Feasability of Nanospray-LC-MS/MS for the absolute quantification of peptides.

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Introduction

Sensitive determination of neuropeptides is necessary, certainly in view of the low concentrated, low sample volumes obtained by e.g. microdialysis. Although RIA provides high sensitivity, it has limited specificity. Capillary zone electophoresis is a simple and fast separation technique combining high separation efficiency with low sample requirement and high absolute sensitivity. One of the major disadvantages of CE compared to LC is, however, the limited loading capacity resulting in low concentration sensitivity.

The advent of the LC-MS interface, in combination with nanospray techniques effectively improved the sensitivity limits to the picomolar, even femtomolar range. Moreover, tandem MS is attractive because it offers the possibility of detecting peptides with sequence specificity and can be used, in principle, for any peptide. We have used this approach for the analysis of leucine-enkephalin (leu-enk).

As sensitivity was the most important requisite, a nano LC system was used. We opted for a commercially available 75 µm nano column. On such a system, sample injection volumes are limited to ~10 nL, which is not practical for the analysis of biological extracts containing low levels of analytes. In order to be able to inject large sample volumes on a nano column, on-column analyte focusing or a column switching setup is necessary. Since on-column focusing needs a prohibitively long time at a flow in the nanoliter-per minute level, the column switching setup was evaluated. Sample injection is in this case made off line at a flow rate of 10 µL/min, the trapping column is then connected to the analytical column by switching a valve. The extra advantage of this approach is desalting and preconcentration. For optimum sensitivity and selectivity, the mass spectrometric analysis was performed in multiple reaction monitoring (MRM) on a triple quadrupole instrument. This is necessary for the quantification of peptides in the lower concentration range, i.e. the picomolar range.

A relatively complex back-flushing system of this kind should not only be sensitive. In order to routinely and absolutely quantitate, it should also be linear, robust and 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 this case the neuropeptide leu-enk. Special attention has been paid to the overall robustness of the nano-spray system.

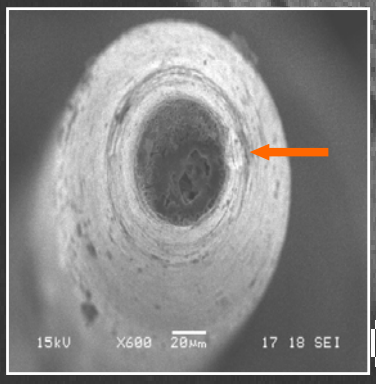

Figure 1. Scanning electron microscopy image of employed nanospray emitter.
Stainless steel emitter after 48 h of use, the arrow indicates a damage / difference with the non-used tip, probably due to manufacturing difficulties or electric degradation.

Materials and methods

Chromatography

- Working standard solutions of leu-enk were prepared in the concentration range 10 fmol/mL – 10 pmol/mL by dilution with 0.1% formic acid in water. The internal standard [glu1]-fibrinopeptide was present in a final concentration of 1 pmol/mL.
- 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

Mass Spectrometry

- Mass Spectrometer: Micromass Ultima triple quadrupole mass spectrometer
- Ion Source: orthogonal nanospray source (Z-spray®) in positive ion mode
- Nanospray: Picotips, New Objective, USA; Alternative gold coated emitters were a kind gift of Nanoseparations, the Netherlands. Stainless steel emitters were purchased from Proxeon ,Denmark.
- The mass spectrometer was operated in the MRM modus using argon as collision gas. Transitions of the doubly charged 786,21>480,49 and 786,21>684,49 for [glu¹]-fibrinopeptide and the singly charged 556,41>278,2 and 556,41>397,31 for leu-enk were recorded.

Results and discussion

Linearity

- The MRM method permitted the construction of linear response curves (weighted regression factor 1/X, between 50 fmol/mL or 500 amol on column and 10 pmol/mL, respectively, 100 fmol on column) (Figure 2). Correlation coefficients of this weighted linear regression were between 0,9928 and 0,9997 (n=6).
- The limit of detection and limit of quantitation were established at 16 fmol/ml and 54 fmol/ml. Within day precision for the various standards (50 fmol/ml – 10 pmol/ml) did not exceed 25%.

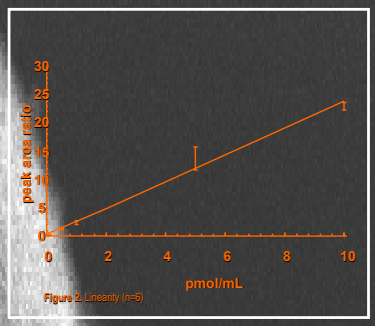

Figure 2. Linearity (n=6)

Nanospray performance

- Durability of the SilicaTip™ emitters is crucial for routine quantitative measurements. Average life of these tips is about one week.
- Optimal spray usually lasts for aproximately two days. The main reason for spray instability is droplet formation at the tip orifice. This can be temporarely adjusted by increasing the capillary voltage but no solution is offered because of its non-feasible characteristics. Applying desolvation gas does not inhibit any droplet formation and effectively reduces MS signal.
- Gold-coated fused silica tapered tips and non-tapered nano-bore stainless steel emitters were evaluated as alternatives. Non tapered stainless steel tips are normally less susceptible to clogging. Continuous infusion of leu-enk and [glu¹]-fibrinopeptide resulted in comparable responses in view of sensitivity, for both alternative emitters. When coupled to an LC-system, similar droplet formation and variable spray appeared. Even the non tapered nano-bore stainless steel emitters suffered from the formation of droplets at the tip orifice after only 24 hr at 2500V.
- Drawbacks of nanospray emitters, such as fragility, causing a high propensity to fracture the sharp end of the tip, corrosion of the conductive coating, clogging and manufacturing difficulties (the orifice of the nanotip is less uniform than in conventional ES) make their use very difficult in quantitative analysis. (cfr. SEM, Figure 1). The poor quality and thus short life-time of the metal coatings has also been reported in CZE-nano-ESI with sheathless interfaces. The nanospray is most sensitive due to the most efficient ionization process, however, robustness is lost due to difficulties in generating a stable spray. The lifetime of metal coatings is often short varying from minutes to days, due to electrochemical / electrical degradation.
- We can conclude that droplet formation, durability, clogging and spray instability of the ESI emitters effectively reduce the feasibility of routine nanospray in absolute quantitative measurements.

Conclusion

It is possible, with a conventional nano-LC-MS system in the column switching setup, to determine peptides as low as 100 amol on column. Although linearity is good in a dynamic range of almost three orders of magnitude, within day precision variability reveals the in essence non robustness of the nano-LC-MSMS system. Robustness of the nano-ionisation process should effectively be improved in the future to make nano-LC-MS/MS a workable instrument to routinely quantify peptides in the picomolar range. Isotopically labeled internal standards might be essential to obtain this goal.

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