Evaluation of Nano-LC-MS/MS in a column switching setup as a tool for the absolute quantification of peptides in the picomolar range

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1. Introduction

The aim of our work is to develop a sensitive determination of neuropeptides. Very low sensitivity is a prerequisite in view of the small sample volumes obtained by e.g. microdialysis. Due to its inherent specificity we use LC-MS/MS. However, to obtain the aimed sensitivity, miniaturisation is a key factor. Theoretically seen, diminishing the column diameter gives a quadratically improvement in sensitivity as calculated by the following equation: $(F=ID_{conventional}/ID_{reduced})^2$ [1]. Finally, this results in nano-LC. In order to be able to inject larger sample volumes on a nano column, a column switching setup is necessary. The extra advantage of this approach is desalting and preconcentration. Nevertheless, such a relative complex back-flushing system should not only be sensitive. In order to absolutely quantitate, it should also be linear, reproducible ... As such, we wanted to evaluate the possibility of a standard nano LC-MS/MS system in a column switching setup, as generally used in proteomics, to absolutely quantify peptides, in our case the neuropeptide leu-enkephalin.

2. Aim

Evaluation of nano LC-MS/MS in a standard column switching setup as a tool to absolutely quantitate peptides in the picomolar range.

3. Materials and methods

HPLC Conditions:

- Trapping Column: PepMap C18 (5 µm, 100Å, 300 µm I.D. x 1mm; (1))
- Loading pump / switching device: Switchos II (1), loading time 3 min. @ 10µl/min
- Column: PepMap C18 (3 µm, 100Å, 75 µm I.D. x 15 cm; (1), 150 nl/min
- HPLC: Ultimate Micro Pump HPLC System (1)
- Mobile Phase: (A) 0.1% (v/v) formic acid in water, (B) 0.1% (v/v) formic acid in 80/20 acetonitrile/water mixture, both (A) and (B) were filtered through an Alltech 0.2 µm membrane.
- Gradient: 0-3 min 6% B (loading of precolumn), 3-46 min linear gradient to 75% B, 47-56 min 100% B, 58-73 min equilibration at starting conditions 6% B
- Autosampler: FAMOS (1), 10 µl loop
- (1): LC Packings- A Dionex Company, The Netherlands

MS Conditions:

- Mass Spectrometer: Micromass Ultima triple quadrupole mass spectrometer
- Ion Source: orthogonal nanospray source (Z-spray®) in positive ion mode
- Nanospray: Picotips, New Objective, USA
- Capillary voltage: 2500V
- Cone: 65V
- Collision energy: 25 eV for glufibrinopeptide, 20 eV for leu-enkephalin

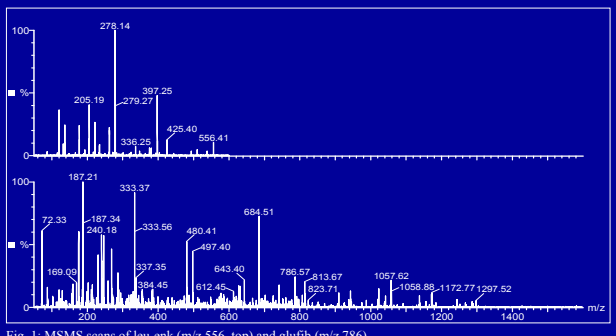

Fig. 1: MSMS scans of leu-enk (m/z 556, top) and glufib (m/z 786)

Injection parameters were optimised in the microliter pick up mode, meaning that exactly 10 µl of the standards could be injected, without any sample loss. This method is particularly suited for limited sample volumes. Standards were loaded on the trapping column in 0.1% (v/v) aqueous HCOOH solution at 10 µl/min. After 3 min, the valve was switched, the analyte was back-flushed onto the analytical column and the mobile phase gradient was started.

The triple quadrupole instrument was operated in the MRM modus, selecting both protonated molecular ions, *m/z* 786,21 for glufib and *m/z* 556,41 for leu-enk. Transitions of the doubly charged 786,21>480,49 and 786,21>684,49 for glufib and the singly charged 556,41>278,2 and 556,41>397,31 for leu-enk were recorded in the MRM method.

Working standard solutions of leu-enk were prepared in the concentration range 10 amol/µL – 10 fmol/µL by dilution with 0.1% formic acid in water. The internal standard glufib was present in a final concentration of 1 fmol/µL.

4. Results and discussion

The MRM method permitted the construction of linear response curves (weighted regression factor 1/X, between 50 amol/µL or 500 amol on column and 10 fmol/µL, respectively, 100 fmol on column). Correlation coefficients of this weighted linear regression were between 0,9928 and 0,9997 (n=6).

Linearity						
n=6	R^2	slope	intercept			
average	0,9962	2,53	-0,0214			
CV%		10,17%				
Precision						
conc fmol/µl	0,05	0,1	0,5	1	5	10
CV%	23,30%	19,85%	11,45%	13,73%	11,13%	2,67%

Table 1. Linearity and total reproducibility data

The 95% confidence interval of the y-intercept of the linear response curve always included zero, corresponding with significant P-values. The limit of detection and limit of quantitation were established at 16 amol/µl and 54 amol/µl, as determined by the formular $LOD=3S_{dev\ intercept}/m$, respectively $LOQ=10S_{dev\ intercept}/m$. Within day precision for the various standards (0.05 fmol/µl – 10 fmol/µl, Table 1) did not exceed 25%.

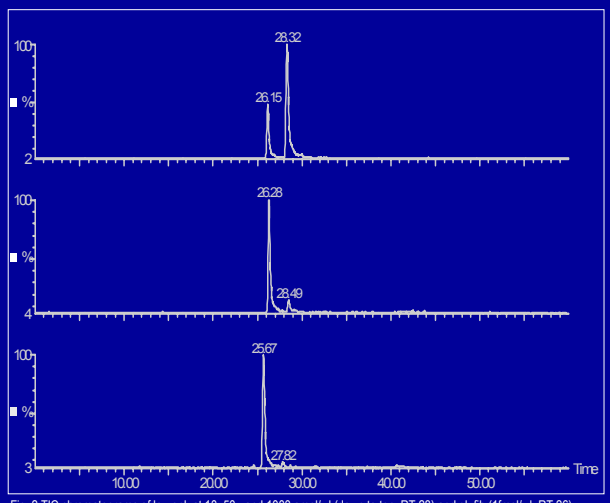

Fig. 2 TIC chromatograms of leu-enk at 10, 50, and 1000 amol/µl (down to top, RT 28) and glufib (1fmol/µl, RT 26)

Retention time stability appeared to be a crucial problem in nano-LC. Continuous sparging of the mobile phases with helium was the only way to prevent the formation of air bubbles in the pump. Sparging with nitrogen was not efficient and resulted consequently in large shifts in retention times (max. 3 minutes).

5. Conclusion

It is possible, with a conventional nano-LC system in the column switching setup, to determine peptides as low as 100 amol on column in MS/MS (MRM). Linearity is good in a dynamic range of almost three orders of magnitude.

However, within day precision variability already reveals the in essence non robustness of the nano-LC-MSMS system. Tuning and continuous infusion of various peptides also revealed signal instability of the Picotip nanospray system. Internal standardisation with another peptide, in this case glufib, is not really sufficient to solve this problem. Especially the nano source and electrospray ionisation process, are far from stable.

Nano-electrospray is a technique with great advantages concerning sensitivity, but it is quite unstable. Improving this stability would effectively enlarge its analytical possibilities.

Stability of the nano-ionisation process should effectively be improved in the future to make nano-LC-MS/MS a « workable » instrument to routinely measure peptides in the picomolar range. Isotopically labeled internal standards might be essential to obtain this goal.

6. References

[1] Varesio et al., 2002

7. Acknowledgements

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