these substances were reasonably detected at MRPL concentration, it was judged to be suitable for doping test. This SRM method works well both qualitative and quantitative analysis, and the analysis speed is faster. Since this method analyzes by designating the target substances, the sensitivity is higher than non-targeted method. However, this targeted qualitative method collects

information on only the designated substances, it cannot discover unknown substances. Furthermore, in the case of some prohibited drugs which cannot obtain standard products, it is actually impossible to set up analysis method. And this method has no sustainability, it requires frequent modification when the new drugs were added at prohibited list (Table 10). Therefore, in this study, targeted qualitative method was developed into a non-targeted qualitative method using full and vDIA mode. No later modification is required in this method since the vDIA mode method analyzes the full spectrum of data in fixed *m/z* ranges (*m/z* 100-200, 200-300, 300-400, 400-500, and 500-800). Because of these features it can discover unknown substances and trace new drug information from accumulated data. In the ddMS/MS method which is commonly used for existing unknown screening, there was a limit that it was hard to detect if the substance intensity was low but, in the vDIA mode, it was able to obtain information on all the MS/MS data within the fixed range so it is suitable for qualitative analysis than ddMS/MS or SRM (Table 10). As for non-target qualitative method, this study simultaneously applied 111 substances including small chemical drugs and small peptides, then collected the resulting data according to ISO/IEC 17025 and WADA guidelines. The method was validated in terms of selectivity (no interferences), recovery (29-131%), matrix effect (35-237%), intra- and inter-day precision (%CV lower than 25%) at all of target substances, and LOD was 0.0002 to 100 ng/mL. This method was confirmed via application of EQAS samples and the results demonstrates that non-targeted qualitative method of this study can become a powerful tool for unknown target analysis for next generation doping test.

PART 2 is the quantitative analysis of IGF-1. There are various methods such as immune assay and LC-MS/MS assay for IGF-1 analysis. In the case of the immunoassay method, there is a disadvantage that it greatly affects the result value of each laboratory depending on the difference in the used antibody, extraction method and different approaches to calibration. On the

other hand, the quantitative analysis method of IGF-1 using LC-MS/MS is more accurate than the immunoassay. Thus all doping laboratories around the world are going to apply these LC-MS/MS based assays to achieve less variation than immunoassay method. In this study, IGF-1 was divided into peptide state via tryptic enzyme digestion step and analyzed with nanoLC-HRMS. However, even with this method, there was a problem that variations occur depending on the type of rat serum used for calibration curve and the type of equipment. To solve this problem, WADA distributed one point calibrator to 33 doping laboratories which is human serum samples known at a certain concentration and tried to minimize the quantitative deviation from the laboratories. This study also validated the quantitative analysis method using these calibrator and the results of validation, there are no interferences in selectivity step. And all fragment matrix effect were 90-94%, carry-over were lower than 5%, LOD was 10 ng/mL, and intra- and inter-day precision were lower than 15%. Thus, the method of this study can be used in Korea doping laboratory for quantification of IGF-1. However, analysis of IGF-1 for doping control has the additional weakness that it can be highly elevated in people with disease such as acromegaly, so further research and data accumulation to overcome this problem are needed.

Table 10. Summary of targeted and non-targeted method

	Targeted method	Non-targeted method
Quantitative analysis	● ● ●	● ● ○
Qualitative analysis	● ● ○	● ● ●
Analysis speed	● ● ●	● ● ○
Sensitivity	● ● ●	● ● ○
Discoverable of unknown compounds	-	● ● ●
Traceability	-	● ● ●
Sustainability	-	● ● ●

V. CONCLUSION

This study tried to develop the analysis method for small peptide drugs (GHRPs, GHSs), small chemical drugs, and IGF-1. Qualitative analysis of GHRPs and GHSs by targeted qualitative method was established and it is used for actual doping test. Also, qualitative analysis of GHRPs, GHSs, and small chemical drugs by non-targeted qualitative method was developed. This method has powerful advantages that are useful for the discovery of unknown compounds, an open method for tracking new drugs in accumulated samples and not required additional modification of the method, therefore it can be the beginning of the method of new doping analysis. And quantitative analysis of IGF-1 was established that can be used for actual doping analysis through evaluation of effectiveness. These doping test will eradicate the athletes who abuse doping drugs and lead to a fair sports competitions.

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ABSTRACT(IN KOREAN)

액체 크로마토그래피 질량분석기를 이용한 도핑 약물의 표적, 비표적 분석법 개발

<지도교수 유제욱, 손정현>

연세대학교 대학원 의과학과

한보영

성장호르몬 방출 펩타이드, 성장호르몬 분비촉진물질, 화학약물, 인슐린 유사 성장인자-1은 세계 반 도핑기구에서 지정한 금지약물 국제 표준에 기재되어있는 불법약물이다. 본 연구에서는 성장호르몬 방출 펩타이드, 성장호르몬 분비촉진물질, 화학약물, 인슐린 유사 성장인자-1의 분석법을 개발하고 validation 하고자 하였다. PART 1에서는 성장호르몬 방출 펩타이드, 성장호르몬 분비촉진물질의 targeted-qualitative method를 확립하고 validation 하였다. 과거의 GHRPs, GHSs 분석은 주로 부상 회복 관련 pathway, mechanism을 밝히기 위한 것이 대부분이었지만 최근 GHRPs와 GHSs를 사용한 bio-doping 방식이 급부상하고 있다. 운동선수들은 성장호르몬 분비를 촉진시킬 목적으로 성장호르몬 방출 펩타이드, 성장호르몬 분비촉진물질을 도핑에 악용하고 있다. 따라서 doping control을 목적으로 LC-MS/MS를 이용한 분석법이 개발되고 있다. 이 분석법은 선택성 (간섭 없음), 직선성 (R^2 >0.9986), 매질효과

(50.0-141.2%), 회수율 (10.4-100.8%), 일내 (2.8-16.5%), 일간 (7.0-22.6%) 정확성, 검출한계 (0.05-0.5 ng/mL) 항목이 포함된 유효성 평가를 수행하여 검증되었다. 더 나아가 기존의 화학약물 분석 방식을 GHRPs, GHSs와 통합시키고 non-targeted qualitative 이라는 새로운 방식을 도입하였다. 이 방식에서는 서로 다른 특성을 갖는 GHRPs, GHSs와 같은 small peptide와 small chemical drugs를 하나의 전처리법으로 통일하여 전처리 시간을 감소시켰다. 또한, 추가된 약물에 대한 별도의 optimization 없이 바로 적용 가능한 non-targeted method를 사용하여 next generation of doping control test에 이바지하고자 하였다. 이 분석법 역시 선택성 (간섭 없음), 매질효과 (35-237%), 회수율 (29-131%), 일내, 일간 정확성 (%CV 25% 이내), 검출한계 (0.0002-100 ng/mL) 항목이 포함된 유효성 평가를 수행하여 검증되었다. PART 2에서는 혈청시료에서 IGF-1을 분석하는 연구를 진행하였다. IGF-1은 내인성 단백질로, 운동선수가 성장인자를 주입하였을 때 증가하기 때문에 운동선수가 성장인자를 주입하였는지 판단할 수 있는 bio marker가 될 수 있다. 내인성인 IGF-1은 정량곡선을 사용한 정량분석법으로 검출한다. 이 분석법은 IGF-1의 peptide level인 T1, T2를 목적성분으로 하여 실제 도핑테스트에 이용될 수 있도록 선택성 (간섭 없음), 매질효과 (90-94%), 잔효성 (5% 이내), 일내, 일간 정확성 (%CV 25% 이내), 검출한계 (10 ng/mL) 항목이 포함된 유효성 평가를 수행하여 검증되었고 실제 도핑 테스트에 활용될 것이다.

핵심되는 말 : 성장호르몬, 성장호르몬 방출 펩타이드, 성장호르몬 분비 촉진물질, 인슐린 유사 성장인자-1, 생체유래 약물, targeted, non-targeted, doping, 유효성확인

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