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Probing for the presence of semenogelin in human urine by immunological and chromatographic-mass spectrometric methods in the context of sports drug testing

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Abstract

Rationale: An increasing number of adverse analytical findings (AAFs) in routine doping controls has been suspected and debated to presumably result from intimate contact with bodily fluids (including ejaculate), potentially facilitating the transfer of prohibited substances. More precisely, the possibility of prohibited drugs being present in ejaculate and introduced by sexual intercourse into the vagina of an athlete and, subsequently, into doping control urine samples, was discussed.

Methods: Two testing strategies to determine trace amounts of semenogelin I, a major and specific constituent of semen, were assessed as to their applicability to urine samples. First, the testing protocol of a lateral flow immunochromatographic test directed against semenogelin was adapted. Second, a liquid chromatography/tandem mass spectrometry (LC-MS/MS)-based method was established, employing solid-phase extraction of urine, trypsinization of the retained protein content, and subsequent detection of semenogelin I-specific peptides. Sensitivity, specificity, and reproducibility, but also recovery, linearity, precision, and identification capability of the approaches were assessed. Both assays were used to determine the analyte stability in urine (at 3 µL/mL) at room temperature, +4°C, and -20°C, and authentic urine samples collected either after (self-reported) celibacy or sexual intercourse were subjected to the established assays for proof-of-concept.

Results: No signals for semenogelin were observed in either assay when analyzing blank urine specimens, demonstrating the methods' specificity. Limits of detection were estimated with 1 µL and 10 nL of ejaculate per mL of urine for the immunochromatographic and the mass spectrometric approach, respectively, and figures of merit for the latter assay further included intra- and interday imprecision (4.5-10.7% and 3.8-21.6%), recovery (44%), and linearity within the working range of 0-100 nL/mL. Spiked urine tested positive for semenogelin under all storage conditions up to 12 weeks, and specimens collected after sexual intercourse were found to contain trace amounts of semenogelin up to 55-72 h.

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1 | INTRODUCTION

Analytical approaches in sports drug testing undergo continuous refinement[1] and have been optimized for utmost retrospectivity and, consequently, sensitivity especially for those substances that are prohibited by the World Anti-Doping Agency (WADA) at-all-times.[2] In consideration of the existing limitations in testing frequency, it has been desirable to extend the detection windows for banned substances by means of most modern analytical instrumentation combined with dedicated research into metabolic biotransformation processes, providing the lowest detection limits and long-term traceability of drugs and their metabolites in blood, urine, and other matrices.[3] While the rationale of this strategy is comprehensible and logical, the question whether adverse analytical findings (AAFs) are the result of deliberate doping or originate from inadvertent and/or unknown exposure to drug residues has been raised lately at numerous occasions.[4] Addressing this question analytically is a challenging task, and a specific situation that concerns the possibility of drug/drug metabolite transfer through intimate contact and exposure to ejaculate has received much attention, which warrants further research and investigation to assist decision-making processes in anti-doping.

Drugs have been determined in semen in various studies, mostly concerning antibacterial therapeutics but also regarding antiarrhythmic and 'social' drugs such as methadone and amphetamine,[5] as well as aza-steroids.[6,7] More recently, naturally produced hormones were analyzed and quantified in seminal plasma and blood serum, including a variety of androgens, estrogens, corticoids, and so on, and concentrations were predominantly lower in seminal fluid than in blood plasma.[8,9] The ejaculate is largely composed of fluids secreted from the prostatic gland (ca. 30%) and the seminal vesicles (ca. 60%), plus contributions from the epididymis, the ampullae, the bulbourethral and the urethral glands. This is relevant insofar as the respective pH of the constituents affects the blood plasma/seminal fluid ratio of drugs. Assuming a blood plasma pH of 7.4 and a prostatic fluid pH of 6.6, weak bases can concentrate in the prostatic fluid; conversely, with vesicular fluid exhibiting an alkaline pH of 7.8, drugs would partition at approximately equal concentration, if no additional factors would apply. Those additional factors however exist, including plasma protein binding, lipophilicity, metabolic biotransformations, and so on.[10,11] As a result, only a limited amount of information exists concerning the range of drug and drug metabolite concentrations in the ejaculate.

A hypothetical scenario through which a urine sample could be contaminated with prohibited substances would be the presence of these compounds in ejaculate, which enters the athlete's vagina during sexual intercourse, and fractions of which subsequently drip into or flush out with (and into) the urine sample of a routine doping control test. In such cases, markers specific for semen such as the highly abundant protein semenogelin I (Figure 1) would be present in the doping control urine sample; if absent, the claim of ejaculate containing prohibited substances being the reason for an athlete's AAF is not supported. In forensic casework, semenogelin I (which prevails in human ejaculate at approximately 20-350 µM/1-17.5 mg/mL,[12] respectively 4-68 mg/mL[13]) is routinely detected by rapid lateral flow immunochromatography and liquid chromatography-mass spectrometry.[14–20] Hence, these two strategies were adapted for anti-doping follow-up investigations in order to sensitively determine the presence of semenogelin I in urine, representing options for a rapid initial testing (ITP) as well as a confirmatory (CP) procedure. Following assay characterization, semenogelin I was analyzed from spiked urine after storage at RT, +4°C, and -20°C over a period of up to 12 weeks.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

The Rapid Stain Identification (RSID™)-semen field test was obtained from Independent Forensics (Lombard, IL), and the human semenogelin I ELISA kit was purchased from Abbkine (Wuhan, China). Acetonitrile and methanol (both HPLC grade) were from VWR International GmbH (Darmstadt, Germany), formic acid (p.a.) from Fluka (Darmstadt, Germany), and dimethylsulfoxide (99.9%) from Alfa Aesar (Kandel, Germany). Ammonium bicarbonate (99%), dithiothreitol (DTT, 99.5%), hemoglobin from bovine blood (lyophilized powder), and MQuant non-bleeding pH indicator strips were purchased from Merck-Millipore (Steinheim, Germany), Chromabond HLB solid-phase extraction (SPE) cartridges (60 mg, 3 mL) were obtained from Macherey-Nagel (Düren, Germany), and modified trypsin (sequencing grade) was from Promega Corporation (Madison, WI).

2.2 | Samples

Following ethical approval by the local ethics committee of the German Sport University Cologne (#101/2020) and informed written consent, the required collective of biological material was obtained.

A total of 20 blank urine samples was collected from 20 different healthy females (age: 20-50 years) after an abstinence from sexual intercourse for at least 72 h. The pH (4-8) and protein content (9-290 mg/mL) of all blank urine samples was estimated by means of pH indicator strips and microvolume spectrophotometry (NanoDrop One, Thermo Fisher Scientific, Dreieich, Germany), respectively. Further, for proof-of-concept analyses, one set of 14 urine samples was obtained from one volunteer, composed of one pre- and 13 post-coital urine specimens collected up to 70 h after sexual intercourse.

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Chemistry Europe
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QKGGSKGRLP SEFSQFPHGQ KGQHYSGQKG KQQTESKGSF SIQYTYHVDA NDHDQSRKSQ
QYDLNALHKT TKSQRHLGGS QQLLHNKQEG RDHDKSKGHF HRVVIHHKGG KAHR**GTQNPS**
QDQGNSPSGK GISSQYSNTE ERLWVHGLSK EQTSVSGAQK GRKQGGSQSS YVLQTEELVA
NKQQRETKNS HQNKGHYQNV VEVREEHSSK VQTSLCPAHQ DKLQHGSKDI FSTQDELLVY
NKNQHQTKNL NQDQQHGRKA NKISYQSSST EERRLHYGEN GVQKDVSQSS IYSQTEEKAQ
GKSQK**QITIP SQEQEHSQK**A NKISYQSSST EERRLHYGEN GVQKDVSQRS IYSQTEKLVA
GKSQIQAPNP KQEPWHGENA KGESGQSTNR **EQDLLSHEQK** GRHQHGSHGG LDIVIIEQED
DSDRHLAQHL NNDRNPLFT

FIGURE 1 Primary structure of semenogelin I (UniProtKB - P04279 (SEMG1_HUMAN)). Target tryptic peptide sequences for LC-HRMS/MS analysis are indicated in bold letters

Semen samples were obtained from five healthy males (age: 24-31 years), who indicated no use of any medication within the last seven days, and the semenogelin I content (8.3-11.2 mg/mL) was determined employing a commercially available and target analyte-specific sandwich ELISA.

All samples were stored frozen (-20°C) until preparation for immediate analysis or preparation of the stability/traceability study that was conducted under different storage conditions (vide infra).

2.3 | Lateral flow immunochromatographic analysis

One approach to test urine samples for the presence of semenogelin and, thus, contamination through ejaculate employed a commercially available and highly sensitive lateral flow immunochromatographic strip test utilizing two monoclonal anti-semenogelin I antibodies.[21] Predominantly developed for forensic applications, the manufacturer's sample preparation and analysis protocol was slightly modified for the purpose of testing doping control urine samples for seminal fluid residues. Positive and negative controls were prepared and analyzed as recommended; however, instead of reconstituting stain residues (*e.g.* from swabs) in the provided universal buffer (which contains an undisclosed and proprietary mixture of salts, chelating agents, proteins, detergents, and preservatives[22]), 20 µL of urine were merely mixed with 80 µL of the buffer, and 50 µL were applied to the sample window of the test cassette. Ten min after application, the result (presence or absence of semen) was photographically documented.

2.4 | Liquid chromatography-tandem mass spectrometry

Probing for the presence of semenogelin I by liquid chromatography-tandem mass spectrometry (LC-MS/MS) was conducted using a Thermo Scientific (Dreieich, Germany) Vanquish™ UHPLC system coupled to a Thermo Scientific Orbitrap Exploris™ 480 mass spectrometer.

Urine samples prepared for analysis were injected onto an Accucore Phenyl/Hexyl trapping column (3 × 10 mm, 2.6 µm, Thermo Fisher Scientific, Dreieich, Germany) using formic acid (0.1%) for isocratic loading for 2 min at 400 µL/min. After 2 min, the effluent was directed to the analytical column (Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm, Agilent Technologies, Santa Clara, CA) employing the eluents A (0.1% formic acid and 1% DMSO in water) and B (0.1% formic acid and 1% DMSO in ACN) at a flow rate of 0.45 mL/min. Gradient elution was conducted from 1% of B to 40% B in 8 min, followed by an increase of B to 80% B within 4 min, and re-equilibration at starting conditions for 4 min, yielding an overall runtime of 18 min. The LC was interfaced by electrospray ionization (ESI) in positive mode to the mass spectrometer, applying an ionization voltage of 3 kV and an auxiliary gas and transfer capillary temperature of 300°C. Full scan (*m/z* 400 to 1700 at a resolution of 60000 full width at half maximum) as well as a targeted MS^2 experiments (*m/z* 100 to 1700; quadrupole isolation window: 2 *m/z*; resolution 30,000 FWHM) for proteotypical peptides were conducted, applying normalized collision energies of 25-30 as detailed in Table 1. Nitrogen for auxiliary and collision gas supply was obtained through an N_2-generator (CMC, Eschborn, Germany), and the instrument was calibrated according to the manufacturer's instructions on a weekly basis.

2.5 | Sample preparation for LC-MS/MS analysis

In order to test for residues of semenogelin I by LC-MS/MS, urine samples were subjected to different sample preparation steps. First, a volume of 0.5 mL of urine was fortified with 1 µg of bovine hemoglobin as internal standard (ISTD, 10 µL of an aqueous solution containing 100 µg/mL), and the sample was further diluted with 0.5 mL of 50 mM aqueous ammonium bicarbonate. The mixture was loaded onto a preconditioned (3 mL of ACN followed by 3 mL of deionized water) SPE cartridge, and the resin was washed with 2 mL of deionized water before elution with 1.5 mL of ACN/water (80/20, v/v) into a 2 mL protein low-bind Eppendorf tube. The solvent was evaporated to dryness in a vacuum centrifuge, and the residue was re-dissolved in 50 µL of 50 mM ammonium bicarbonate. Second, the recovered proteins were subjected to a standard trypsinization protocol.[23] In brief, 5 µL of 1 M DTT was added and the sample incubated at 60°C in a heating block agitated at 900 rpm. After 60 min, the solution was cooled to RT, and 7 µL of ACN and 400 ng of trypsin (10 µL of a 40 µg/mL solution) were added prior to incubation at 37°C for 12 h and agitation at 500 rpm. The hydrolysis was terminated by the addition of 5 µL of glacial acetic acid, and the samples were transferred to LC vials for analysis.

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TABLE 1 Characteristics of proteotypical peptides of semenogelin I and the internal standard used in the LC-MS/MS assay

Analyte	Peptide	Amino acid sequence	Sum formula	Precursor Ion (*m/z*)	Qualifier ion 1 (*m/z*)	Qualifier ion 2 (*m/z*)	NCE (%)
Semenogelin I (human)	T_{21}	GTQNPSQDQGNSPSGK	$C_{62}H_{100}N_{22}O_{28}$	801.36	1201.54	388.22	25
	T_{46}	QITIPSQEQEHSQK	$C_{69}H_{113}N_{21}O_{26}$	826.92	1197.55	214.16	30
	T_{57}	EQDLLSHEQK	$C_{51}H_{83}N_{15}O_{20}$	613.80	628.31	741.39	25
Hemoglobin (bovine)	T_6	FFESFGDLSTA1DAVMNNPK	$C_{93}H_{136}N_{22}O_{31}S$	1045.48	1147.55	1432.70	30

Abbreviation: NCE, normalized collision energy.

2.6 | Assay characterization

Both approaches offering applications as ITP and CP, respectively, were characterized with regards to specificity, sensitivity, and reproducibility. For the CP, also recovery, linearity, precision, and identification capability were assessed.

The characteristics of the RSID test system were extensively tested and documented earlier.[21] Therefore, for the application to diluted urine, the lateral flow immunochromatographic ITP's specificity was determined using 10 different blank urine samples (female volunteers), which were analyzed and assessed for interfering signals. The assay's LOD was estimated by spiking six different blank urine samples with 10 µL of seminal fluid (containing semenogelin I at ca. 11.6 mg/mL) and subsequent dilution of the samples with the corresponding blank urine to yield a concentration of 1 µL/mL. The samples were then analyzed using the established protocol to probe for the method's capability to detect lowest concentrations of semen (i.e. 1 µL/mL), where the reproducible detection of bands for the target analyte and the control was considered as sufficient. The assay reproducibility was tested by spiking six urine samples to 5 µL/mL and preparing and analyzing one aliquot each per day on three consecutive days (n = 18).

The characterization of the CP was conducted analogously. The specificity was assessed by analyzing ten different blank urine samples for interfering signals at expected retention times and diagnostic precursor/product ion pairs with regards to the proteotypical peptides of semenogelin I (Table 1). The method's LOD was estimated from a dilution series (1 µL/mL, 10 nL/mL, and 1 nL/mL) employing 10 different urine samples. If the identification of at least two tryptic peptides meeting the identification criteria of WADA's technical document TD2021IDCR[24] was accomplished, then semenogelin I was considered as confirmed. Peak areas of extracted ion chromatograms obtained from spiked urine samples (containing seminal fluid at 5, 10, 20, 50, and 100 nL/mL) were used to probe for the linearity of the method in the defined working range, and assay imprecision (n = 6), reproducibility on three consecutive days (n = 3+3+3), recovery of solid-phase extraction (n = 3), and identification capability (n = 10) were determined at a urinary concentration of seminal fluid of 100 nL/mL.

2.7 | Analyte stability

In order to probe for the capability of both test methods (ITP and CP) to detect semenogelin I in urine samples stored under different conditions, three different urine samples were spiked to a concentration of 3 µL/mL, and aliquots of 1 mL were prepared in protein low-bind Eppendorf tubes (1.5 mL) for storage at room temperature (20°C), refrigerated (+4°C), and frozen (-20°C). Simulating doping control urine sample transport and storage scenarios, the following testing protocol was applied: after one week of storage, aliquots of all three urine samples kept at all storage conditions were analyzed, refrigerated and frozen samples were further assayed after 4 weeks of storage, and frozen samples were additionally analyzed after 12 weeks of storage.

3 | RESULTS AND DISCUSSION

3.1 | Lateral flow immunochromatographic analysis

All blank urine samples analyzed with the lateral flow immunochromatographic test yielded negative results, demonstrating the specificity and lack of interference caused by urinary matrix components (Figure S1). All six blank urine samples spiked with semen and diluted to 1 µL/mL were tested positive for semenogelin, corroborating the assay's capability to detect minute contaminations of urine with semen and allowing for estimating a LOD of 1 µL/mL (or better, Figure S2). Also, result reproducibility was shown for three sample replicates of six specimens, prepared on three consecutive days each (Figure S3).

3.2 | Liquid chromatography-tandem mass spectrometry

Confirmation of the presence of semenogelin in an athlete's urine sample is also accomplished by LC-MS/MS, and a summary of the assay characteristics is presented in Table 2. Three target tryptic peptides were used to identify semenogelin I, namely T_{21} (GTQNPSQDQGNSPSGK), T_{46} (QITIPSQEQEHSQK), and T_{57} (EQDLLSHEQK), which were monitored by diagnostic precursor/product ion pairs as summa-

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TABLE 2 Assay characterization results

Parameter	Concentration of seminal fluid in urine (nL/mL)	Target peptide		
		T_{21}(precursor at $m/z = 801$)	T_{46}(precursor at $m/z = 826$)	T_{57}(precursor at $m/z = 613$)
Specificity (n = 10)			OK	
Linearity	0-100	$R^2 = 0.96$	$R^2 = 0.99$	$R^2 = 0.92$
Limit of detection	10	10/10	10/10	9/10
(n = 10, detection rate)	1	7/10	10/10	8/10
Precision (n = 6)	100	CV = 10.7%	CV = 4.5%	CV = 4.5%
Reproducibility, 3 days (n = 3)	100	CV = 21.6%	CV = 4.6%	CV = 3.8%
Recovery (n = 3)	100		44%	
Identification capability (n = 10)	100		OK	
Stability (n = 3)				
1 Week	+20°C	3000	OK	
	+4°C	3000	OK	
	-20°C	3000	OK	
4 Weeks	+4°C	3000	OK	
	-20°C	3000	OK	
12 Weeks	-20°C	3000	OK	

rized in Table 1. Employing these peptides, a target analyte-specific assay was developed, offering a LOD of 10 nL/mL (comparable to literature data), a recovery of 20-58%, an intraday imprecision of 4.5-10.7%, a reproducibility of 3.8-21.6%, and an identification capability at 100 nL/mL. Representative extracted ion chromatograms of a blank urine and a urine sample spiked with seminal fluid at 0.1 µL/mL are illustrated in Figure 2 along with the product ion mass spectra of the target peptides. Of note, T_{46} and T_{57} were found to yield better precision and reproducibility data than T_{21}, tentatively attributed to higher analytical responses of T_{46} and T_{57}.

3.3 | Analyte stability

The degradation of proteinaceous analytes over time in urine samples has been reported for selected target analytes in doping controls. Whether or not such degradation processes also affect semenogelin I and/or the test methods applied in this study was investigated concerning different storage conditions (room temperature, refrigerated, and frozen) for a period up to 12 weeks. Under the chosen scenarios, both test methods detected semenogelin I in all urine samples, suggesting that the target analyte was not (substantially) affected by transport or storage conditions that commonly exist in the context of doping controls (Table 2).

3.4 | Application to pre- and post-coital urine samples

The CP was applied to a set of urine samples collected from a volunteer before and up to 70 h after unprotected sexual intercourse. Similar to Figure 2, two extracted ion chromatograms are depicted in Figure 3 using T_{46} and T_{57} to demonstrate the absence of semenogelin I in the pre-coital sample and the presence of both peptides in the urine sampled 55 h after sexual intercourse. T_{46} was detected also in a urine sample collected 17 h later (i.e., at 72 h), but not T_{57}, which would not fulfil the identification criteria for semenogelin applied in this study (data not shown).

3.5 | Doping controls

Detecting urine contamination with semen at 1 µL/mL appears adequate for most sports drug testing purposes considering the reported drug residues in ejaculate, if the "flush-out" scenario is to be verified or falsified. A hypothetical assessment as to the utility of the presented test methods for result management in anti-doping requires the consideration of average volumes of doping control urine samples, reported drug concentrations in semen, and applicable minimum required performance levels and/or minimum reporting limits in anti-doping.

A suitable urine volume for routine doping controls is 90 mL, which is distributed with 60 mL into the A- and 30 mL into the B-sample container.[25] Further, as an exemplary compound, amphetamine was reported in an earlier study to be present in a user's semen specimen at 54 ng/mL,[5] and it is a substance that is prohibited in sport in-competition only, for which a reporting limit of 50 ng/mL applies.[26] In order to introduce amphetamine into 90 mL of urine at an amount that results in urinary concentrations in excess of 50 ng/mL, 4500 ng of amphetamine are required. With an amphetamine semen concentration of 54 ng/mL, more than 80 mL of ejaculate would be required. Assuming a 100-fold higher concentration of amphetamine

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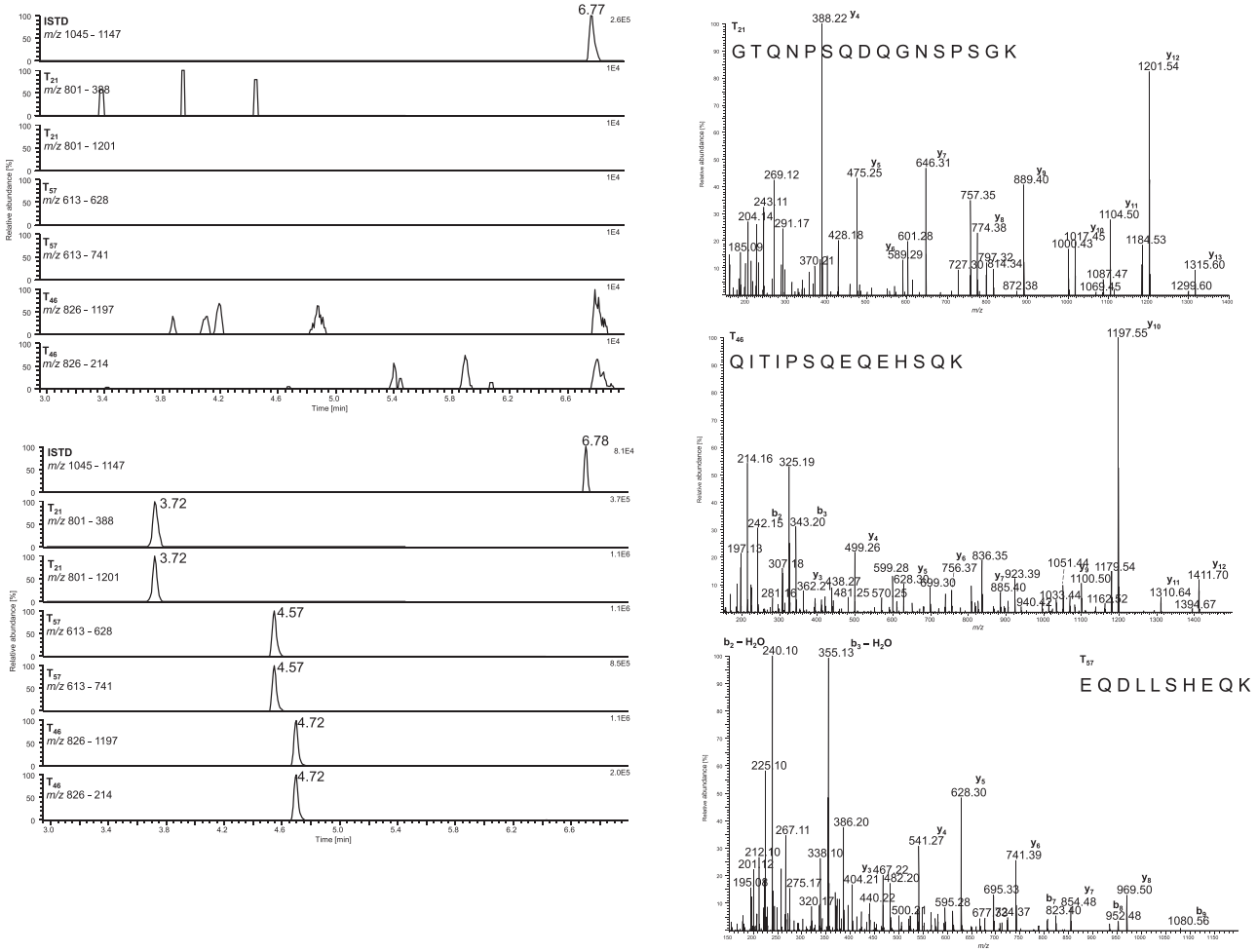

FIGURE 2 Left, extracted ion chromatograms of a blank urine (top) and a urine specimen containing 100 nL of semen (bottom); right, product ion mass spectra of characteristic peptides of semenogelin I (T_{21}, T_{46}, and T_{57}, top to bottom)

in seminal fluid, the presence of 800 µL of semen in 90 mL of urine would be necessary, representing a contamination of 9 µL/mL, which both test methods (ITP and CP) reported herein would readily identify.

The accomplished assay sensitivity has been considered relevant in particular as, currently, it cannot be excluded that non-threshold substances such as anabolic agents, for which extremely sensitive anti-doping analytical methods exist, are present in semen at the ng/mL level. If an anti-doping test method allows for the detection of 5 pg/mL of an anabolic agent, the presence of the drug at 5 ng/mL in semen in conjunction with a contamination of 90 mL of urine with 100 µL of that semen could result in an AAF. Here, testing for seminal fluid at 1 µL/mL would be necessary to provide further data in support of the result management.

4 | CONCLUSION

The potential of drug contamination scenarios involving intimate contact has been repeatedly discussed and argued in various cases of AAFs. Amongst several others, one such scenario, the introduction of ejaculate via "flush-out" or "dripping" into a doping control urine sample, can be verified or falsified by probing for the presence of semenogelin, which was successfully accomplished using lateral flow immunochromatographic and mass spectrometric tests. Obviously, the mere presence of semenogelin in urine does not provide unequivocal evidence as to how a drug or its metabolites were introduced in an athlete's urine sample; however, the absence of semenogelin essentially excludes the aforementioned "flush-out"/"dripping" scenario as the main reason for drug (metabolite) detection. Future research will be required to facilitate assessing the plausibility of AAFs through intimate contact in general. For instance, investigating the drug (metabolite) concentration ranges of anabolic agents in semen would substantially furnish the data necessary to assess AAFs arguably resulting from sexual intercourse, and also the passage of time since presumed drug exposure or the relevance of absorption site(s) might need to be factored in into result interpretation processes.

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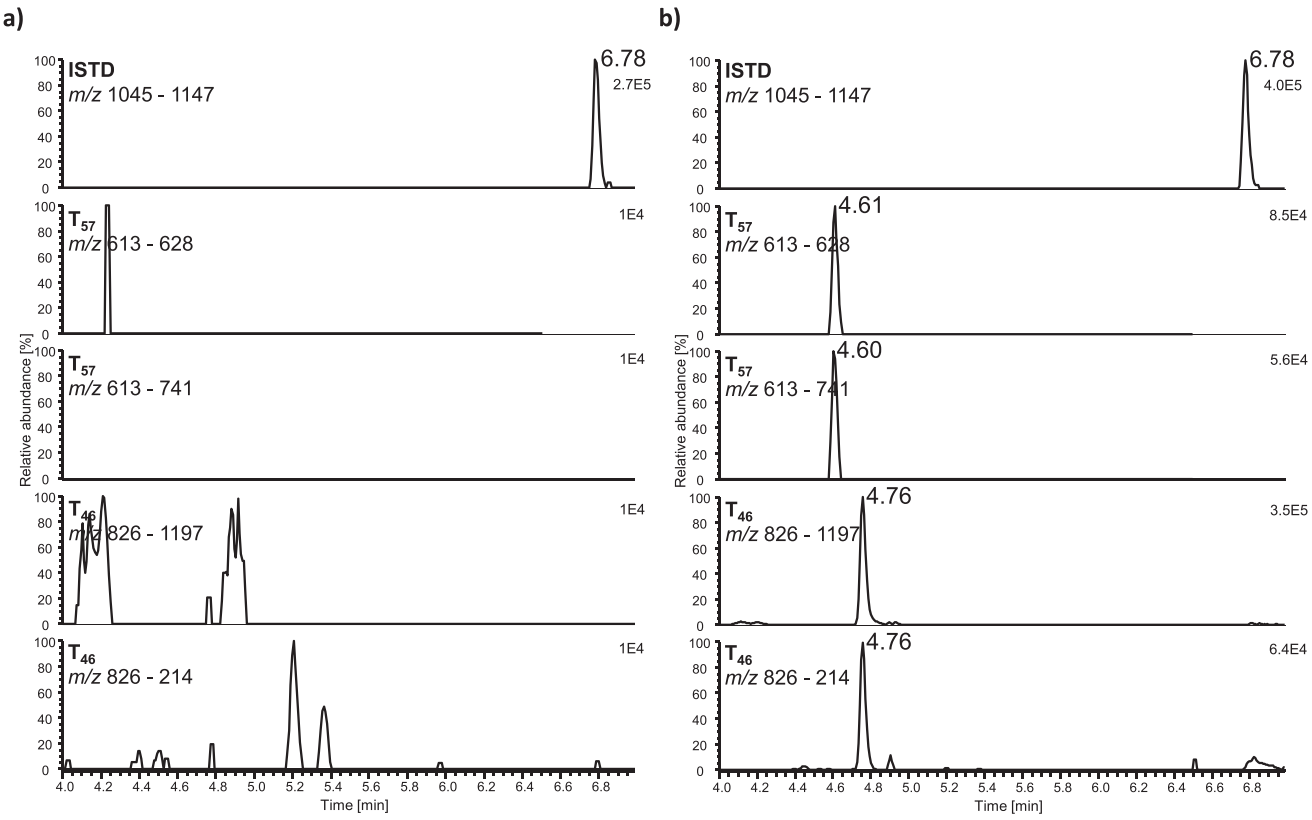

FIGURE 3 Extracted product ion chromatograms of urine samples tested for the presence of semenogelin I collected pre- (a) and 55 h post-coital (b). Two diagnostic peptides are identified post-coital with T_{46} and T_{57}

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AUTHOR CONTRIBUTIONS

Johanna Breuer and **Andreas Thomas** were associated with methodology, investigation, and formal analysis; **Hans Geyer** and **Mario Thevis** were associated with conceptualization, supervision, wrote the original draft, and reviewed and edited the final manuscript.

ETHICS STATEMENT

Ethical approval was granted by the ethics committee of the German Sport University Cologne (Nr. 101/2020)). All volunteers gave their informed consent for participation and publication in anonymized form.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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