A new automated approach for the determination of peptides in human plasma using on-line SPE – LC – MS/MS

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Overview

- A new automated sample extraction technique for the determination of peptides through Mixed Mode Strong Anion exchange is developed.
- The new method is tested with 3 peptides with different molecular masses: 2100, 5900 and 6200 amu.

Introduction

Sample preparation for analytes in biological matrices is a critical part of successful LC MS/MS analyses. Analysis of peptides is particularly challenging due to some unique properties such as size, carry-over, polarity differences and poor solubility, which can affect extraction performance. All these properties have a severe impact on the recovery of large peptides from biological matrices like plasma and serum. A new extraction approach for peptides is evaluated: integrated clean-up and analysis of peptides using an on-line SPE anion exchange procedure with a mixed mode strong anion exchange cartridge. Several peptides drugs from different suppliers were investigated with this procedure.

Experimental conditions

Samples were prepared by spiking pooled human plasma with a peptide. Prior to extraction, all plasma samples were diluted 1:4 with water. A diluted sample (100 µL) was directly placed into the autoasampler and analyzed fully automated. Online SPE is performed on a disposable cartridges: each sample uses a fresh SPE cartridge. After extraction, the peptides are eluted from the cartridge onto a reverse phase analytical column utilizing the HPD focusing mode.

Online –SPE-LC (Symbiosis™ Pico, Spark Holland)

LC

Column:	Phenomenex Jupiter 4µ Proteo 50x2 mm (p/n:00B-4396-B0)
Mobile phase 1:	0.1% Formic Acid in water
Mobile phase 2:	95% Methanol and 5% (0.1% Formic Acid in water)

SPE

Cartridge:	Oasis® MAX 30 µm 10x1 mm (Waters corporation)
Conditioning:	1 mL Methanol at 5 mL/min
Equilibration:	1 mL 20% Methanol in 1% NH4OH water at 5 mL/min
Sample injection:	100 µL spiked plasma
Sample extraction:	500µL 20% Methanol in 1% NH4OH water at 0.5 mL/min
Cartridge wash 1:	1 mL 20% Methanol in 1% NH4OH water at 5 mL/min
Cartridge wash 2:	1 mL 80% Methanol in 1% NH4OH water at 5 mL/min
HPD Focus elution:	300 µL Methanol at 100µL/min
Clamp flush:	1 mL 80/20 acetonitrile/water 0.2% FA at 5 mL/min

Gradient:

time (min)	flow (mL/min)	A %	B %
00:01	0.4	100	0
03:00	0.4	100	0
03:02	0.5	100	0
03:40	0.5	0	100
04:40	0.5	0	100
05:00	0.5	100	0
06:00	0.5	100	0

on-line SPE HPD focus elution

ESI-MS (API4000, Applied Biosystems)

ESI-MS/MS conditions (positive mode)

MS-settings

Compound Mass	Q1 mass	Q3 mass	DP	CE	CXP
2100	698.5	110.2	76	101	20
5900	1184.3	454.6	51	57	14
6200	1239.7	731.6	86	51	22

General settings *IS 5500* *TEM 400* *CAD 7* *CUR 15* *GS1 75* *GS2 40* *EP 10* *Dwell 150*

Results and Discussion

The sample clean-up is optimized by increasing the percentage organic modifier in the wash solvent. Figure 1 shows the wash optimization for the 6200 AMU peptide. The other two used peptides showed similar results.

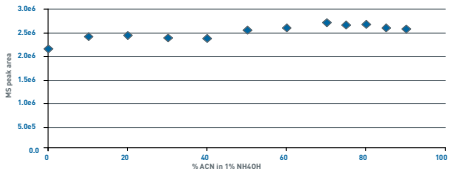

Figure 1: wash optimization for peptide 6200. peptide 5900 and 2100 showed same results

As shown in figure 1 the mixed mode anion exchange cartridge allows for a high organic wash without reducing the analyte recovery. For optimal clean-up a first wash step with 20% ACN was programmed to remove interfering proteins (no denaturing on SPE cartridge), followed by a second high organic wash at 80% ACN to remove non-polar matrix compounds (see chromatograms below).

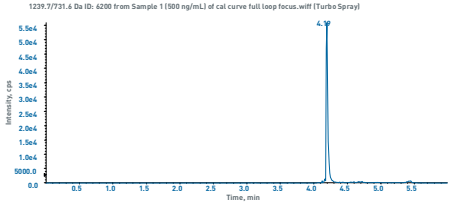

Figure 2: 6200 AMU peptide. Spiked plasma sample extracted on Oasis MAX (10X1 mm)

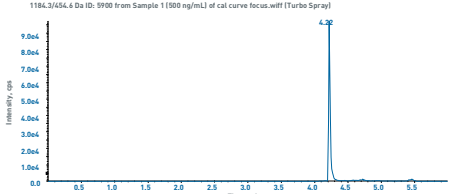

Figure 3: 5900 AMU peptide. Spiked plasma sample extracted on Oasis MAX (10X1 mm)

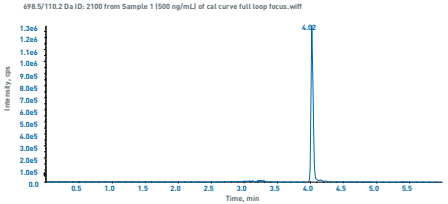

Figure 4: 2100 AMU peptide. Spiked plasma sample extracted on Oasis MAX (10x1 mm)

As shown in figure 2-4 clean and reproducible chromatograms were obtained. Subsequently, calibration curves were determined by injecting a full set of calibration standards ranging from 0.5 to 500 ng/mL spiked human plasma standards. The calibration curves are calculated with a 1/X weighting. The curves for the peptides 6200, 5900 and 2100 are displayed below (r > 0.996 for all three peptides).

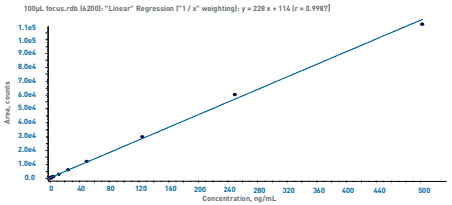

Figure 5: 6200 AMU peptide. Spiked plasma calibration curve from 0.5 to 500 ng/mL

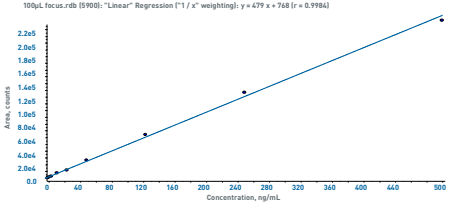

Figure 6: 5900 AMU peptide. Spiked plasma calibration curve from 0.5 to 500 ng/mL

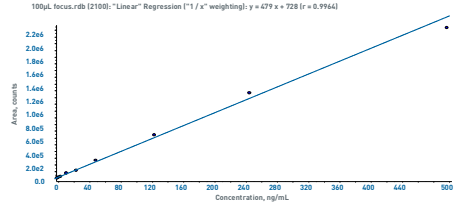

Figure 7: 2100 AMU peptide. Spiked plasma calibration curve from 0.5 to 500 ng/mL

The method was evaluated using spiked human plasma samples. The developed XLC-MS/MS method has an absolute recovery of more than 90%. The accuracy is ranging from 89-117% and the precision 2.1-7.9 %CV. The estimated quantitation limits for the peptides are more than sufficient to allow the analytical method to be used for therapeutic drug monitoring.

Conclusion

- **A simple sample preparation method was developed for the determination of three peptides in plasma.**
- **In this concept 100 µL of a spiked plasma sample is loaded on a mixed mode anion exchange cartridge.**
- **The developed concept of automated on-line SPE – LC – MS/MS delivers clean and reproducible results.**
- **Good linearity was obtained for all 3 peptides in human plasma over the 0.5-500 ng/mL range (r > 0.996)**
- **More than 100 µL sample could be loaded onto the cartridge to achieve even lower detection limits.**
- **The Symbiosis™ Pico method is currently used routinely in several pharmaceutical labs (various companies) for analyzing different size of peptides (< 10K amu) with similar results.**

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