Application of online automatic filtration and filter back-flush solid phase extraction in routine doping control analysis

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INTRODUCTION

Nano-liquid chromatography-mass spectrometry (nanoLC-MS) is a powerful tool for a variety of applications in (bio)analytical chemistry. Combining the use of columns with reduced inner diameter and the use of column switching systems allows measurements with increased sensitivity. However, a major challenge in the implementation of such setups is clogging of columns and connections, which requires labour intensive sample preparation (1) or the use of high dilution factors (2) to obtain clean extracts.

In order to avoid system clogging and the need for extra, time-consuming offline clean-up steps, online automatic filtration and filter back-flush (AFFL) was introduced by Svendsen et al. (3). It has been demonstrated that placing a filter-union upstream relative to the trapping cartridge and analytical column greatly reduced backpressure buildup and did not affect chromatographic performance during the analysis of protein precipitated plasma samples.

The focus of this current study was to assess the ruggedness of this technique in routine doping control analysis. As a first application, the hyphenation of dilute-and-shoot sample clean-up with online AFFL-nanoLC-MS was investigated for the analysis of small peptide hormones (MW < 2 kDa). The evaluation of ruggedness was based on pressure monitoring both over the trapping cartridge and the analytical column during ~ 300 injections of diluted urine samples. The practical potential in routine doping control analysis was shown by evaluating the obtained data based on the World Anti-Doping Agency (WADA) proposed minimum required performance level (MRPL), and chromatographic / mass spectrometric identification criteria (TD2015IDCR) (4-5).

EXPERIMENTAL

Sample preparation (final conditions): To 125 µL of urine (n=10) (spiked with peptides (Table 2) at 2 ng/mL), 122.5 µL dilution mix (98% H_2O, 2% HOAC, 1 ppm bovine insulin) and 2.5 µL ISTD (100 ng/mL) were added and centrifuged. From the supernatant, 25 µL was added to an LC-MS vial containing 175 µL dilution mix.

Instrumental analysis: For chromatographic separation, a nano-flow liquid chromatography system equipped with a high pressure gradient nano-flow pump and a low pressure gradient micro-flow loading pump was used (Dionex UltiMate 3000 RSLCnano). A PepMap100 C8 trapping column (5 µm, 300 µm x 5 mm) and a PepMap RSLC C18 analytical column (5 µm, 300 Å, 75 µm x 15 cm, thermostated at 35 °C) from Thermo Fischer Scientific were used for the preconcentration and separation of the peptides. The samples were filtrated online through a 1 µm stainless steel filter / screen (Valco). The schematic view of the switching valve is shown in Figure 1.

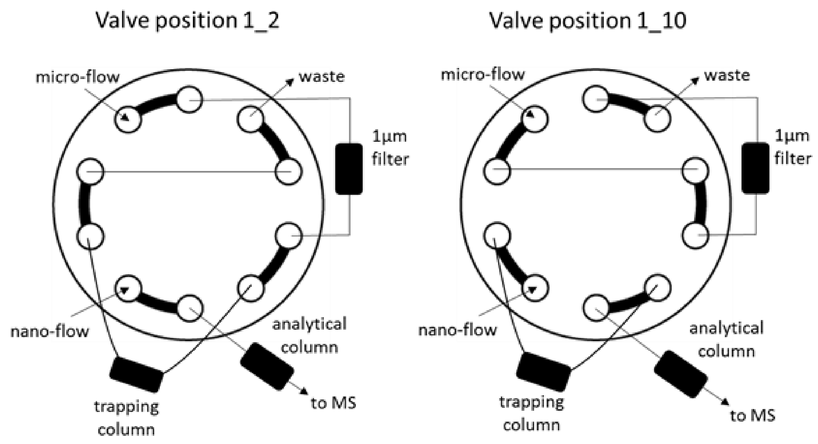

Figure 1

The loading mobile phase consisted of: A: 98 % H_2O, 2 % ACN, 0.05 % TFA; B: 90 % ACN, 10 % H_2O. The mobile phase delivered by the nano pump consisted of A: 98.8 % H_2O, 1 % DMSO, 0.2 % HCOOH; B: 80 % ACN, 18.8 % H_2O, 1 % DMSO, 0.2 % HCOOH. The injection volume was 6 µL. The applied gradients and programming of the positions of the 10 port switching valve are demonstrated in Table 1.

Table 1

Micro-flow pump			Nano-flow pump			Valve position	
Time (minutes)	Flow rate (µL/minutes)	A (%)	Time (minutes)	Flow rate (nL/minutes)	B (%)	Time (minutes)	Position
0	20	100	0	300	1	0	1_2
2	20	100	2	300	1	1.5	10_1
3	100	100	26	300	40	34	1_2
10	100	100	27	300	40		
10.1	100	0	30	300	99		
14	100	0	35	300	99		
14.5	50	0	36	400	99		
25	50	0	39	400	99		
25.1	50	100	39.1	400	1		
39	50	100	47	400	1		
39.1	20	100	48	300	1		
50	20	100	50	300	1		

The LC-system was coupled with a Q-Exactive Plus mass spectrometer, equipped with a Thermo Scientific™ EASY-Spray™ electrospray ionization source. The parameters of the ion source were: spray voltage 1.7 kV, capillary temperature 300 °C, S-lens 70. The instrument was set to operate in full scan (FS) and in parallel reaction monitoring (PRM) mode. The settings were resolution 35000 (at m/z 200), AGC target 1e5, maximum ion injection time 110 ms, isolation width 2.2 m/z, loop count 8 and resolution 70000 (at m/z 200), AGC 1e6, injection time 200 ms for the PRM and for the FS modes, respectively. The selected, characterized ions (precursor / product ions) and applied collision energies are listed in Table 2. Mass extraction width was 5 ppm.

Table 2

	Peptides	Precursor ion (monoisotopic mass)	Product ion 1 (monoisotopic mass)	Product ion 2 (monoisotopic mass)	(N)CE (eV)
Vasopressin analogues	Lys(8)-Vasopressin	528.7230 (+2)	226.1550 (b8-b6)	300.2030 (y3)	25
	Arg(8)-Vasopressin	542.7262 (+2)	276.1343 (b4-b2)	328.2092 (y3)	20
	(deamino Cys1, Val 4, D-Arg8) AVP ISTD 1	1040.4441 (+1)	399.1373 (b3)	311.1826 (y3-NH_3)	35
	Desmopressin	1069.4342 (+1)	276.1343 (b4-b2)	311.1826 (y3-NH_3)	35
	Terlipressin	614.2552 (+2)	203.1503 (y2)	300.2030 (y3)	25
	Felypressin	520.7257 (+2)	203.1503 (y2)	300.2030 (y3)	25
	Desmopressin_(1-7)	856.3116 (+1)	641.2388 (b5)	742.2323 (b6)	25
GnRH analogues	Peforelin	420.5283 (+3)	249.0982 (b2)	172.1081 (y2)	25
	LHRH	591.7914 (+2)	435.1775 (b3)	172.1081 (y2)	20
	LHRH_(2-10)	536.2778 (+2)	934.4894 (y8)	748.4101 (y7)	25
	LHRH_(1-3)OH	453.1881 (+1)	221.1033 (a2)	249.0982 (b2)	30
	Leuprolide	605.3300 (+2)	221.1033 (a2)	249.0982 (b2)	30
	Leuprolide_(1-3)OH	453.1881 (+1)	221.1033 (a2)	249.0982 (b2)	30
	Leuprolide_(5-9)	344.7289 (+2)	249.1598 (a2)	412.3031 (y3)	10
	Goserelin	635.3285 (+2)	221.1033 (a2)	249.0982 (b2)	25
	Lecirelin	605.3300 (+2)	221.1033 (a2)	249.0982 (b2)	30
	Triptorelin	656.3227 (+2)	249.0982 (b2)	328.2092 (y3)	20
	Buserelin	620.3353 (+2)	221.1033 (a2)	249.0982 (b2)	25
	Deslorelin	641.8276 (+2)	249.0982 (b2)	299.2190 (y2)	25
	Nafarelin	661.8251 (+2)	249.0982 (b2)	328.2092 (y3)	20
	Nafarelin_(5-10)	401.2245 (+2)	333.1598 (a2)	441.2932 (y4)	15
	Histrelin	662.3409 (+3)	221.1033 (a2)	249.0982 (b2)	35
GHRPs	Alexamorelin	479.7560 (+2)	181.1084 (a2)	209.1030 (b2)	40
	GHRP-1	478.2505 (+2)	181.1084 (a2)	209.1039 (b2)	40
	GHRP-1_(2-4)OH	424.1979 (+1)	307.1548 (a2)	335.1493 (b2)	25
	GHRP-1_(3-7)	374.20248 (+2)	241.1335 (a2)	269.1285 (b2)	10
	GHRP-2	409.7210 (+2)	170.0964 (a1)	241.1335 (a2)	30
	GHRP-2_(1-3)OH	358.17613 (+1)	198.0913 (b2-b1)	287.1390 (y2)	15
	13C, 15N GHRP-2_(1-3)OH ISTD 2	362.1820 (+1)	170.0964 (a1)	241.1335 (a2)	25
	GHRP-3	328.2056 (+1)	272.1394 (b2)	384.2718 (y3)	10
	GHRP-4	608.2979 (+1)	444.2038 (b3)	351.1816 (y2)	20
	GHRP-5	771.3613 (+1)	421.1870 (b3)	334.1550 (z2)	25
	GHRP-6	437.2296 (+2)	129.1022 (Lys der.)	324.1455 (b2)	25
	GHRP-6_OH	437.72157 (+2)	395.1826 (b3)	324.1455 (b2)	25
	GHRP-6_(2-6)	368.7001 (+2)	230.1288 (a2)	479.2765 (y3)	15
	GHRP-6_(2-6)OH	369.1926 (+2)	258.1237 (b2)	294.1812 (y2)	25
	GHRP-6_(2-5)OH	609.28237 (+1)	352.1656 (y2)	335.1390 (z2)	20
	Hexarelin	444.2374 (+2)	129.1022 (Lys der.)	110.0713 (His-imm.)	20
	Hexarelin_(4-6)	479.2765 (+1)	306.1601 (a2)	146.1288 (y1)	25
	Hexarelin_(1-3)OH	427.2088 (+1)	310.1662 (a2)	338.1612 (b2)	25
	Hexarelin_(2-5)OH	623.2981 (+1)	352.1656 (y2)	335.1390 (z2)	20
	Hexarelin_(2-6)OH	376.1999 (+2)	258.1237 (b3-b1)	294.1812 (y2)	25
Ghrelin mimetics	Ibutamoren	529.2470 (+1)	263.1390	267.1162	20
	Tabimorelin	529.3173 (+1)	280.1332	252.1383	30
	Anamorelin	547.3391 (+1)	174.1277	276.2076	40
	Capromorelin	506.2762 (+1)	263.1390	244.1444	18
	Ipamorelin	356.7001 (+2)	129.1022 (Lys der.)	223.1195 (b2)	40
	Ipamorelin_(1-4)OH	585.2820 (+1)	223.1195 (b2)	420.2036 (b3)	30
Thymosin β4 fragments	TB-500	445.2531 (+2)	248.1241 (y2)	606.3093 (y5)	25
	TB-500_(1-5)OH	330.7002 (+2)	377.2031 (y3)	505.2980 (y4)	25
GH fragments	AOD-9604	605.6274 (+3)	136.0757 (Tyr-Imm.)	223.1077 (y2)	20
	AOD-9604_(7-16)	521.7077 (+1)	820.3076 (b8)	877.3291 (b9)	20

RUGGEDNESS

The ruggedness of the method was tested by continuous pressure monitoring over the trapping and the analytical column (Figure 2). No backpressure increase (related to clogging) was observed, even when two-fold diluted urine samples were injected. However, several parameters, including the dilution factor had to be optimized to be compliant with the TDICR2015 (5).

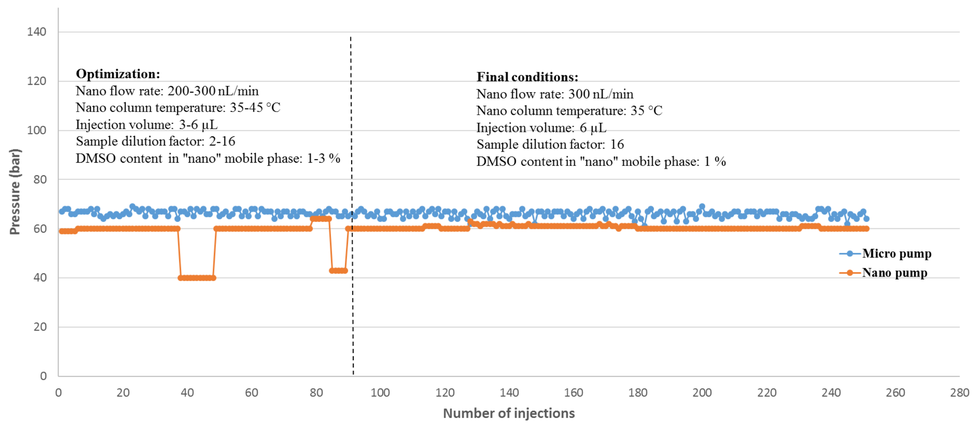

Figure 2

Conclusion

In this study, AFFL-nanoLC-MS was tested in a routine doping control setting. No pressure increase was observed either over the trapping or the analytical column in the course of this ongoing study, and both the MRPL and identification criteria were met for all peptides at the investigated concentration level. The future plans include a full validation of the method for the confirmatory analysis of small peptides, and further testing of the ruggedness of the setup by the injection of different matrices, subjected to different sample clean-up procedures.

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