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Urinary excretion profile of higenamine in females after oral administration of supplements – Doping scenario

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ABSTRACT

In 2017, higenamine was added to the *World Antidoping Agency's* (WADA) Prohibited list under group S3: beta-2 agonists and it is banned for athletes both in – and out of competition. Aim of this study was to characterize the urinary excretion profile of higenamine and its metabolite coclaurine after oral administration of multiple doses of higenamine capsules. For this purpose, an administration study including female basketball players was performed.

For the detection of higenamine and cocalurine in the collected urine samples, a new, fast, and highly sensitive quantitative on-line SPE LC HRMS method was developed and validated. The method was applied for the quantification of higenamine and cocalurine in urine and their excretion pattern was defined. Results obtained show substantial inter-individual differences in the excretion profile of higenamine and coclaurine. For higenamine, half-lives were estimated to be between 4 and 27 h, and for coclaurine between 5 and 25 h. Furthermore, the data indicate that the elimination of coclaurine is rate-limited by its formation. Higenamine could be detected at a urine concentration above 10 ng/mL for at least 20 h after the last application for all study participants.

1. Introduction

Higenamine, a stimulant found in plants, has beta-agonist activity with chronotropic and inotropic properties [1]. It occurs naturally in different plants such as *Nandina domestica*, *Aconitum carmichaelii*, *Asarum heterotropioides*, *Galium divaricatum*, *Annona squamosa*, *Nelumbo nucifera* etc. In 2017, higenamine was added to the WADA (World Antidoping Agency) Prohibited list under Beta-2 agonists as a banned substance at all times [2]. According to TD2022MRPL [3], higenamine urine concentrations lower than 10 ng/ml are not to be reported as adverse analytical findings (AAFs).

Several urinary excretion studies related to higenamine misuse in sport have been published [4–11]. In these studies, higenamine has been determined in urine samples after consumption of throat lozenge [4], *Plumula Nelumbinis* capsules [5], traditional Chinese medicine [6], tablets [7], Annona fruit [8,9] or vegetable beetroot [10]. Okano et al [4] also investigated coclaurine, the main metabolite of higenamine, while Zhao et al [7] fully identified and characterized several higenamine metabolites. In one recent publication [8] authors present synthesis and characterization of sulfo conjugate metabolites of higenamine but also suggested complementary biomarkers which could support discrimination between different sources of urinary higenamine. The most common applied method for sample preparation is dilute and shoot (DaS) [5–8,11]. Hydrolysis with β-glucuronidase followed by solid phase extraction (SPE) [4] or acid hydrolysis in combination with double liquid–liquid extraction (LLE) [11] were also included in sample preparation protocols.

It is worth noting that higenamine is present in different plant extracts or supplements [1]. For example, higenamine was determined as an ingredient in a Thai antihypertensive herbal recipe [12], as well as in *Plumula Nelumbinis* as an alkaloid [13], in Annona fruit [8,9], in vegetable beetroot [10] and in different nutritional supplements [14,15]. In a recent review [16] authors gave an overview of plants containing higenamine. Previous work shows that specific plant extracts did not

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lead to AAFs but of course this is depending on consumption dose [4,8–10]. On the other hand, supplements usually have a higher amount of substance (20 mg/ serving or more) and could clearly lead to AAFs. Hence, administration of higenamine supplements could be considered as intentional doping. In the present research, focus is on intentional doping and on the urinary elimination profile of higenamine and its metabolite coclaurine after oral intake of supplements. For that purpose, a fast and sensitive online solid phase extraction – liquid chromatography high resolution mass spectrometry (online SPE -LC/HRMS) method was developed and validated. Further on, the goal of the presented study was to define the detection window for higenamine and its metabolite coclaurine after multiple dose application of supplements, as well as to define interindividual variations in the higenamine elimination profile.

2. Experimental

2.1. Design of the clinical study

Six female subjects – 1st 35 years old, 60.7 kg, 2nd 31 years old, 76 kg, 3rd 35 years old, 72.3 kg, 4th 34 years old 75 kg, 5th 29 years old 71.1 kg and 6th 34 years old 69.2 kg, Caucasian race, healthy, and no medication were volunteers for the presented excretion study. We selected a homogenous study group to minimize the impact of confounding variables on assessing differences in elimination kinetics between individuals. The participants received *per os* doses of Higenamine capsules (1 capsule contains 25 mg of higenamine, Singular Sport, United States) three times a day for three weeks. Samples were collected before administration (0 h) and daily for three weeks. After the last application, all urine samples were collected for 48 h. The sampling time and volume of all subjects were recorded. The urine samples were sealed and stored at −20 °C until analysis. Before analysis, samples were thawed, and specific gravity was measured.

The study was conducted in accordance with the International Conference on Harmonization guidelines for Good Clinical Practice and was compliant with the ethical principles described in the current version of the Declaration of Helsinki. Prior to study initiation, all protocols were approved by the local ethics committee of the Sports Medicine Association of Serbia (Belgrade, Serbia).

2.2. Chemical and reagents

Higenamine hydrobromide and (-) coclaurine hydrochloride were purchased from Toronto Research Chemicals (Toronto, Canada). Formoterol hemifumarate-13C,2H3 (internal standard) was provided by Alsachim (Illkirch-Graffenstaden, France). Methanol of HPLC grade was obtained from Chem Lab (Zedelgan, Belgium) and water of HPLC grade was provided by Merck (Darmstadt, Germany). Formic acid (99 %, ULC/MS-CC/SFC) was purchased from Bisolve (Valkenswaard, The Netherlands).

2.3. Sample preparation

To an aliquot of 500 μL urine, 10 μL of a formoterol hemifumarate-13C, 2H3 internal standard solution (25 μg mL^{-1}) and 500 μL of solvent were added. The solvent was 0.1 % formic acid in water. The sample was mixed and an aliquot of 10 μL was injected into the LC-HRMS instrument.

2.4. Online SPE - liquid chromatography – High resolution mass spectrometry (LC-HRMS)

For online SPE, an Accucore Phenyl-Hexyl, 10 × 3 mm extraction column with 2.6 μm particle size and 80 Å pore size was used (Fischer Scientific, Loughborough, UK). The analytical HPLC column was an XTerra MS C18 (100 mm × 2.1 mm, 3.5 μm particle size) (Waters, Milford, MA, USA). Mobile phase A was water with 0.2 % of formic acid and mobile phase B was methanol with 0.1 % formic acid. A constant flow rate of 0.33 mL min^{-1} was applied with the following gradient: 0 % B (0–2 min), 0 % → 100 % B (2—–8.6 min), 100 % B (8.6–10.4 min), 0 % B (10.4–13 min). For the first 2 min, the valve was set to a position that directed the solvent through the extraction column and into a waste container. During this process, matrix compounds such as proteins and salts are flushed into waste, while analytes are trapped in the column. After 2 min, the valve was adjusted to a second position and the gradient started, resulting in the elution of the analytes from the extraction column to the chromatography column, and finally to the mass detector. The chromatography column temperature was maintained at 25 °C and the temperature in the autosampler was set to 4 °C.

Measurements were performed on a Vanquish Horizon UHPLC + System coupled to a Q-Exactive Orbitrap high-resolution mass spectrometer (Thermo Fisher, Austin, Texas, USA in positive electrospray ionization mode (ESI +) using the following settings: spray voltage was set to 3.8 kV, and capillary temperature was 350 °C. Nitrogen was used as sheath gas (pressure 25 units) and as auxiliary gas (pressure 8 units, temperature 310 °C). Sweep gas flow rate was set to 0 and s-lens radio frequency (RF) level was 55. A mass resolution of 17,500 at *m/z* 100 and automatic gain control (AGC) to 2 x 10^5 ions was used. Parallel reaction monitoring (PRM) was chosen as measuring method. Selected transitions for higenamine were *m/z* 272.13 → *m/z* 107.0493, *m/z* 272.13 → *m/z* 161.0593, *m/z* 272.13 → *m/z* 255.1008 and *m/z* 272.13 → *m/z* 255.1008 and the collision energy was 30 eV. Selected transitions for coclaurine were *m/z* 286.14 → *m/z* 107.0484, *m/z* 286.14 → *m/z* 175.0734, *m/z* 286.14 → *m/z* 237.0882 and *m/z* 286.14 → *m/z* 269.1140 and the collision energy was 30 eV. For quantitative determination selected transitions were *m/z* 272.13 → *m/z* 107.0493 and 286.14 → *m/z* 107.0484 for higenamine and coclaurine, respectively. The selected exact mass for formoterol hemifumarate-13C, 2H3 (internal standard) was *m/z* 349.20348. Selected masses for the sulfo conjugates were *m/z* 352.0818 and 366.097 for higenamine and coclaurine, respectively. Additionally, Full scan was recorded 100 to 600 *m/z*.

2.5. Validation

The analytical method was validated according to current WADA guidelines [17]. Investigated parameters included selectivity, matrix interferences, intra- and inter-day precision, carry-over, linearity, limit of quantification (LOQ), limit of identification (LOI) and limit of detection (LOD).

QC samples To assess the stability of the system, a quality control (QC) of 50 ng mL^{-1} for both higenamine and coclaurine was injected into the system in triplicate before each batch to examine the peak area and retention time of both analytes.

Selectivity was tested by investigating the chromatograms of 10 blank urine samples (five female, five male) for interfering signals at the respective retention times.

Carry-over from sample to sample during instrumental analysis was evaluated by injecting a high concentrated spiked urine sample (200 ng mL^{-1} for higenamine and coclaurine) prior to the injection of three consecutive blank urine samples.

Linearity of the method was tested for higenamine and coclaurine in the range 2 ng mL^{-1} – 200 ng mL^{-1} (2 ng mL^{-1}, 10 ng mL^{-1}, 20 ng mL^{-1}, 50 ng mL^{-1}, 75 ng mL^{-1}, 100 ng mL^{-1}, 150 ng mL^{-1} and 200 ng mL^{-1}). For every calibration point three independent samples were prepared and injected in triplicate. Samples were prepared in blank urine and obtained results evaluated according to the European Medicines Agency (EMEA) Guideline on bioanalytical method validation [18].

For linearity, correlation coefficients were evaluated ($R^2 > 0.99$). Ratios of the chromatographic peak areas of higenamine and coclaurine to internal standard were plotted against concentration.

Matrix effect was investigated by comparing peak area substance/ peak area internal standard obtained in linearity testing with

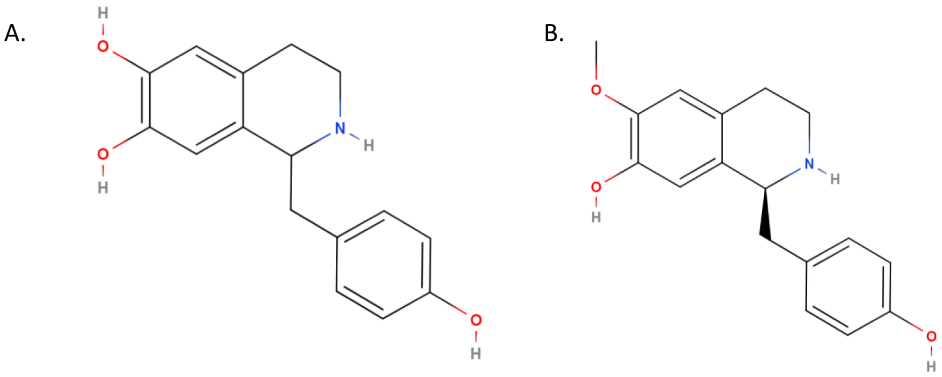

Fig. 1. Higenamine (A) and coclaurine (B) structures.

corresponding water standard solutions prepared in the same concentrations.

For **LOI determination** 6 different urine blank samples (3 male and 3 female) were spiked at three concentration levels 2.5 ng mL^{-1}, 5 ng mL^{-1} and 10 ng mL^{-1}. LOI estimation was based on criteria defined in TD2023IDCR [19].

Limit of quantification (LOQ) was determined as the lowest concentration level with a signal to noise ratio > 10:1 and RSD% for five repetitions less than 20 %. Limit of detection (LOD) was estimated as the lowest concentration level with a signal to noise ratio > 3:1. LOQ and LOD were calculated with the calibration curve method using MS Excel based on the standard deviation of the intercept (σ) and slope (s) of the calibration curves prepared with the concentrations 1 ng mL^{-1}, 1.5 ng mL^{-1}, 2 ng mL^{-1} and 4 ng mL^{-1} for higenamine and coclaurine.

Repeatability of injection was tested at a concentration of 50 ng mL^{-1} for higenamine and coclaurine by injecting 10 times the same sample.

Intra-day precision was evaluated at three different concentration levels (2 ng mL^{-1}, 50 ng mL^{-1} and 200 ng mL^{-1}) with 10 independent spiked urine samples on each level. The analysis was performed on the same day.

Inter-day precision was assessed by repeating the experiments for intra-day precision on two consecutive days.

Accuracy was assessed by spiking 10 urine samples with concentrations of 50 ng mL^{-1} for higenamine and coclaurine. Accuracy was expressed as *Recovery* (determined concentration/nominal concentration × 100 %).

2.6. Pharmacokinetics

Half-lives of higenamine and coclaurine were estimated for each subject from urinary excretion rate data. Semilogarithmic plots of renal excretion rates versus time were prepared for the elimination phase, post administration of higenamine. The elimination rate constant (k) was determined from the slope of the plots, and elimination half-lives were calculated from k.

3. Results and discussion

Previous studies show that consumption of some plant extracts, especially some traditional Chinese medicines containing higenamine, could result in AAFs [5,6]. Some cases might be inadvertently doping, especially if the preparations are not adequately labelled. Adequate knowledge about supplementation and products on the market is necessary to recognize potentially harmful preparations. From another side, there are many products which clearly state that they contain higenamine in quite high doses (20 mg per serving or more). Hence, the goal of this study was to define excretion patterns of higenamine after advertently use in expected doping doses. To support data for higenamine misuse, the excretion profile of coclaurine, its metabolite, was also investigated. Even though coclaurine does not have pharmacological activity like higenamine, its elimination pattern could be used as additional evidence of higenamine misuse.

Chemically higenamine (or norcoclaurine) is (1-[(4-hydroxyphenyl) methyl]-1,2,3,4-tetrahydroisoquinoline-6,7-diol, while coclaurine is (1S)-1-(4-hydroxybenzyl)-6-methoxy-1,2,3,4-tetrahydroisoquinolin-7-ol (Fig. 1). They can both be eliminated from the body as glucuronide and sulphate conjugates, but sulpho-conjugates are significantly more abundant. Detail metabolic pathway along with corresponding chemical structures for metabolites are described in the literature [7]. According to WADA TD2022MRPL [3] the cut-off level for higenamine is 10 ng/mL based on the parent form. Hence, it is very important to have adequate separation between the parent compound and conjugate forms. Gruzsa at all [11] pointed out that a compound peak with a molecular weight of 80 Da larger than higenamine was found in the urine samples of athletes, which was presumed to be a sulfonated metabolite of higenamine. Additionally, Guo at all [6] showed that the sulpho-group of higenamine-sulphate is very easily separated from the higenamine skeleton after entering the mass spectrometer, and the remaining skeleton is higenamine. However, the position of the sulphate moiety is unknown, but the elimination of higenamine in the sulpho-conjugated form is evident. Due to structural similarity the same behavior in mass detector could be expected for coclaurine.

In this study, special attention was put on separation between parent molecules and sulpho-conjugates. This is to avoid misinterpretation of obtained results due to potential co-elution of the parent compound and the sulphated form. Therefore, as the reference material for sulphate metabolites are not commercially available, exact masses were added in the full scan. Selected exact masses were *m/z* 352.0818 and 366.0974 for higenamine sulphate and coclaurine sulphate, respectively. Sulpho conjugated forms were followed in full scan mode while parent compounds were followed in full scan and MS/MS modes.

To obtain adequate separation between parent compound and sulphate products, different chromatographic columns and organic modifiers were tested. Considering physico-chemical properties of the analytes, C18 as stationary phase was selected at first. Even small changes in column characteristics (length, particle size, diameter) led to inadequate separation of either higenamine and its sulphate derivative or coclaurine and its sulphates. In most of the experiments, a gradient with methanol as organic modifier was used. Replacement of methanol with acetonitrile led to better peak shape but inadequate separation between parent compounds and sulphate conjugates. Finally, the best

Table 1
Validation data for higenamine and coclaurine.

Validation parameter	Substance	
	Higenamine	Coclaurine
Calibration curve	**Y = 0.000188x + 0.000795**	**Y = 0.000766x + 0.005194**
Linearity (2–200 ng/mL)	$R^2 = 0.9977$	$R^2 = 0.9972$
LOQ (ng/mL)	0.6	0.8
LOI (ng/mL)	2.5	2.5
LOD (ng/mL)	0.2	0.24
Repeatbility (RSD%) (50 ng/mL)	7.3	8.8
Intermediate precision (RSD%)		
2 ng/mL	3.4	3.4
50 ng/mL	1.1	0.1
200 ng/mL	4.2	6.0
Matrix interference (%)	54	49
Accuracy (%)	97	104

separation was obtained on an XTerra MS C18 column (100 mm × 2.1 mm, 3.5 μm) using methanol as organic modifier in a gradient program. All above mentioned scouting experiments were done using a dilute and shoot method combined with online SPE. This was shown to be a fast and efficient cleaning step. In all experiments, samples with 0 % of organic modifier were sent through a nonpolar Phenyl-Hexyl column. After 2 min, the pre-cleaned samples were then directed to the chromatography column.

The optimized online SPE LC-HRMS method was validated according to current WADA guidelines [17]. The results of the method validation are summarized in Table 1.

By analysing ten different blank urine samples, a good selectivity was demonstrated with no interfering signals observed at the higenamine and coclaurine retention times.

The method proved to be linear over the concentration range studied with a correlation coefficient of (R2) > 0.99. Nominal concentrations in all calibration's points fulfilled the criteria defined in [18] for linearity testing. Obtained equations for calibration curves as well as coefficient of determination are presented in Table 1. A comparison to the calibration curve prepared in water indicated matrix interferences at around 50 %.

Furthermore, higenamine and coclaurine could easily be detected according to WADA criteria [19] in the six urine samples spiked at a concentration above 2.5 ng/mL. Criteria were met for all 4 transitions listed in the Experimental part. LOQ and LOD were calculated using the calibration curve. Selected transitions for quantification were *m/z* 272.13 → *m/z* 107.0493 and 286.14 → *m/z* 107.0484 for higenamine and coclaurine, respectively.

The obtained values for LOQ, LOI and LOD are presented in Table 1 and confirmed that the developed method is sensitive enough for both doping analysis and the pharmacokinetic study. Additionally, obtained results for LOQ meet the criteria defined in [18]. Nominal concentration determined LOQ values were less than 20 % and 16 % for higenamine and coclaurine, respectively.

The calculated values of RSD for intra – and inter-assay precision, summarized in Table 1, are within an acceptable range (below 10 %). Furthermore, there is no sample carry-over.

Obtained *recovery* values for accuracy testing are presented in Table 1.

With respect to stability, the samples from the clinical study have been analysed three times over the past three years, employing slightly different methods. In all experiments, we obtained consistent results. This demonstrates evidence of stability through freeze–thaw cycles and long-term storage at −20 °C.

In conclusion, on the basis of the evaluated validation parameters, the analytical assay proved to be fit for purpose.

The validated method was applied for the determination of the

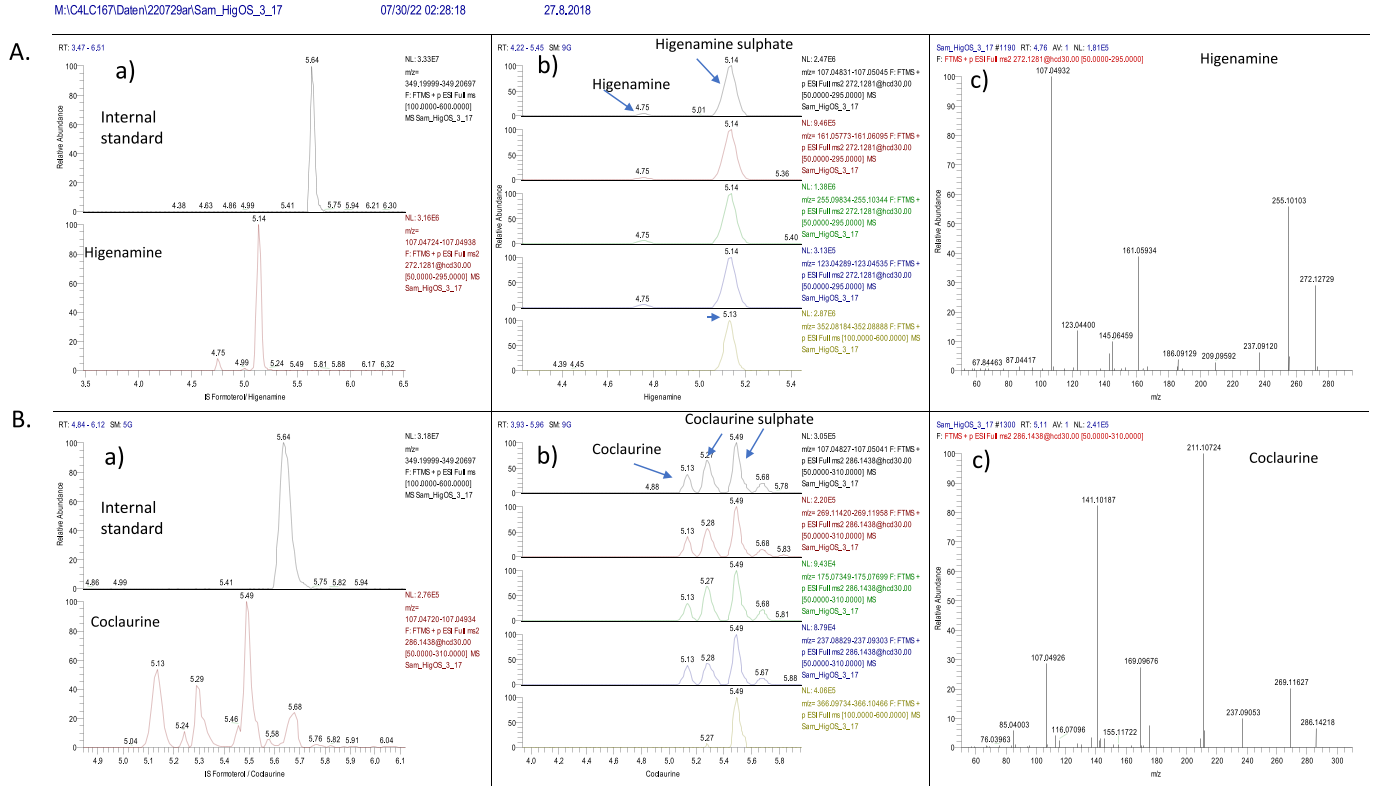

Fig. 2. Representative chromatograms - subject 3, day 16. A. Higenamine a) internal standard (full scan) and higenamine (product ion scan) b) real sample c) mass spectra. B. Coclaurine a) internal standard (full scan) and coclaurine (product ion scan) b) real sample c) mass spectra.

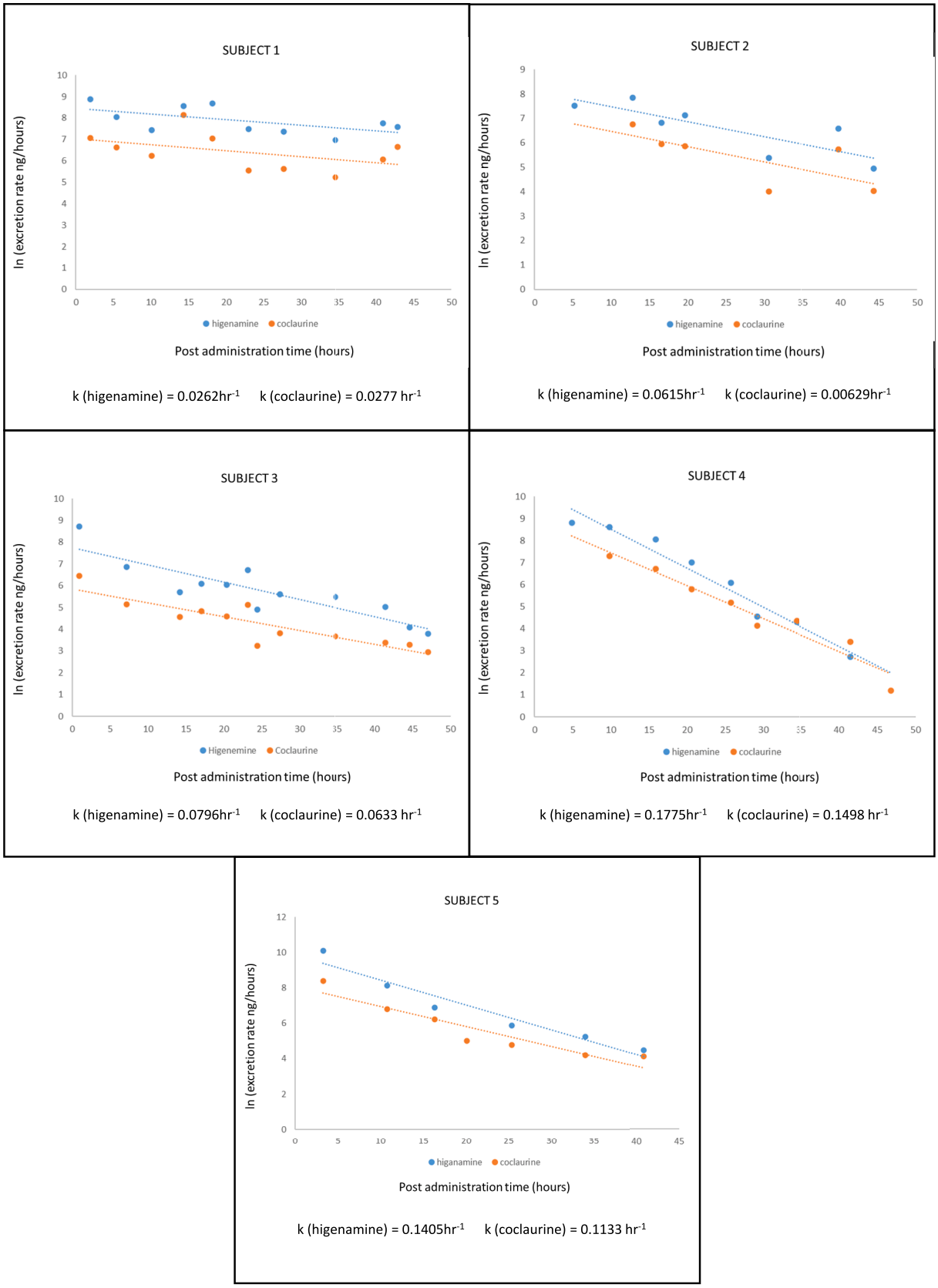

Fig. 3. Semilogaritmic plot of the excretion rate of higenamine and coclaurine after multiple administrations of higenamine (p.o.), subject 1–5.

Table 2
Calculated half-lives for higenamine and coclaurine.

	Higenamine	Coclaurine
Vol	$t_{1/2}$ (h)	$t_{1/2}$ (h)
1	26.5	25.0
2	11.3	11.0
3	8.7	11.0
4	3.9	4.6
5	4.9	6.1
6	–	–
Average	**11.1**	**11.5**

concentration of higenamine and coclaurine in urine samples from six subjects after multidose *per os* application of Higenamine capsules.

Representative chromatograms are presented in Fig. 2. As shown, a base line separation was obtained for higenamine and the metabolites higenamine sulphate, coclaurine, and coclaurine sulphates. Two sulphated forms of coclaurine were detected. Due to the lack of reference material, an unambiguous identification of the sulphated metabolites could, however, not be performed. Nevertheless, the chromatographic separation of higenamine and coclaurine from its sulphated forms is essential for the correct quantification of the free form since they share the same mass transitions. The WADA reporting cut-off of 10 ng/ml is based on the free form of higenamine [3].

3.1. Clinical study

All pharmacokinetic parameters were estimated from urine data. Urine samples are highly relevant for doping analysis representing a non-invasive sample collection. For the calculation of pharmacokinetic data, however, the uncertainty of complete bladder emptying and need to collect urine over short intervals might represent some limitations on the accuracy of the excretion rate data. Nevertheless, such data can provide a good basis for the estimation of half-lives and identification of excretion patterns.

Obtained urine concentrations of higenamine and coclaurine were corrected for specific gravity and urinary excretion rates were calculated and graphically displayed. Semilogaritmic plots of the elimination excretion profiles obtained from five subjects are shown in Fig. 3. Elimination half-lives for higenamine and coclaurine were estimated from the data obtained. The half-lives are summarized in Table 2. One of the subjects was excluded from the calculations because the associated excretion profile clearly indicated the intake of higenamine after day 21. Hence, no elimination phase could be analysed and evaluated.

Significant inter-individual differences in half-lives were observed, for both higenamine and coclaurine. The shortest half-life was estimated to be 3.9 h and 4.6 h for higenamine and coclaurine, respectively (subject 5). The longest half-life was 27 h for higenamine and 25 h for coclaurine (subject 1). Interestingly, the data indicate that the elimination of coclaurine is either rate-limited by its formation, or the true half-lives of higenamine and coclaurine are similar. This is supported by the observation of similar elimination speed of drug and metabolite in all subjects. Thus, when the elimination rate constant for a drug is smaller than that of the metabolite, metabolite elimination is formation rate-limited, and the metabolite will decline with the same speed as the drug.

According to WADA regulations, higenamine urine concentrations above 10 ng/ml are reported as adverse analytical findings. During the application period, this concentration was exceeded for all subjects. Furthermore, the concentration exceeded 10 ng/mL for at least 20 h after the last administration of higenamine for all subjects. As an example, the excretion profile for subject 3 is shown in Fig. 4.

4. Conclusion

In the presented study an on-line SPE - LC/HRMS method was applied for the characterisation of excretion profiles of higenamine and its main metabolite coclaurine, after higenamine supplement administration. Pharmacokinetic excretion profiles and elimination half-lives were defined for both substances, and substantial interindividual differences were observed. Furthermore, the time window in which urine samples would be positive according to WADA regulations was investigated, and higenamine could be detected above the cut-off (10 ng/mL) for at least 20 h after the last supplement application.

CRediT authorship contribution statement

B. Stojanovic: Methodology, Investigation, Validation, Formal analysis, Writing – original draft, Visualization. **J. Rasic:** Conceptualization, Investigation, Resources. **M. Andjelkovic:** Conceptualization, Investigation, Resources. **N. Dikic:** Conceptualization, Investigation, Resources. **N. Dragicevic:** Investigation. **B. Djordjevic:** Supervision. **G. Forsdahl:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Visualization, Supervision. **G. Gmeiner:** Conceptualization, Supervision, Writing – review & editing, Project administration.

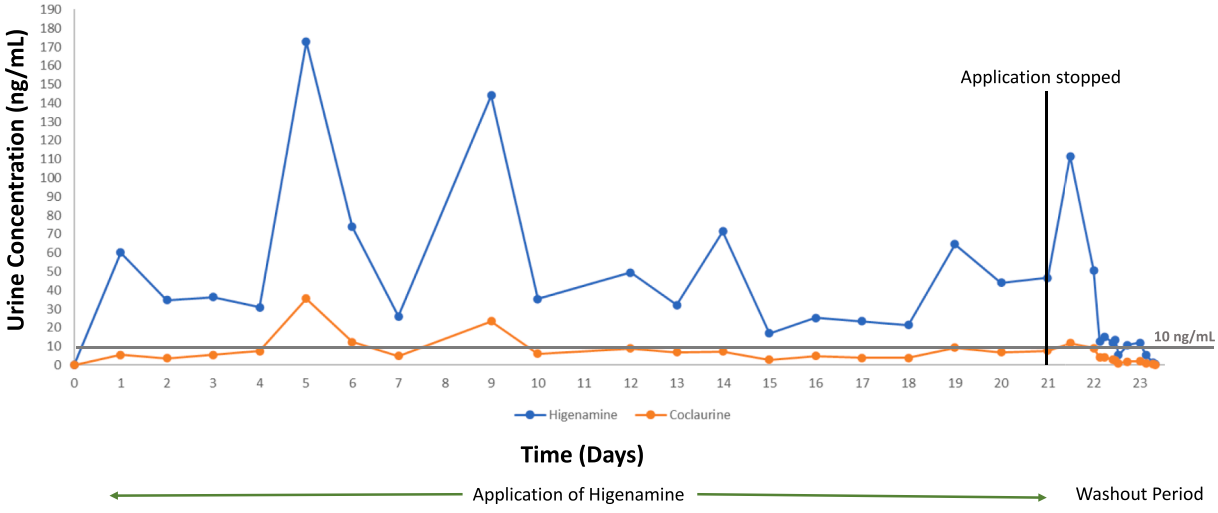

Fig. 4. Excretion profile of higenamine and coclaurine (subject 3).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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