Capillary LC-MS/MS for the quantification of enkephalins

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1. Introduction

Sensitive determination of neuropeptides is necessary, certainly in view of the low concentrated, low sample volumes often obtained and the low concentrations present in biological fluids. 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. Due to robustness problems with nano-LC-MS, this approach was not preferred [1]. Instead, a capillary LC approach was evaluated, in combination with a standard electrospray source equiped with a small spray capillary.

For optimum sensitivity and selectivity, the mass spectrometric analysis was performed in multiple reaction monitoring (MRM) on a triple quadrupole instrument. We have used this approach for the analysis of methionine-enkephalin and leucine-enkephalin.

2. Aim

Evaluation of a capillary LC-MS/MS system with on column analyte focusing for the absolute quantification of enkephalins.

3. Materials and methods

Chromatography

A Waters 2790 HPLC system was equipped with a home made flow splitting device. All inner tubing of the HPLC system has been replaced by narrow bore capillaries, to effectively reduce dead volumes as much as possible. The resulting flow was adjusted at 6.5 µL/min. A 20 µL loop was installed, which resulted in minimal extra dead volume, compared with a 10 µL loop. Partial loop injections of 10 µL were performed.

Column: C18 µ-Guard™ trapping column (5 µm, 100Å, 300 µm I.D. x 1mm) + Pepmap® C18 analytical column (3 µm, 100Å, 300 µm I.D. x 15 cm) at 38°C (Dionex, The Netherlands).

Mobile phases: (A) 0.1% HCOOH (B) 80/20 CH_3CN/H_2O with 0.1% HCOOH

Gradient: 0-5 min 100% A (loading of precolumn), 5-20 min linear gradient to 40% B, 20-23 min 40% B, 23-35 min equilibration at starting conditions 100% A.

The gradient consisted of an initial 5 minutes at 100% A, in which the neuropeptides are trapped on the trapping column. Desalting and a certain level of sample cleanup is achieved in this way. The gradient profile was optimised taking dead time phenomena into consideration, and evaluated (UV, 258 nm) by adding 0.5% aceton to eluent B.

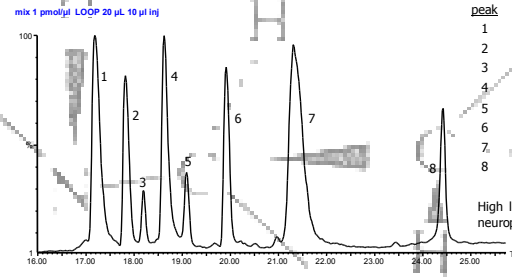
Mass Spectrometry

Detection was performed with a Quattro Ultima triple quadrupole instrument (Micromass, Wythenshawe, UK) equipped with an orthogonal electrospray source (Z-spray®) in the positive ion mode. A 65 µm ID spray capillary was used. 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, the singly charged 556,41>278,2 and 556,41>397,31 for leu-enk and the singly charged 574.25>277.98 and 574.25>397.34 for met-enk were recorded.

4. Results and discussion

Chromatography

The gradient was optimized to give an optimal separation of a standard mixture of 8 neuropeptides (figure 1). Within day and between day reproducibility of retention times was maximally 0.73%, respectively 2.34%.



peak	peptide	PWHH (min)
1	somatostatin 28	0,19
2	substance P	0,14
3	methionin enkephalin	0,09
4	galanin	0,15
5	leucin enkephalin	0,12
6	somatostanin 14	0,14
7	neuropeptide Y	0,31
8	neurokinin B	0,13

High loading MS scan of a mixture of eight neuropeptides (1 pmol/µL; 10 pmol o.c.)

Figure 1

Linearity

Linearity was evaluated by analyzing calibration curves of met-enk (n=5) and leu-enk. [glu1]-fibrinopeptide was used as internal standard at a concentration of 50 fmol/µL.

The MRM method permitted the construction of linear response curves for aequous standards of met-enk (weighted regression factor 1/X) between 500 amol/µL or 5 fmol on column and 100 fmol/µL, respectively, 1 pmol on column. Correlation coefficients of this weighted linear regression were between 0,995 and 0,998 (n=5).

Validation

Accuracy and precision were evaluated with two quality control samples (QC1 and QC2, respectively 5 and 100 fmol on column). Accuracy was below 30%.

(n=5)	Conc (fmol/µL)	PRECISION WD	PRECISION total	ACCURACY mean	ACCURACY stdev
QC1	0,5	16,74%	6,37%	-22,13%	8,37%
QC2	10	5,05%	7,55%	-22,70%	4,22%

The limit of detection (LOD = 0.5 fmol/µL) and limit of quantification (LOQ = 1 fmol/µL) were estimated at a signal-to-noise ratio of 3 and 10, respectively.

5. Conclusion

Capillary LC-MS/MS enables the determination of enkephalins in a linear dynamic range of between 2 and 3 orders of magnitude. LOD and LOQ are in the low femtomole range. However, at this stage, i.e. without isotopically labeled internal standardisation, validation results offer room for improvement

6. Acknowledgements

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7. References

[1] Sinnaeve B. and Van Bocxlaer, J. Chromatogr. A, Accepted for publication

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