

## Abstract

The poor stability of biopharmaceuticals along with their limited ability to cross biological barriers restricts their therapeutic potential. A special class of peptides, named cell-penetrating peptides (CPPs), has the ability to translocate across cellular membranes and to transport other molecules such as potential drug candidates with them. Often CPPs are investigated in *in vitro* cell-based assays or biophysical/biochemical model assays where several degradation products may be formed. In the present study, a hyphenated technique comprising high-performance liquid chromatography, high-resolution mass spectrometry, solid-phase extraction, and nuclear magnetic resonance (HPLC-HRMS-SPE-NMR) was investigated for analysis of complex biological mixtures containing peptides. The technique has not previously been applied for analysis of larger molecules such as peptides; and five cationic polar model peptides with diverse polarities was used as test compounds for the method development process. To determine the optimal chromatographic conditions while simultaneously allowing for sufficient signal detection with HPLC-HRMS-SPE, a range of mobile phase additives and different columns were investigated.

The combination of a column with a wide pore size (Supelco Discovery Wide Pore C<sub>18</sub>) and a mobile phase acidified with 0.01% TFA was found to be the best compromise allowing for optimal chromatographic performance with minimal ion-suppression. The most efficient trapping of peptides was achieved with an SPE cartridge containing a silica-based C<sub>18</sub> sorbent material and the highest recovery upon elution of the peptides was obtained with 50% or 75% acetonitrile in water depending on the peptide. As proof of concept the optimized conditions were applied in the hyphenated HPLC-HRMS-SPE-NMR technique using a cell extract obtained from penetratin (a well-studied CPP) exposed to the surface of a Caco-2 cell monolayer. The peptide sequence of a selected degradation product was identified by LC-MS in agreement with the partial assignment of several signals in the <sup>1</sup>H NMR spectrum. However, it was apparent that a higher amount of eluted degradation product was necessary to enable a full NMR structure elucidation by 2D NMR experiments. An experiment comprising incubation of a fluorinated version of penetratin (F-penLeu) with the enzyme trypsin - allowing for a higher peptide concentration than an *in vitro* cell-based assay - was therefore used as proof-of-concept for the applicability of HPLC-HRMS-SPE-NMR for analysis of mixtures of degraded peptides. Several enzymatic degradation products were separated and detected by LC-HRMS, but trapping, elution as well as full structural identification by 2D NMR was not possible within this project period

due to the lack of a functioning SPE unit available for on-line trapping. However, these experiments are scheduled to be performed in week 38 after repair of the instrumentation.

Frequently, *in vitro* studies carried out with CPPs comprise chemical modifications to the parent sequence in order to e.g. achieve increased stability or interactions with the cellular membrane to facilitate translocation. However, further modifications to the peptide sequence, such as fluorophore-conjugation to the model CPP, are often necessary to enable fluorescence-based detection of CPP uptake. As conjugation of a fluorophore to a CPP is hypothesized to alter the physicochemical properties of the CPP, several fluorophore-CPP conjugates as well as unlabeled peptides were synthesized and tested in different *in vitro* studies as part of an interdisciplinary research project. The results obtained from these studies clearly showed that fluorophore conjugation to the model peptide penetratin increased cytotoxicity as well as membrane damage and disturbance. Preferably, peptide labeling should not affect the functionality of the CPP, which is a problem with current methods relying on fluorophore labeling of these peptides. ***Thus, it is hypothesized that using fluorine as an alternative label for CPPs will result in minor changes to the overall physicochemical properties of the CPP and allow for the use of  $^{19}\text{F}$ -NMR to detect internalized peptide.*** Different fluorine ( $^{19}\text{F}$ )-labeled analogs of penetratin was synthesized, enabling  $^{19}\text{F}$ -NMR measurements of apparent uptake and degradation occurring during exposure of the peptides to Caco-2 cells. Diverse degradation patterns were observed among penetratin analogs and results obtained from this study indicated that a commonly used fluorophore 5(6)-carboxytetramethyl rhodamine (TAMRA) contributes to an increased CPP uptake. These results are supported by previous studies and emphasize the importance of using alternative labels, e.g. fluorine, to assess cellular uptake of CPPs.

In conclusion, the results from the current studies showed that enzymatic CPP degradation products can be separated, trapped and eluted from an on-line HPLC-HRMS-SPE system. However, this will require an initial CPP concentration above 200  $\mu\text{M}$  when NMR is used as the detection method, which can be achieved from incubation with trypsin. However, by the use of  $^{19}\text{F}$ -NMR it was possible to detect differences in uptake among different  $^{19}\text{F}$ -labeled analogs and also for a  $^{19}\text{F}$ -labeled analog conjugated to a fluorophore. Thus, these findings showed that alternative labeling strategies, not based on fluorophore-labeling, may expand the repertoire of analytical techniques in the assessment of CPP uptake.