Received: 12 September 2019 | Revised: 10 October 2019 | Accepted: 11 October 2019

DOI: 10.1002/dta.2717

SHORT COMMUNICATION

WILEY

The occurrence of putative cognitive enhancing research peptides in seized pharmaceutical preparations: An incentive for controlling agencies to prepare for future encounters of the kind

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Abstract

At the end of 2017 and 2018 two different unknown suspicious preparations were encountered and were subjected to a plethora of different analyses in order to identify, if present, any bioactive compound. It turned out that these samples contained the assumedly cognitive enhancing research peptides Selank and Semax, which, to our knowledge, have not completed any clinical trials. Moreover, an online search, excluding the dark web, demonstrated that these kinds of nootropic research peptides are freely available either as lyophilized powder for injection purposes or are present in nasal sprays. It stands to reason that controlling laboratories need to anticipate the uprising of these types of potentially dangerous molecules and must therefore be able to correctly identify these compounds. Therefore, these findings served as an incentive to develop a novel combined liquid chromatography tandem mass spectroscopy (LC–MS/MS) methodology, applicable to both hydrophilic or more hydrophobic peptides, which was utilized to analyze a total of 10 putative cognitive enhancing polypeptides, with variable biochemical characteristics, that are currently being sold online. The screening rationale, complying to the recommendation paper of the General European Official Medicines Control Laboratory (OMCL) network on the interpretation of screening results for unknown peptides by mass spectrometry, was also validated in different matrices as required by ISO 17025.

KEYWORDS

falisified medicine, forensic analysis, illegal peptide

1 | INTRODUCTION

It is a truth almost universally acknowledged that illegal peptide drugs for human use can easily be acquired from illicit internet pharmacies whether or not disguised as "research companies". Numerous reports demonstrate the popularity of these peptides, as reviewed by Janvier et al.[1] These products are mainly used to improve sports performance or to strive to comply to the current cultural and societal ideals (e.g. the use of weight loss enhancers and the use of tanning peptides in Western and Northern Europe). The danger of the illegal usage of these polypeptides resides in the fact that these products are not necessarily produced under a controlled environment and consequently could contain the wrong ingredient, the wrong dosage, pathogenic bacteria, heavy metals, etc.,[1-4] which may result in severe health issues. Furthermore, for many of these polypeptide drugs, there is no, or limited, knowledge of their effects

and/or side-effects (long and short term) hence clinical studies have not even been initiated, are ongoing, or were given a negative advice due to the overall potential adverse health effects. Therefore, global actions are on-going to protect the sometimes unaware public from these malign activities. In Belgium, the Federal Agency for Medicines and Health Products (FAMHP) is responsible for this task and upon their request unknown pharmaceutical preparations suspected to contain illegal drugs or "research chemicals" are regularly subject to analysis in our official medicines control laboratory (OMCL).

At the end of 2017, an unknown unlabeled pharmaceutical preparation was submitted by the FAMHP for analysis by means of liquid chromatography–tandem mass spectrometry (LC–MSn). Based on the interpretation of the LC–MSn data, we were not able to identify this substance as being one of these "classical" peptides described in the plethora of case reports or present in our in-house library.[5] Further analysis by full scan high-resolution, accurate-mass (HRAM) MS/MS combined with a newly developed LC gradient, applicable to both hydrophobic and hydrophilic peptides, de novo peptide sequencing, digestion by specific proteases, and final confirmation by custom synthesized reference standard showed that the substance present in the sample corresponded to a polypeptide with sequence TKPRPGP, known as Selank.[6] Moreover, in 2018, another more hydrophobic unknown peptide was encountered in a seized sample, that, upon some mass spectral similarities (of the MS/MS spectrum) with Selank and subsequent de novo sequencing, followed by confirmation with a reference standard, confirmed the identity of this polypeptide, with the sequence MEHFPGP, as Semax.[7]

Both these peptides are sold online and are probably taken to improve mental performance. Selank is a synthetic analog of the endogenous tuftsin peptide (a fragment of the human immunoglobulin G heavy chain) which was elongated at its C terminus by the addition of a tripeptide with the sequence PGP, in order to improve its metabolic stability.[8] It has been shown that this heptapeptide has a cognition enhancing or nootropic action and positively influences the formation of memory and learning processes in rats.[7,9] A similar effect was also attributed to Semax, another synthetic heptapeptide, consisting of a fragment of adrenocorticotropic hormone (ACTH4–7) C-terminally fused to the tripeptide PGP. Additionally, immunomodulatory and neuropsychotropic effects have also been ascribed to these molecules.[7,10,11] Currently and to the best of our knowledge, no clinical studies with these peptides have been terminated or were given a positive advice by the European Medicines Agency. Therefore, the use of these research peptides on humans has not been approved by the European Union and can therefore be considered illegal.

Nevertheless, a simple internet search by means of some popular legal web search engines, demonstrated that these nootropic peptides, including Selank, Semax, their related substances or other research peptides intended for cognition enhancing effects, are freely available online. They are either sold as lyophilized powder for injection purposes or present in nasal sprays. Unsettlingly, their use is also openly discussed on the different internet fora and on legal video sharing websites. Based on the way they are perceived through these communication channels, it is very likely that these molecules have the potential to become very popular in the future. It stands to reason that the controlling laboratories need to anticipate the uprising of these illegal compounds and must have handled tools to correctly identify these compounds. Therefore, we have subjected eight additional, either custom made or commercially purchased, putative cognitive enhancing polypeptides that were available online, to the newly developed full scan LC–MS/MS methodology (see section 3.2). Our results clearly demonstrate that the utilized screening methodology is able to distinguish between these 10 peptides, based on the criteria put forward in the recommendation paper of the General European OMCL network on the interpretation of screening results for unknown peptides mass spectrometry.[12] Moreover, the screening rationale was also validated in different matrices as required by ISO 17025.

Taken together, with this manuscript we demonstrate not only the applied reasoning to come to the unambiguous identification of two different unknown peptides but also how this encounter served as an incentive to develop and validate a screening method applicable to other illegal nootropic peptide preparations that are already available online and which are currently being purchased by the sometimes unaware public.

2 | EXPERIMENTAL

2.1 | Chemicals and reagents

Water was obtained using a milliQ-Gradient A10 system (Millipore, Billerica, USA). Reference standards for noopept (lot 104M4738V), tuftsin (lot GR147149–9), Semax (lot 035M4701V), orexin A (lot 019M4753V), and orexin B (lot CS0198) were bought from Sigma Aldrich (St Louis, MO, USA). The remaining peptides were custom synthesized to a HPLC purity grade that exceeds 95% (Thermo Fisher Scientific, Ulm, Germany).

The products used to generate the different matrices or the buffer solutions, utilized during the digestion with either sequencing grade trypsin or with sequencing grade Arg-C or rLys-C (Promega, Madison, WI, USA), were purchased from Sigma Aldrich (St Louis, MO, USA).

Both acetonitrile (UPLC/MS grade) and formic acid (analytical grade), were purchased from Biosolve (Valkenswaard, the Netherlands).

2.2 | Preparation of the reference standards to compare with the unknown suspicious samples

All reference standards were solubilized in water at a final concentration of 0.5 mg/mL. These stock solutions were then diluted with water to 0.1 mg/mL and acidified to 1% formic acid prior to injection onto the LC–MS/MS system. For comparison of the retention time of the peptides with the sequences HEMFPGP and MEHFPGP and the suspicious samples, the reference standards were spiked into the acidified prepared sample at a final concentration of 0.01 mg/mL.

2.3 | Preparation of the suspicious samples

An aqueous solution of the samples was prepared by adding 500 μL of water to the vials of sample A (seized at the end of 2017) and sample B (seized in 2018), both containing a white, flocculent powder (see Figure 1A and Figure 3A). The powder dissolved almost immediately upon brief swirling. A 10 × dilution of these solutions was made in water and formic acid was added to a final concentration of 1%. Next, the samples were subjected to centrifugation for 15 minutes at 20238 × g in a micro centrifuge prior to analysis by means of either our standard LC–MS^n methodology[5] or the newly developed HRAM LC-MS/MS methodology (see further).

2.4 | Digestion of the suspicious polypeptide with Arg-C or rLys-C

A 20 μL aliquot of the intact suspicious polypeptide solution was subjected to digestion with either sequencing grade Arg-C or rLys-C, according to the manufacturer's recommendations. Briefly, for the digestions with Arg-C, the intact peptide was first diluted 5 times in incubation buffer (final concentration of 50 mM Tris–HCl pH 7.8, 5 mM $CaCl_2$, and 2 mM EDTA) in anticipation of the enzymatic overnight digestion at 37°C with 1 μg activated sequencing grade Arg-C. The latter was obtained by adding 1,4-dithiothreitol (DTT) to the mixture to a final concentration of 2 mM.

For the digestion with rLys-C, the intact peptide was diluted 5 times in a buffer with final concentration of 25 mM Tris, 1 mM EDTA, pH 8.5 prior to the overnight digestion at 37°C with 1 μg sequencing grade rLys-C (Promega, Madison, WI, USA). Subsequently, the digests were acidified with 10% formic acid, dried (stored if applicable at −20°C for a maximum of 1 week) and reconstituted in 100 μL 1% formic acid just prior to analysis by HRAM LC–MS/MS.

2.5 | HRAM LC–MS/MS for hydrophilic and hydrophobic nootropic peptides

High resolution accurate mass (HRAM) LC–MS/MS analyses were carried out on a Thermo Scientific™ Vanquish™ ultra-high performance liquid chromatography (UHPLC) system coupled to a Q Exactive™ focus orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany).

The unknown samples and the reference standards (injection volume: 1 μL) were separated on an Acquity UPLC HSS T3 column (100 mm × 2.1 μm, 1.8 μm particle size) (Waters, Milford, MA, USA). The LC methodology was as follows: isocratic elution for 1 minute of 100% mobile phase A (0.1% of formic acid in water) at a constant flow rate of 0.4 mL/min and a column temperature of 40°C. The gradient started from 0 to 24% B (0.1% formic acid in acetonitrile) in 9 min, increased to 60% B in the next minute, then was kept at 60% B for 3 min, and finally was increased to 98% B for 1 minute, followed by a 2 minute re-equilibration step (total run time: 17 minutes).

The Q-Exactive focus mass spectrometer was operated in positive ion mode with alternating full MS scans of the precursor ions and all ion fragmentation scan (AIF) in which the peptides were fragmented by higher energy collisional dissociation (HCD). Both scan types were performed with 70,000 resolution (at m/z 200) with the maximum ion injection time set at 50 milliseconds. The m/z range for the full MS scans was 200–1000, and the m/z range for AIF scans was 100–1000. The target value for the full MS scans was 10^6 ions and the target value for the AIF scans was 3×10^6 ions. The normalized HCD collision energy was set at 30%. The heated electrospray ionization (HESI) conditions were as follows: spray voltage: 3.5 kV; sheath gas flow rate: 53 arb; auxiliary gas flow rate: 14 arb; heated capillary temperature: 269°C; S-lens RF level: 50 V. Nitrogen was used for spray stabilization and as the collision gas in the C-trap.

All data were collected in profile mode and were acquired and processed by using the Thermo Xcalibur 4.0 software (Thermo Fisher Scientific, Bremen, Germany). In order to visualize the total ion chromatograms obtained for all the reference standards, the raw data files were processed by mzmine 2.[13]

The MS and MS/MS queries were performed using the amino acid sequences of the respective peptides or by means of de novo sequencing by PEAKS X (Bioinformatics Solutions Inc., Waterloo, Canada). A 10 ppm precursor tolerance for full MS spectra and a 0.03 Da fragment tolerance for the MS/MS spectra were accepted. No non-specific cleavage or modifications were allowed.

2.6 | Preparation of the reference standards for the screening method and subsequent validation

Stock (0.5 mg/mL) solutions were made in pure water and working (0.1 mg/mL) solutions were made in 1% formic acid, prior to the determination of the retention time (RT), the most intense precursor ion and the average relative intensity of the fragment ions (at least 20 measurement points were averaged).

Moreover, for the validation of the screening method, as required by ISO 17025, we applied the same strategy as described by the guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed,[14] which has been successfully utilized for these kinds of products in the past.[5,15] Briefly, the peptides were diluted at the chosen screening detection limit (SDL = 10 μg/mL), into the three different matrices. Matrix 1 mimicked a basic nasal spray (150 mM NaCl), while matrix 2 (40 mM Tris pH 7.4 and 150 mM NaCl) and matrix 3 (matrix 2, supplemented with 5% mannitol), mimicked possible excipients that might be present in the lyophilized material. The SDL is the lowest concentration for which it has been demonstrated that a given analyte can be correctly identified in at least 95% of the samples.

For the identification of the peptides, we applied the criteria described in the recommendation document, generated by the general European OMCL network, on the interpretation of screening results for unknown peptides and proteins by mass spectrometry based methods.[12] In short, a combinatory utilization of the retention time

(RT), the full scan HRAM-MS spectrum (see Figure 5) of the precursor ions, and the full scan HRAM-MS/MS spectrum of the chosen most intense or discriminatory b or y fragment ions resulted in a specific identification of the peptides of interest (see Table 1) and this by largely exceeding the minimal required identification points.[12] The error tolerated on the relative retention time was ±0.15 minutes, and the errors accepted for the either the MS and MS/MS spectra were set at 10 ppm and 0.03 Da, respectively.

3 | RESULTS AND DISCUSSION

3.1 | Identification of the mysterious peptides present in the seized samples

Prior to the mass spectrometric analysis both the samples were analyzed by means of SDS-PAGE and subsequent Coomassie staining. In both cases the migration pattern of these samples revealed the presence of a polypeptide.

3.1.1 | Identification of the peptide present in sample A

Next to the abovementioned analysis, this sample was also subjected to our routine peptide identification methodology.[5] Unfortunately, the polypeptide was not retained by the applied reversed phase LC-gradient and resulted in the presence of a doubly charged precursor ion with an average m/z of 376.7, which did not match with the peptides present in our in-house database. However, a Russian manuscript on doping drugs, published at the end of 2017, described a multi-parametric targeted LC–MS/MS and HRAM LC–MS approach targeting doping peptides, including the Russian research peptide Selank. This peptide, with sequence TKPRPGP, was detected as a doubly charged

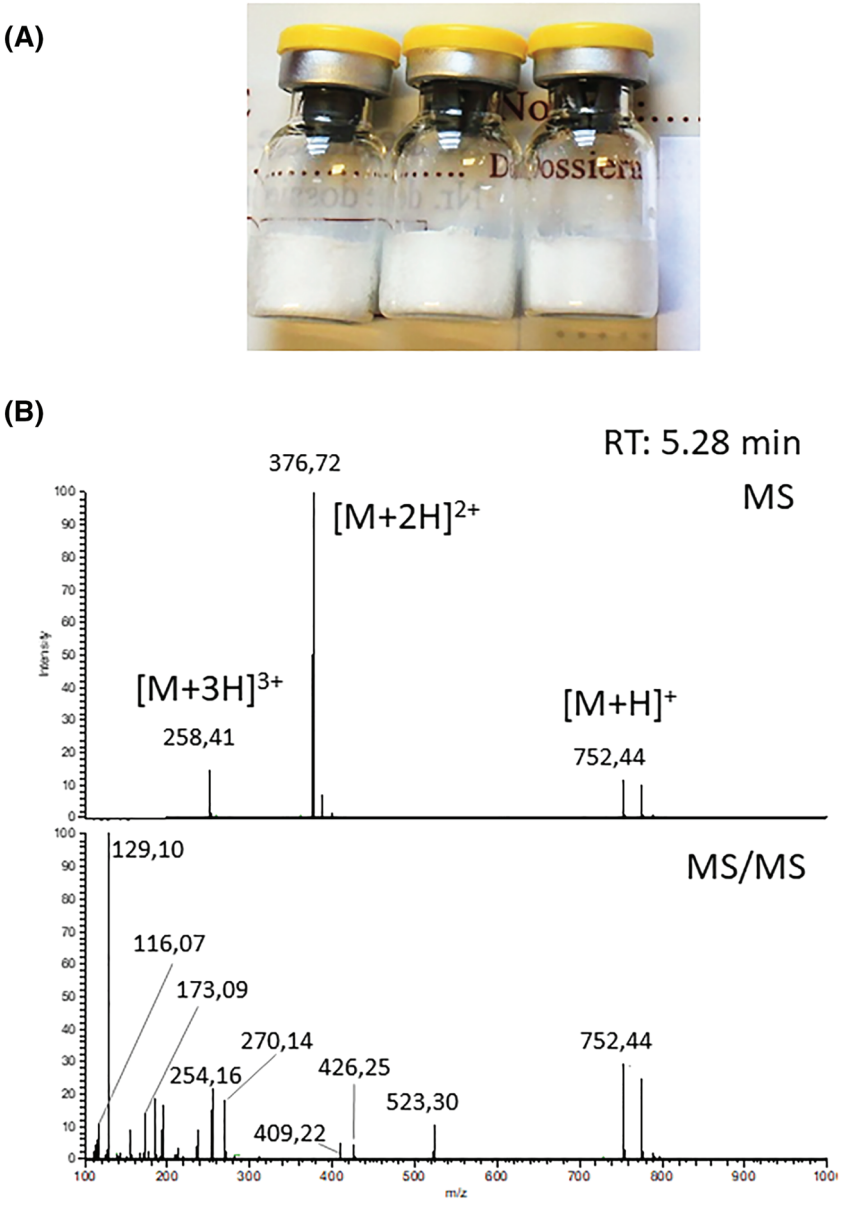

FIGURE 1 Picture of the unlabeled vials intercepted in 2017 by the FAMHP (A). Full scan HRAM MS and MS/MS spectrum of the component present in the unknown sample and eluting at 5.28 minutes (B)

ion with m/z 376.724 (HRAM-MS data).[6] Moreover, our low resolution MS/MS spectrum obtained also demonstrated that the two most intense fragment ions with an m/z of 129.1 and m/z of 254.1 correlated with the m/z that were encountered in the Russian study.

However, since the outcome of our analysis can be used in court, the presence of the suspected peptide needs to be unambiguously proven. In order to do so, we utilized a combination of HRAM LC–MS/MS approaches while waiting for the custom synthesized references standard as recommended.[12] Initially, we first determined the exact monoisotopic mass of the peptide by means of a newly developed MS-compatible LC-methodology that can be utilized for both polar and hydrophobic peptides (see materials and methods). Next, we performed

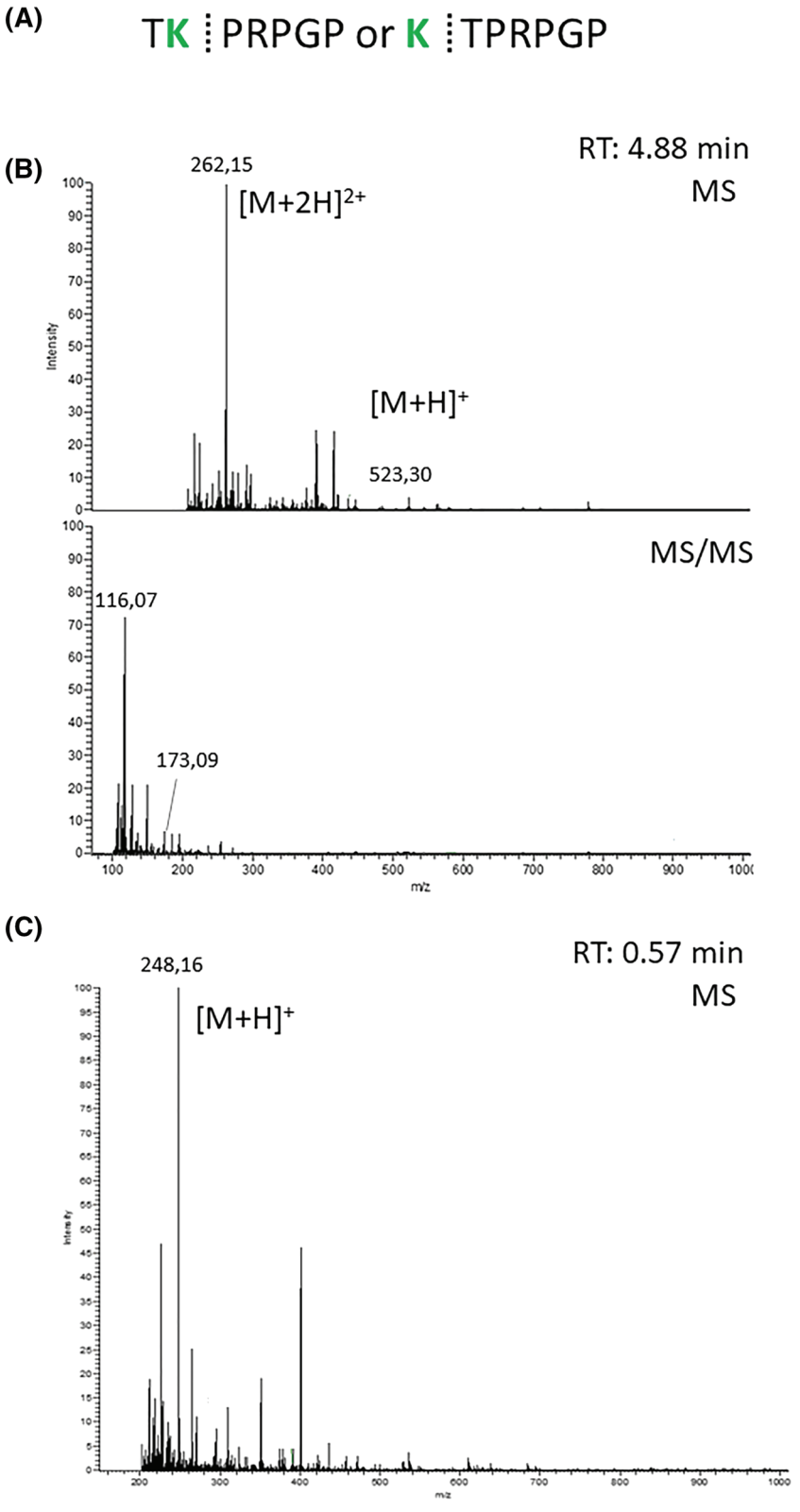

FIGURE 2 One letter amino acid sequences of the two potential in silico hits and the rLys-C cleavage site enabling discrimination between these two peptides (A). Full scan HRAM MS and MS/MS spectra of the two peaks present in only the total ion chromatograms (TIC) of the digested unknown peptide sample. The peptide eluting at 0.57 minutes corresponds to dipeptide TK (B) while the pentapeptide eluting at 4.88 minutes reflects the sequence PRPGP (C)

an enzymatic digestion of this peptide with Arg-C and rLys-C and analysis of the digested peptide parts by full scan HRAM LC–MS/MS.

The full scan HRAM MS spectrum of the intact unknown peptide revealed that the doubly charged molecule had an m/z of 376.724, as observed in the Russian study, and eluted at 5.28 minutes. Also, a triply charged molecule with an m/z of 251.484 and a single charged molecule with an m/z of 752.44 were visible in the MS spectra. The most intense peaks in the MS/MS spectrum with an m/z of 129.102 corroborated with the most intense peak described in the Russian paper for this specific polypeptide (see Figure 1B). Next, these MS and MS/MS data were subjected to de novo sequencing queries and, based upon our acceptance criteria (see material and methods), only two possible very similar polypeptide sequences, KTPRPGP and TKPRPGP, were generated and this with a precursor mass error of

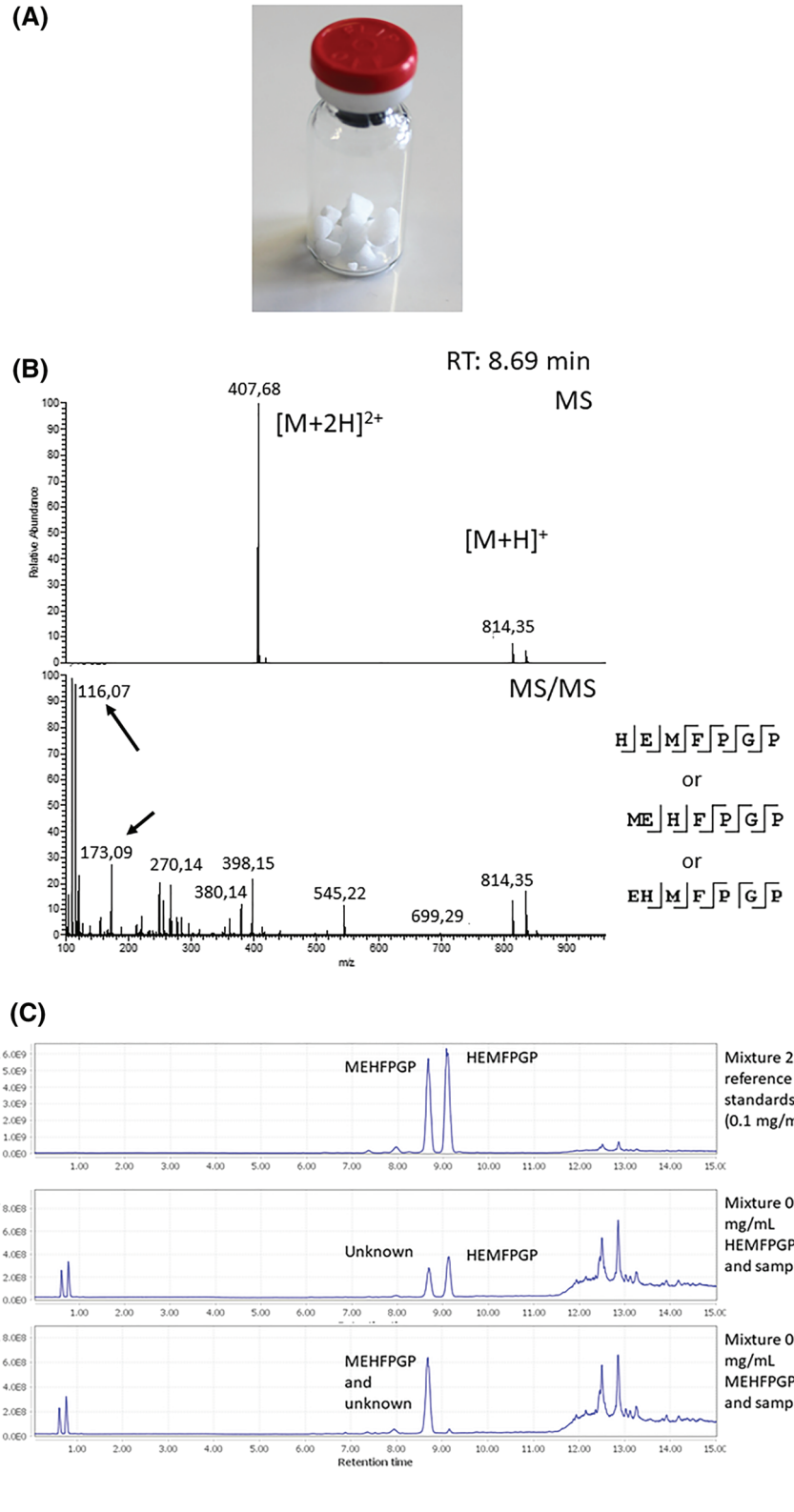

FIGURE 3 Picture of the unlabeled vials intercepted in 2018 by the FAMHP (A). Full scan HRAM MS and MS/MS spectrum of the component present in the unknown sample and eluting at 8.69 minutes (B). The arrows indicate the occurrence of the same fragment ions as has been seen for the tripeptide PGP or the pentapeptide PRPGP. Injection of 0.1 mg/mL of the custom synthesized reference peptides demonstrate a difference in the retention time of the two reference standards. Also a difference in RT between the unknown sample and the peptide HEMFPGP occurred while the unknown sample co-eluted with Semax (C)

1.5 ppm (see Table S1). In order to verify if the sequence of the suspected peptide indeed corresponded to one of the in silico obtained sequences, two digestions were performed in attendance of the ordered reference. The digestion with Arg-C should result in the generation of two peptides, KTPR or TKPR with a monoisotopic mass of 500.307 Da and the peptide PGP, with a monoisotopic mass of 269.138 Da (see Figure S1A). Indeed, the MS and MS/MS spectra of the digest with Arg-C resulted in the presence of a two additional peaks that were not present in the blank control, eluting at 0.72 min (see Figure S1B) and 3.00 min (see Figure S1C). The MS spectra at 0.72 min demonstrated the occurrence of a doubly charged precursor ion with an m/z of 251.160 which could be in agreement with the $[M + 2H]^{2+}$ ion of both peptides TKPR and KTPR. Unfortunately, the de novo sequencing of both the MS and MS/MS spectra was not able to distinguish between the two possible sequences. Additionally, the ion present at 3.00 minutes corresponded to a single charged precursor ion with an m/z of 270.144 and resulted in two intense fragment ions with an m/z of 116.071 and an m/z of 173.092, which very likely corresponded to the y_1 and y_2 of the peptide PGP (see Supplemental Figure 1C). Subsequently, a digestion with rLys-C was also performed in order to make a distinction between the two possible sequences.

If the sequence of the unknown peptide was indeed consistent with the sequence of Selank, the digestion should result in the generation of two peptides, a peptide PRPGP with monoisotopic mass of 522.294 and the dipeptide TK with monoisotopic mass of 247.153. Alternatively, the digestion of the sequence KTPRPGP, should produce the hexapeptide TPRPGP with monoisotopic mass of 623.339 and free lysine (see Figure 2A). Indeed, two peaks appeared in the MS chromatogram of the digested peptide, which were not present in the blank control. The initial peak, eluting with the solvent peak at 0.57 minutes, contained an ion with an m/z of 248.160 while the other peak, eluting at 4.88 minutes, reflected a doubly charged precursor ion with an m/z of 262.153 and which upon MS/MS fragmentation gave rise to the previously observed m/z of the y_1 and y_2 ions of the tripeptide PGP (Figure 2B and 2C). Taken together, all the above mentioned data clearly concur with the presence of the peptide Selank in the suspicious sample. This finding was then later corroborated by the subsequent analysis of the custom synthesized reference standard.

3.1.2 | Identification of the peptide present in sample B

The polypeptide present in the second sample, received in 2018, also contained an unknown doubly charged precursor ion with an average m/z of 407.64, which was not present in our in-house database or described in the available case reports on suspected illegal peptide drugs. Therefore, again this sample was subjected to HRAM MS/MS and our LC methodology applicable to both hydrophilic and hydrophobic peptides. The full MS scan of the intact peptide revealed the presence of a doubly charged molecule with an m/z of 407.681 that eluted at 8.69 minutes. The MS/MS of this molecule showed similarities to the MS/MS spectra obtained for the peptide fragment PGP, used to stabilize peptides (see Figure 3B). Additionally, the de novo sequencing, also suggested the possibility of three very similar peptide sequences containing a C-terminal PGP with an acceptable precursor mass error of 1.8 ppm (see supplemental table). From those sequences only the sequences HEMFPGP and MEHFPGP were retained since the third possibility, the sequence EHMFPGP, was not deemed stable due to the N-terminal glutamate that should have resulted in the formation of pyroglutamate upon heating, which did not occur (data not shown). An online search demonstrated that one of these sequences, MEHFPGP, is identical to the research peptide, Semax, developed in Russia. Unfortunately, due to the lack of affordable specific peptidases, we could not perform one or multiple digestions to come to a preliminary conclusion and therefore had to wait for the generation of the custom synthesized reference peptides to provide a final result to our stakeholders. Analysis of these reference standards showed a significant difference in the retention time since the reference standard of Semax eluted at 8.70 min, while the reference peptide HEMFPGP eluted at 9.06 min (see Figure 3C). Moreover, although the obtained MS/MS spectrum was quite similar between the reference standards, the MS/MS spectrum of HEMFPGP showed the presence of an additional intense ion with an m/z of 267.108, which was not present in the MS/MS spectrum of the Semax reference peptide or of the sample (see Figure S2). This specific fragment ion very likely corresponds to the b_2 ions of this peptide. It stands to reason that the obtained data clearly indicate the presence of the peptide Semax in the suspicious sample.

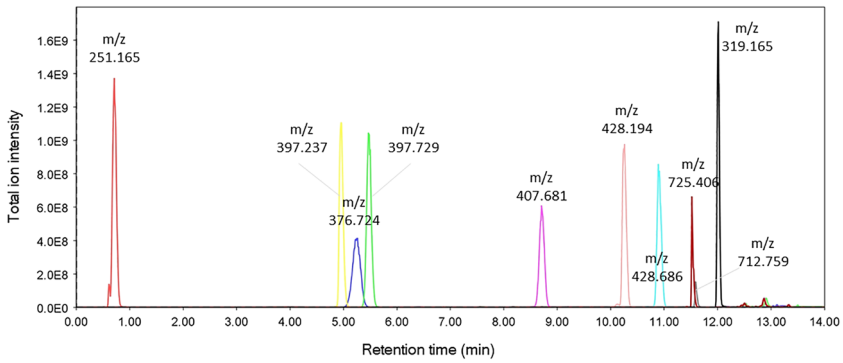

FIGURE 4 Total ion chromatograms of the full scan mass spectrum of the ten different peptides encountered online. Each m/z value corresponds to the measured precursor ion with the maximum intensity

TABLE 1 Characteristics of the different peptides: Name, monoisotopic mass, chemical formula, amino acid sequence, retention time, dominant charge state of the precursor ion, fragment ions, their relative intensities (above 5%) the corresponding possible identified fragments obtained during MS/MS. The underlined m/z of the fragment ions correspond to those ions chosen for identification purposes. Ac, N-acetyl; et, C-terminal ethyl ester; PheAc, N-phenylacetyl; Pyr, pyroglutamate

Name	Mono – Isotopic mass (Da)	Chemical formula	Amino acid sequence	RT (min)	Dominant precursor ion	Daughter ions and their relative intensities (MS_2)	Possible identified fragment ions (MS_2)
Noopept (or SGS 111 or GVS 111)	318.1580	$C_{17}H_{22}N_2O_4$	PheAc-PG-et	12.01	319.165 [M +H]	188.11 (100), 216.10 (17)	$C_{14}H_{14}NO^*$ $C_{13}H_{14}NO_2{}^*$
Tuftsin	500.3071	$C_{21}H_{40}N_8O_6$	TKPR	0.70	251.160 [M +2H] $^{2+}$	129.10 (100), 158.09 (29), 175.12 (73), 255.14 (29), 272.17 (16)	Immonium ion K y_1-$NH_3{}^+$ $y_1{}^+$ y_2-$NH_3{}^+$ $y_2{}^+$
Selank (or TP-7)	751.4341	$C_{33}H_{57}N_{11}O_9$	TKPRPGP	5.25	376.724 [M +2H] $^{2+}$	116.07 (13), 129.10 (100), 173.09 (13), 270.14 (15), 409.22 (5), 426.25 (5) 523.30 (6)	$y_1{}^+$ Immonium ion K $y_2{}^+$ $y_3{}^+$ y_4-$NH_3{}^+$ $y_4{}^+$ $y_5{}^+$
N-acetyl selank	793.4446	$C_{35}H_{59}N_{11}O_{10}$	Ac-TKPRPGP	5.47	397.729 [M +2H] $^{2+}$	116.07 (19), 129.10 (100), 173.09 (14), 254.16 (30), 270.14 (24), 409.22 (5), 523.30 (15)	$y_1{}^+$ Immonium ion K $y_2{}^+$ b_2-H_2O^+ $y_3{}^+$ y_4-$NH_3{}^+$ $y_5{}^+$
N-acetyl selank amidate	792.4606	$C_{35}H_{60}N_{12}O_9$	Ac-TKPRPGP-NH_2	4.94	397.237 [M +2H] $^{2+}$	115.09 (32), 129.10 (100), 172.11 (10), 254.16 (26), 269.16 (21), 408.23 (6), 522.31 (17)	$y_1{}^+$ Immonium ion K $y_2{}^+$ b_2-H_2O^+ $y_3{}^+$ y_4-$NH_3{}^+$ $y_5{}^+$
Semax	813.3480	$C_{37}H_{51}N_9O_{10}S$	MEHFPGP	8.70	407.681 [M +2H] $^{2+}$	104.05 (22), 110.07 (93), 116.07 (100), 120.08 (21), 173.09 (25), 270.14 (10), 380.14 (9), 398.15 (14), 545.22 (6)	Immonium ion M Immonium ion H $y_1{}^+$ Immonium ion F $y_2{}^+$ $y_3{}^+$ b_2-H_2O^+ b_3 b_4

(Continues)

TABLE 1 (Continued)

Name	Mono – Isotopic mass (Da)	Chemical formula	Amino acid sequence	RT (min)	Dominant precursor ion	Daughter ions and their relative intensities (MS_2)	Possible identified fragment ions (MS_2)
N-acetyl semax	855.3585	$C_{39}H_{53}N_9O_{11}S$	Ac-MEHFPGP	10.89	428.686 [M +2H] $^{2+}$	104.05 (32), 110.07 (100), 116.07 (89), 120.08 (6), 146.06 (18), 173.09 (21), 174.06 (6), 440.16 (16), 587.23 (27)	Immonium ion M Immonium ion H y_1^+ Immonium ion F a_1^+ y_2^+ b_1^+ b_3^+ b_4^+
N-acetyl semax amidate	854.3745	$C_{39}H_{54}N_{10}O_{10}S$	Ac-MEHFPGP-NH_2	10.26	428.194 [M +2H]$^{2+}$	104.05 (14), 110.07 (100), 115.09 (55), 146.06 (10), 412.16 (5), 440.16 (18), 587.23 (18)	Immonium ion M Immonium ion H y_1^+ a_1^+ a_3^+ b_3^+ b_4^+
Orexin A (human)	3558.7104	$C_{152}H_{243}N_{47}O_{44}S_4$	PyrPLPDCCRQKTCSCRLYELLHGAGNHAAGILTL-NH_2 (sulfide bridge 6-12 and 7-14)	11.59	712.759 [M +5H]$^{5+}$	110.07 (100), 131.12 (47), 214.16 (21), 726.58 (16), 776.35 (19), 853.90 (44)	Immonium ion H y_1^+ y_2-H_2O^+ b_{26}^{+4} b_{29}^{+4} b_{32}-H_2O^{+4}
Orexin B (human)	2897.5882	$C_{123}H_{212}N_{44}O_{35}S_1$	RSGPPGLQGRLQRLLQASGNHAAGILTM-NH_2	11.52	725.406 [M + 4H]$^{4+}$	104.05 (59), 110.07 (100), 556.81 (15), 606.58 (44), 628.10 (19), 683.88 (16), 684.13 (25), 696.39 (41), 697.06 (28), 966.48 (24)	Immonium ion M Immonium ion H b_{21}^{+4} b_{24}^{+4} b_{26}+H_2O^{+4} b_{27}-H_2O^{+4} b_{27}-NH_3^{+4} b_{20}^{+3} y_{20}-H_2O^{+3} y_{10}-H_2O

3.2 | Screening method for the identification of 10 nootropic research peptides offered online

The two abovementioned examples clearly illustrate that the illegal peptide market is diversifying rapidly and is currently no longer limited to potential doping peptides or physical appearance enhancing peptides, but contains also alleged mind doping or cognition enhancing research peptides. Indeed, a simple internet search for nootropic peptides has already given several hits leading to suspicious rogue internet pharmacies. Moreover, in addition to the different internet fora that openly discuss the benefits of these products, there are also several movies available (on the legal video sharing websites) that demonstrate how to use these substances. Based on this online information we have already encountered 10 different peptides that are possibly already offered and sold online as nootropic peptides, including Selank, Semax, and noopept.[16] These peptides are sold either as a lyophilized powder for injection purposes or are present in a nasal spray.

In order to anticipate any future encounters with these research peptides, we subjected either the custom synthesized peptides or the commercially available research peptides to the developed HRAM LC–MS/MS approach and verified its utility as a full scan screening methodology for these 10 peptides. For the identification of the peptides, we applied the criteria described in the recommendation document, generated by the general European OMCL network, on the interpretation of screening results for unknown peptides and proteins by mass spectrometry based methods.[13] Briefly, a combinatory utilization of the retention time (RT), the full scan HRAM-MS spectrum of the product ion, and the full scan HRAM-MS/MS spectrum of the most intense or discriminatory b or y fragment ions (two fragment ions for noopept; minimum three fragment ions for the other peptides) resulted in a specific identification of the peptides of interest (see Figure 4 and Table 1).

In compliance with the validation procedure (see material and methods), the screening method should not only be able to identify and distinguish the different peptides from each other, but also from the matrix ingredients frequently present in lyophilized peptides or in nasal sprays (e.g. mannitol, salts, buffer).[3] This has to be demonstrated for a certain concentration level, the screening detection limit (SDL) for which the respective peptide can be correctly identified in 95% of the samples and does not generate false-positives since the outcome of the screening methodology can be used in court cases.[5,16] Hereto, the validation involved the injection at the SDL of the 10 different peptides before and after spiking in the three different matrices described in the material and methods section. The value of the SDL was chosen to be higher than the highest limit of detection, determined by serial dilutions and with a signal to noise ratio (S/N) ≥ 3.3 of the chosen fragment ion with the lowest intensity (see Table 1), which was obtained for Orexin A (2 μg/mL). Moreover, all of the illegal products claim to contain at least 0.1 (nasal spray) to 10 mg (injectable) of the peptide in question. Consequently, the chosen concentration is situated between 10 to 1000 times lower than the amount typically present in the commercially available products or claimed to be present in these online available products. Therefore, the SDL of 10 μg/mL for all peptides is well below the amount of peptide present or claimed to be present in the sample and is thus appropriate for these studies.

Analysis of the abovementioned 30 different samples (the 10 peptides in the three different matrices) showed that the method was selective for all 10 peptide SDL and no false-positives or false-negatives were found. This is in compliance with the predefined limits for false-positives and false-negatives, indicating that our full scan HRAM LC–MS/MS screening methodology is fit for purpose, namely the screening and identification of suspicious unknown samples putatively containing nootropic peptides.

4 | CONCLUSION

Two unknown pharmaceutical preparations were submitted by the FAMHP for analysis in our OMCL laboratory and initially remained enigmatic with our routine methodology. Next, a plethora of different methodologies was utilized, including de novo sequencing, the use of specific proteases (if applicable), and confirmation with either custom synthesized or commercially available reference peptides, to come to an unambiguous identification that can stand in court. The sequences were consistent with the putative cognition enhancing research peptides Selank and Semax. These cases served as an incentive to generate and validate a full scan HRAM LC–MS/MS screening methodology that can be applied for the identification of the 10 putative cognition enhancing peptides, that are already available online through rogue internet "pharmacies" and which will very likely be encountered, if not prevailed upon yet, by other controlling laboratories.

ACKNOWLEDGMENTS

We would like to thank the FAMHP for the fruitful collaboration. Moreover, we would also like to express our appreciation to Magdalena Livadaris and the people of Thermo Scientific™ for their technical assistance when required.

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SUPPORTING INFORMATION

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

How to cite this article: Vanhee C, Francotte A, Janvier S, Deconinck E. The occurrence of putative cognitive enhancing research peptides in seized pharmaceutical preparations: An incentive for controlling agencies to prepare for future encounters of the kind. *Drug Test Anal*. 2020;1–11. https://doi.org/10.1002/dta.2717