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Quantification of pharmaceutical peptides using selenium as elemental detection label

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Abstract

The aim of the present work was to demonstrate how selenium labelling of a synthetic cell-penetrating peptide may be employed in evaluation of stability and in quantitative estimation of cellular uptake by inductively coupled plasma mass spectrometry (ICP-MS). Two analogues of the cell-penetrating peptide, penetratin were synthesized, one with selenomethionine (SeMet) added at the N-terminal of the peptide (N-PenM^{Se}) and one in which the internal methionine (Met) was replaced with SeMet (i-PenM^{Se}). Purity of the synthesized peptides was 92% of N-PenM^{Se} and 89% of i-PenM^{Se} as determined by liquid chromatography (LC) ICP-MS. The selenium-labelled peptides were investigated in cell uptake studies in HeLa WT cells. Stability of the peptides was monitored in water, cell medium and during cell uptake studies. Total uptake of selenium was quantified by Flow Injection (FI) ICP-MS. Speciation analysis of cell samples by LC-ICP-MS showed mainly uptake of the intact peptides, while the amount of intact peptide in cell lysates was semi-quantitatively determined. The selenium-containing penetratin analogues were to some extent degraded in pure cell medium, while extensive degradation was observed during cell uptake studies. The major degradation products were determined by LC electrospray ionization mass spectrometry (ESI-MS). The labelling method in combination with FI-ICP-MS, LC-ICP-MS and LC-ESI-MS techniques provided detailed information on the fate of penetratin in cellular uptake studies. Most pharmaceutical peptides,

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4 including penetratin, are synthetic analogues of endogenous peptides, and incorporation of
5 selenium may improve critical assessment of the native drug or drug delivery candidate early
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7 in the drug development process.
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10 11 12 **Introduction**

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14 Peptides are involved in a large variety of physiological processes and cell functions and
15 several peptide drugs have already been successfully applied for treatment of for instance
16 certain cancer forms and type 2 diabetes. The interest in exploiting peptides as drug
17 substances has increased rapidly during the last decades. Although the annual sale of peptide
18 drugs is only a few percent of the global drug market, it is increasing twice as fast as the
19 market for classical small molecules.¹ In the period 2000-2012, marketing approvals for 19
20 peptides were achieved in USA, six of these were approved in 2012. A large number of
21 peptides are in pipeline and several have reached the Phase II and Phase III clinical trials. The
22 therapeutic areas of the Phase I and II candidates are mainly metabolic diseases and oncology,
23 while the major indications for the Phase III candidates are oncology and infectious diseases.²
24 Peptides often offer high efficacy, selectivity and specificity as drug candidates, and as their
25 degradation products are amino acids and smaller peptides they are associated with low risk of
26 systemic toxicity. However, peptides exhibit low oral bioavailability owing to their high
27 propensity to enzymatic degradation in the gastro-intestinal tract combined with low intestinal
28 barrier passage.³ Development of drug delivery system is therefore an integrated research
29 area for improving bioavailability of potential drug candidates.⁴ Drug delivery by nanoparticles
30 and development of cell penetrating peptides (CPP) are example of such efforts.⁵
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52 In order to monitor stability, pharmacokinetics and metabolism of pharmaceutical peptides,
53 development of sensitive quantitative analytical methods for peptides are of great importance.
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55 A challenge in monitoring the fate of peptides in complex biological systems, is to obtain the
56 adequate selectivity and sensitivity.
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Pharmaceutical peptides comprising 5-50 amino acids are predominantly produced by solid-phase peptide synthesis (SPPS). This opens the possibility of introducing a label for detection and thereby enhance analytical selectivity.^{3;6}

Quantification of peptides has traditionally been performed by liquid chromatography (LC) with detection by UV absorption⁷⁻⁹ or fluorescence emission.^{10;11} However, UV detection has limited sensitivity and specificity, whereas fluorescence detection demands either inherent fluorescent properties of the biomolecule or labelling with a fluorophore. With development of high-resolution mass spectrometry (MS), this technique is increasingly applied in quantitative peptide analysis.¹² Recently, inductively coupled plasma mass spectrometry (ICP-MS) was proposed as a complementary method to molecular MS for quantification of peptides. The advantages of ICP-MS are low detection limits, a wide dynamic range and in principle matrix-independent sensitivity of different species of the heteroelements. This opens for the opportunity for quantification by using a single inorganic element standard in contrast to species-specific standards needed for molecular MS.^{13;14} However, detection of C, O and N, the main constituents of biomolecules, is not possible by ICP-MS due to use of an open plasma causing interferences from the air. Fortunately, a wide range of proteins naturally contain ICP-MS detectable heteroelements, *e.g.* S in methionine (Met) and cysteine (Cys), Se in selenoproteins, P in phosphoproteins or I in thyroid hormones. The occurrence of selenoproteins, phosphoproteins and thyroid hormones are moderate in number, and sulfur analysis is restricted due to low ionization efficiency and spectral interferences in ICP-MS resulting in lower sensitivity.¹³ In order to enhance sensitivity, several different ICP-MS sensitive labels have been suggested, and these possibilities have been reviewed by Prange and Pröfrock¹³, Kretschy *et. al.*¹⁵ and Wang *et. al.*¹⁶. Two different approaches are generally pursued when labelling biomolecules: pre-labelling and post-labelling. In proteomics, post-labelling is often applied via labelled antibodies targeting the biomolecule¹⁷ or through direct conjugation with derivatizing agents.¹⁸ In the pre-labelling approach the biomolecule is labelled prior to its introduction to a biological system and hence it remains detectable throughout the

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4 experiment. This approach is mainly used when analyzing smaller biomolecules as in the
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6 present work.
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10 The ideal peptide labelling probe should enhance sensitivity and neither affect functionality of
11 the peptide nor the analytical properties. Incorporation of the label should be well controlled,
12 easy to handle and stoichiometrically unequivocal. All these requirements are not readily
13 achieved. Only a few studies on elemental labelling for ICP-MS analysis of therapeutic peptides
14 have been reported, mainly involving chelating agents.^{15;19} Chelating agents typically consist of
15 a lanthanide/metal chelating moiety linked to a reactive group targeting a specific functional
16 group in the peptide, *e.g.* primary amines or thiols in cysteines. Liu *et al.* addressed the
17 importance of monitoring the labelling procedure ensuring 1:1 labelling stoichiometry. They
18 reported that a mixture of labelled bradykinin with peptide:label ratios of 1:1 and 2:1 was
19 obtained by use of a reactive moiety containing two reactive groups, diethylenetriamine-
20 *N,N,N',N'',N'''*-pentaacetic dianhydride (DTPAA), selective for conjugation to primary amines.²⁰
21 In addition, labelling with DTPAA may result in diastereomeric mixtures of the labelled peptides
22 challenging the subsequent analysis.²¹ Labelling reactions have been optimized to avoid
23 formation of diastereomers,²² however, considerations of the possible presence of multiple
24 labelling sites in the peptide is important. Furthermore, subsequent separation of free label
25 and labelled peptide is necessary during pre-labelling.²³ A few studies have demonstrated
26 labelling with indium by use of DOTA (1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic
27 acid), introducing the label during peptide synthesis, ensuring 1:1 labelling stoichiometry.^{24;25}
28 The advantage of lanthanide or metal chelating agents is considerably enhanced sensitivity of
29 the biomolecule during ICP-MS analysis, however, the relatively large chelating moiety will
30 affect peptide structure and thereby physico-chemical properties and potentially cell uptake,
31 which is undesirable in studies employing pre-labelled peptides.
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58 In the present study, we introduce selenium as a label in peptides by substitution of Met with
59 the naturally occurring selenium analogue, selenomethionine (SeMet) during peptide synthesis.
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4 Selenium labelling constitutes an attractive non-interfering method of probing biological
5 systems, while ensuring unambiguous labelling stoichiometry. Sulfur and selenium belong to
6 the group of chalcogens in the periodic table and thus display similar chemical
7 characteristics.²⁶ Due to these comparable properties, it may be assumed, that peptides
8 incorporating SeMet instead of Met will display similar biological effects. This is corroborated by
9 the fact that SeMet may randomly be exchanged with Met in humans as the body cannot
10 distinguish between Met and SeMet.²⁷ Synthetic selenopeptides have been examined for a wide
11 range of applications, for instance synthetic exchange of disulfide bridges in peptides with
12 diselenide or selenosulfo bridges have been reported for a wide range of bioactive peptides in
13 order to improve stability.²⁸ Lang et al. have proposed introduction of SeMet as an improved
14 target for thiophilic labelling with iodoacetamide reagents as compared to Met, since selenium
15 is more nucleophilic as compared to sulfur.²⁹ To the best of our knowledge, selenium has not
16 previously been introduced as a synthetic label for detection in quantitative analysis.

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33 The aim of the present work was to explore whether selenium labelling of a synthetic cell-
34 penetrating peptide may be employed in evaluation of stability and quantitative cell uptake by
35 ICP-MS. Labelling with SeMet was demonstrated for the cell-penetrating peptide (CPP)
36 penetratin, consisting of 16 amino acids including a Met residue, RQIKIWFQNRRMKWKK. The
37 peptide was originally derived from the *Drosophila* antennapedia transcription protein, and this
38 16-residue sequence was shown to be one of the minimal peptide sequences maintaining cell-
39 penetrating properties.³⁰ In addition, penetratin is able to deliver covalently conjugated drug
40 cargoes through cell membranes, thus offering potential opportunities in development of new
41 drug delivery systems.^{5;31} Two selenium-containing analogues were investigated, one with
42 SeMet added at the N-terminal of the peptide (N-PenM^{Se}) and one with the internal Met
43 replaced with SeMet (i-PenM^{Se}).

Experimental

Instrumentation

Inductively Coupled Plasma Mass Spectrometry

All ICP-MS measurements were performed on a PE Sciex ELAN 6000 Inductively Coupled Plasma Mass Spectrometer (ICP-MS) (Perkin Elmer, Norwalk, CT, USA) equipped with a MicroMist glass nebulizer (AHF, Lab Support Hillerød, Denmark) and a PC3 cyclonic spraychamber (Elemental Scientific Inc., Omaha, NE, USA) operated at 4°C. The sampler and skimmer cones were made of platinum. The nebulizer gas flow, lens voltage and ICP RF power were optimized daily with a solution of 100 µg Se per liter in mobile phase. The data acquisition settings for flow injection: dwell time 50 ms; sweeps per reading, 1; and readings per replicate were varied according to number of samples. For speciation analysis a desolvation system (Aridus II, CETAC Technologies) with a 200 µL min⁻¹ nebulizer was applied in order to remove organic solvent from the eluent prior to introduction to the ICP-MS. Desolvation system settings: a spray chamber temperature of 110°C; desolvator temperature of 160°C; sweep gas flow 7 mL min⁻¹, and nitrogen gas flow 6 mL min⁻¹. ICP-MS nebulizer gas flow was 0.9 mL min⁻¹. Lens voltage and ICP RF power were optimized regularly through the desolvation system on a solution of i-PenM^{Se} containing 10 µg Se per liter in mobile phase (50 % methanol). Data acquisition settings for speciation analysis: dwell time 200 ms; sweeps per reading 1; and readings per replicate 1325. ⁷⁷Se⁺, ⁷⁸Se⁺ and ⁸²Se⁺ isotopes were monitored; ⁸²Se⁺ was used for quantitative determination.

Molecular Mass spectrometry

All MS measurements were performed on a Q Exactive Orbitrap mass spectrometer (Thermo Scientific) with 250°C heated electrospray ionization. The sheath gas was set to 30 arbitrary units; auxiliary gas to 10 arbitrary units; capillary temperature to 350 °C; and spray voltage to 3300 V. The MS was run in full scan positive mode. Scan range was 300-3000 *m/z*, with lock mass 445.120 *m/z*, and the mass accuracy was below 1 ppm. The instrument was mass calibrated prior to analysis.

Reagents

Rink amide resin, coupling reagents and amino acid building blocks for solid-phase peptides synthesis as well as supplementary solvents and chemicals were acquired from Iris Biotech (Merkredwitz, Germany). L-Selenomethionine (SeMet) was obtained from TCI Europe. For mobile phases, glacial acetic acid, trifluoroacetic acid (TFA) and methanol were obtained from BDH Prolabo (France).

Procedures

Peptide synthesis and labelling

All peptides were synthesized by Fmoc-based solid-phase peptide synthesis (SPPS) using a microwave-assisted automated CEM Liberty synthesizer (CEM, Matthews, NC, US). H-SeMet-OH was N-protected with N-(9-Fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSU) to give Fmoc-SeMet-OH; ^1H NMR (600 MHz, dimethylsulfoxide- d_6): δ 12.66 (1H, -COOH), 7.89 (2 H, d, $J = 7.5$ Hz, Fmoc Ar-H), 7.72 (1 H, d, $J = 7.5$ Hz, Fmoc Ar-H), 7.72 (1 H, d, $J = 7.5$ Hz, Fmoc Ar-H), 7.67 (1 H, d, $J = 8.2$ Hz, -CONH-), 7.42 (2 H, t, $J = 7.5$ Hz, Fmoc Ar-H), 7.33 (2 H, br t, $J = 7.5$ Hz, Fmoc Ar-H), 4.33-4.27 (2 H, m, Fmoc -CH $_2$ -), 4.23 (1 H, br t, $J = 7.1$ Hz, Fmoc -CH-), 4.09 (1 H, m, H- α), 2.58 (1 H, ddd, $J = 12.4, 8.7$ and 5.1 Hz, H $_A$ - β), 2.51 (1 H, obsc. by solvent peak, H $_B$ - β), 2.04-1.93 (2 H, m, H- γ), 1.94 (3 H, s, -CH $_3$). ^{13}C NMR (150 MHz; dimethylsulfoxide- d_6): δ 173.58, 156.18, 143.84, 143.77, 140.73, 140.72, 127.64 (2C), 127.06 (2C), 125.26, 125.24, 120.12, 120.10, 65.56, 53.61, 46.67, 31.39, 20.96, 3.46. N-terminal labelling of penetratin with SeMet was performed with Fmoc-SeMet-OH (2.5 eq.), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP; 2.5 eq.) and N,N-Diisopropylethylamine (DIPEA; 5 eq.) in DMF at room temperature for 3 h in a teflon reactor (10 mL) equipped with a polypropylene filter. Incorporation of SeMet in i-PenM^{Se} was performed during automated peptide synthesis. Peptides were purified by preparative HPLC (Luna C18(2) column, 5 μm , 250x21 mm; Phenomenex) with UV detection at 280 nm. A linear gradient of 0-35% eluent B (H $_2$ O:MeCN 95:5 with 0.1% TFA) in eluent A (H $_2$ O:MeCN 5:95 with 0.1% TFA) within 20 min was applied. Flow rate was 20 mL min $^{-1}$.

Cell culture

The human cervical cancer cell line HeLa WT was obtained from American Type Culture Collection (ATCC) and maintained in Eagle's minimal essential medium (EMEM) supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), L-glutamine (2 mM), non-essential amino acids (0.1 mM), sodium pyruvate (1 mM) and 10% fetal bovine albumin (FBS) (Fischer Scientific, Slangerup, Denmark). Cells were grown in an atmosphere of 5% CO₂ at 37°C to 80% confluence and then sub-cultured at a ratio of approximately 1:5 twice a week. Cells, $7.6 \cdot 10^4$, were seeded onto 24 well plates (Fischer Scientific, Slangerup, Denmark).

Cell uptake studies

Experiments were performed 22-24 h after seeding of the HeLa WT cells; by then a sub-confluent monolayer (80-90% confluence) was achieved. Peptide solutions (10 µM) in Hanks Balanced Salt Solution (HBSS; Gibco, Paisley, UK) supplemented with 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, SigmaUltra) were prepared immediately prior to use in cell uptake studies from aqueous 200 µM peptide stock solutions prepared within 2 weeks from the uptake experiment and stored at 4°C. Uptake study was initiated by washing the cells once with 37°C phosphate buffered saline (PBS, Sigma), and subsequently addition of 400 µL of peptide solution. Cells were then incubated with peptide solution for 2 h on an orbital shaker (90 rpm) at 37°C. After exactly 2 h, medium was collected and immediately acidified with acetic acid to a final concentration of 2%. Cell uptake was terminated by washing the cells four times with ice-cold PBS, and then the cells were lysed by addition of 100 µL 0.1% Triton-X 100 in 2% acetic acid and kept on ice for 10 min. The lysis mixture was transferred to low-binding Eppendorf tubes (Alpha Laboratories), centrifuged at 25,000 g and the supernatant collected for analysis. All samples were stored at -18°C until analysis.

Determination of total selenium by flow injection analysis

Total selenium was determined by flow injection analysis (FI) using an aqueous carrier fluid of 0.02% trifluoroacetic acid (TFA), 0.1% acetic acid and 5% methanol. The flow rate was 200 $\mu\text{L min}^{-1}$ and the injection volume was 10 μL . Quantification was performed by single standard calibration based on peak areas of a 50 $\mu\text{g L}^{-1}$ solution of certified selenium element standard (Se concentration of 1010 mg L^{-1} , PlasmaCAL, SCP Science, Canada).

LC-ICP-MS (Aridus)

For LC-ICP-MS analysis an Agilent 1100 series HPLC system equipped with a degasser, a quaternary pump, an autosampler and column oven was applied. The column was an Aeris PEPTIDE XB-C18, 3.6 μm , 100 \times 2.1 mm ID column protected by a C18-Peptide SecurityGuard ULTRA cartridge (Phenomenex, SupWare, Denmark). A linear gradient of 20-80% methanol, (with 0.1% acetic acid and 0.05% TFA added) within 10 min was applied with a flow rate of 200 $\mu\text{L min}^{-1}$, a column temperature of 60 $^{\circ}\text{C}$, and injection volume of 5 μL . Semi quantitative peptide uptake was determined by use of a single standard solution of 2.4 μM i-PenM^{Se} standardized for total selenium content with a certified selenium element standard (PlasmaCAL, SCP Science, Canada), concentration of 1010 mg L^{-1} of Se.

LC-ESI-MS

UHPLC-ESI-MS analysis was performed on a Dionex Ultimate 3000 UHPLC (Thermo Scientific) equipped with a degasser, quaternary pump, autosampler, thermostated column compartment and a diode array detector (DAD) and hyphenated to a Q Exactive Orbitrap mass spectrometer. The column and mobile phase described for LC-ICP-MS were applied for this system as well. However, the linear gradient was shortened to 20-62% methanol (with 0.1 % acetic acid and 0.05 % TFA added) within 7 min to reduce time of analysis. MS data were collected between 2.5-10 min.

Results and Discussion

Peptide synthesis

Table 1 Synthetic Se-containing penetratin analogues (i-PenM^{Se}: internal SeMet incorporated. N-PenM^{Se}: SeMet added in the N-terminal).

| Abbreviation | Sequence | M _{monoisotopic} | M _{average} |
|----------------------|--|---------------------------|----------------------|
| i-PenM ^{Se} | RQIKIWFQNR(M ^{Se})KWKK-NH ₂ | 2292.250 | 2292.638 |
| N-PenM ^{Se} | M ^{Se} -RQIKIWFQNRMKWKK-NH ₂ | 2423.290 | 2423.834 |

The obvious approach for labelling a peptide with selenium would be to substitute a sulfur-containing amino acid with its selenium analogue, *i.e.* selenocysteine (SeCys) or selenomethionine (SeMet). Cysteine is usually avoided in peptide synthesis because of high redox potentials and thereby low stability. Selenoamino acids are generally more readily oxidized during synthesis and purification compared to the sulfur analogues; with the reactivity of SeCys exceeding that of SeMet.²⁶ Thus, SeMet was chosen for selenium labelling in the present study. Exchange of Met in penetratin with other neutral amino acids, leucine (Leu) or alanine (Ala), has been reported in literature without significant change of cell uptake in HeLa WT cells and HaCAT cells respectively.^{32,33} Hence, exchange of Met appears to be a convenient labelling site in penetratin.

Synthesis was carried out by employing microwave (MW)-assisted automated solid-phase peptide synthesis (SPPS). In order to test whether it was possible to introduce SeMet in the peptide sequence with a reasonable yield and purity, a penetratin analogue with SeMet added to the N-terminal (N-PenM^{Se}) was initially synthesized (sequence shown in Table 1). As a standard C→N SPPS strategy was applied, the peptide was conveniently modified by introduction of SeMet at the N-terminal subsequently to MW-assisted automatic synthesis. Thereby minimum exposure of SeMet to reagents was ensured, and thus the risk of SeMet oxidation was minimized. Also, it was intended to avoid MW irradiation in this initial labelling experiment as it might not be compatible with SeMet. Furthermore, N-terminal labelling of penetratin has been widely used for introduction of fluorescent dyes and covalent attachment

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4 of drug cargoes.³⁴⁻³⁶ Synthesis and purification of N-PenM^{Se} was accomplished, and purity of
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6 the resulting selenopeptide was determined by LC-ICP-MS to be 92% as illustrated in Figure 1.
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8 It appears from the figure that an impurity, eluting immediately before the desired peptide was
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10 present. By LC-ESI-MS, a signal was observed at m/z 606.831, corresponding to the $[M+4H]^{4+}$
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12 ion of a peptide with mass 2423.293 confirming N-PenM^{Se} (2423.290 Da) as the primary
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14 product ($\Delta M \leq 1$ ppm). A signal from the impurity was seen at m/z 606.326 corresponding to
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16 the $[M+4H]^{4+}$ ion representing a mass of 2421.273 Da ($\Delta M \leq 1$ ppm) which is 2.020 Da less
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18 than that of N-PenM^{Se}. A difference of 2.020 Da might arise from loss of two hydrogen atoms
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20 (2.016 Da) due to cyclization or formation of a double bond.
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25 Introduction of SeMet within the peptide sequence by exchange of Met was then pursued. By
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27 fully automated MW-assisted SPPS, SeMet was incorporated at the original position of Met in
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29 penetratin (i-PenM^{Se}, the sequence is shown in (Table 1). Purity of the synthesized peptide was
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31 determined by LC-ICP-MS to be 89%. LC-ESI-MS revealed a signal at m/z 1147.132
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33 corresponding to the $[M+2H]^{2+}$ ion of the peptide, confirming the identity of i-PenM^{Se}
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35 (2292.250 Da) with a mass of 2292.248 Da ($\Delta M \leq 1$ ppm) as the primary product. The
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37 $[M+2H]^{2+}$ ion originating from the impurity was observed at m/z 1155.131 corresponding to a
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39 mass of 2308.246 Da ($\Delta M \leq 1$ ppm). The mass increase of 15.998 Da, indicated the presence
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41 of an additional oxygen atom (15.995 Da) that may arise from oxidation of the peptide, most
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43 likely in the SeMet residue. Oxidation of SeMet may have occurred during synthesis due to the
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45 exposure to oxygen at elevated temperatures in the synthesizer or during the purification
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47 process.
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52 As the oxidized impurity only was identified for i-PenM^{Se}, mild synthesis conditions appear
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54 important in order to avoid oxidation of SeMet. Furthermore, slightly higher peptide purity
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56 (92%) was obtained for N-PenM^{Se} as compared to i-PenM^{Se} (89%). Purity of i-PenM^{Se} might be
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58 improved in future by manual synthesis of the peptide or by using lower temperatures during
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60 coupling and deprotection steps. However, manual peptide synthesis is considerably more

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4 time-consuming, favoring automated synthesis. Improving the preparative HPLC method might
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6 also result in increased purity of the final product.
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10 The stability of i-PenM^{Se} in water was monitored by LC-ICP-MS during a two-month storage
11 period. The ratio between intact peptide and impurity remained constant throughout the period
12 (data not shown) confirming that the SeMet-labelled peptide was stable in aqueous solution.
13
14 By dilution of the selenopeptides in HBSS medium, a decrease of intact peptide was
15 immediately observed in LC-ICP-MS chromatograms (Figure 1). The mechanism behind this
16 increased degradation is not clarified, but may be due to the higher ionic strength of the HBSS
17 medium. The content of intact i-PenM^{Se} decreased from 89% to 81%, while the content of
18 intact N-PenM^{Se} decreased from 92% to 86%. Only degradation to the formerly identified
19 impurities was observed, thus no oxidation of N-PenM^{Se} was seen, whereas the presence of
20 oxidation product of i-PenM^{Se} increased. This indicated that oxidation may be dependent on the
21 position of SeMet in the peptide sequence.
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34 The stability of fluorescently labelled penetratin has been investigated in a number of studies,
35 as the translocation of the peptide and its function as drug carrier may be dependent on the
36 stability. Tréhin and colleagues have shown considerable degradation of carboxyfluorescein-
37 labelled penetratin (CF-penetratin) in HBSS after 21 days of incubation, since only 76% of the
38 intact peptide remained.¹⁰ In PBS supplemented with 1 g L⁻¹ D-glucose, CF-penetratin was
39 shown to be stable for only 1 h.³⁷ Thus, instability is also observed for the native Met-based
40 penetratin.
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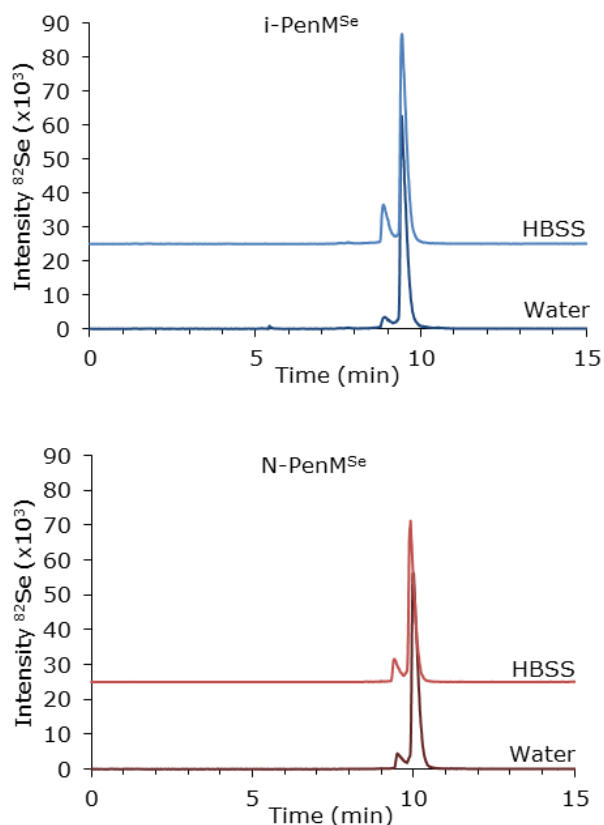


Figure 1 LC-ICP-MS chromatograms of i-PenM^{Se} and N-PenM^{Se} in water and HBSS medium. Amounts of intact peptide determined as the percentage of total selenium in the chromatogram: i-PenM^{Se} in water: 89% and i-PenM^{Se} in HBSS: 81%; N-PenM^{Se} in water: 92% and N-PenM^{Se} in HBSS: 86%.

Cell uptake of selenopeptides

Determination of total selenium by FI-ICP-MS

To test whether the synthesized selenium analogues of penetratin were able to penetrate the cell membrane, a two-hour uptake study in HeLa WT cells was conducted. Several studies have shown translocation of penetratin into eukaryotic cells with HeLa WT cells being one of the most frequently used cell lines.³⁸

The total uptake of selenium from the peptides was determined by flow injection ICP-MS (FI-ICP-MS), (Table 2). SeMet was included as a positive control of the cell uptake experiment

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setup, as extensive cell uptake of SeMet has formerly been reported in *e.g.* the Caco-2 cell line.³⁹ Considerable amounts of i-PenM^{Se} and N-PenM^{Se} were taken up by the cells, 17.4±2.2 pmol/10⁴ cells of i-PenM^{Se} and 25.4±2.9 pmol/10⁴ cells of N-PenM^{Se}, respectively. However, the selenium uptake from the peptides was not as pronounced as from SeMet, 49.6±3.3 pmol/10⁴ cells. Blank lysates contained no selenium.

Table 2 Uptake in HeLa WT cells incubated for two hours with 10 µM of i-PenM^{Se}, N-PenM^{Se} and SeMet. Total selenium in cell lysates was determined by FI-ICP-MS, results obtained from one passage, three independent wells. Uptake of intact peptide determined by LC-ICP-MS, results obtained from one lysate.

| | Start Se | Total Se | Intact peptide |
|----------------------|----------------------------|----------------------------|----------------------------|
| | pmol/10 ⁴ cells | pmol/10 ⁴ cells | pmol/10 ⁴ cells |
| i-PenM ^{Se} | 505 | 17.4±2.2 | 16.1 |
| N-PenM ^{Se} | 484 | 25.4±2.9 | 18.4 |
| SeMet | 508 | 49.6±3.3 | - |

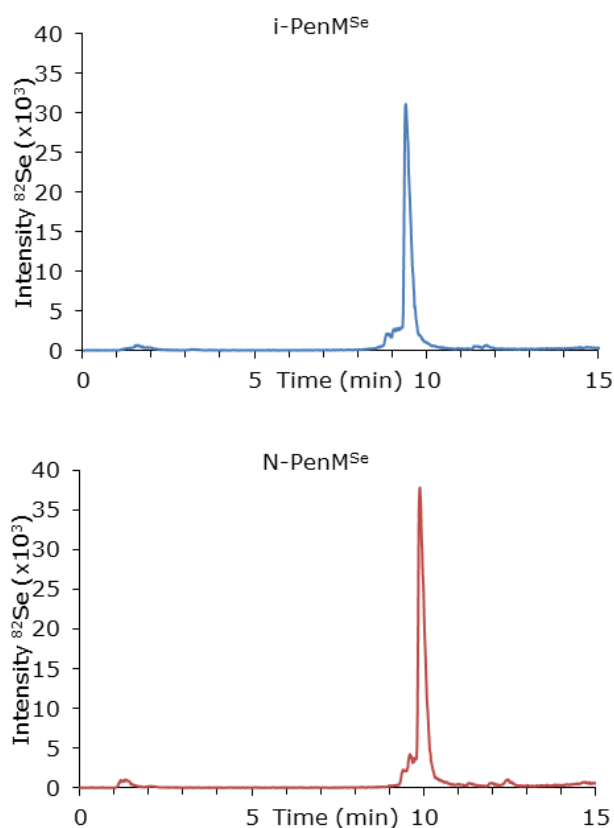
Cellular uptake of penetratin has been studied by several groups in a variety of different human cell lines. The majority of these studies are based on fluorophore-labelled penetratin. Determination of CF-penetratin uptake in four different human carcinoma cell lines by total fluorescence spectrophotometry showed very different degrees of peptide internalization depending on the cell line.^{40;41} Lindgren and colleagues reported 14% uptake of total fluorescence of 2-aminobenzoic acid (Abz)-labelled penetratin in Caco-2 cells after 30 min incubation with 10 µM peptide.³⁴ Sarko *et. al.* investigated cellular uptake of ¹¹¹In-DOTA-labelled penetratin in six different human carcinoma cell lines, where the uptake was determined by a γ-counter. Upon incubation for 30 min with cells, the amount of internalized radioactive label varied remarkably between different cell lines.²⁵ As HeLa WT cells were not among the investigated cell lines, these data are not directly comparable to those obtained in the present work. Bahnsen *et. al.* reported semi-quantitative uptake of CF-labelled penetratin analogues in HeLa WT cells via determination by flowcytometry.³² However, the results were

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4 presented as relative to CF-penetratin uptake and they are thus not comparable to those of the
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6 present work.
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10 **Semi-quantitative determination of peptide uptake in HeLa WT cells by LC-ICP-MS**

11 FI-ICP-MS revealed similar uptake of selenium for i-PenM^{Se} and N-PenM^{Se}. Speciation analysis
12 of the lysates by reversed-phase (RP) LC-ICP-MS allowed estimation of whether penetratin was
13 taken up by HeLa WT cells as the intact peptide or as degradation products. The amounts of
14 peptide are shown in Table 2. Hyphenation of RP gradient systems to ICP-MS is challenging, as
15 the hot plasma in the ICP-MS will extinguish when exposed to high concentrations of organic
16 solvent. This could be overcome by use of narrow bore columns decreasing the flow rate
17 considerably. However the use of high contents of MeOH may result in ICP-MS signal drift.²⁴ In
18 this study, the organic solvent from the mobile phase was removed by use of a membrane
19 desolvation system. Chromatograms of cell lysates (Figure 2) revealed that the main
20 component passing the cell barrier in case of both i-PenM^{Se} and N-PenM^{Se} was the intact
21 peptide. This was confirmed by LC-ESI-MS ($[M+2H]^{2+}$ m/z 1147.127, mass 2292.239, $\Delta M < 5$
22 ppm and $[M+3H]^{3+}$ m/z 808.771, mass 2423.280, $\Delta M < 5$ ppm, respectively). Thus 16.1
23 pmol/10⁴ cells of i-PenM^{Se} and 18.4 pmol/10⁴ cells of N-PenM^{Se} were observed in the
24 respective HeLa WT cell lysates (Table 2). It is not possible from the present data, to clarify if
25 the minor amount of degradation products observed in the lysates entered the cells as such or
26 were produced in the cells during uptake studies. Otherwise they may have been formed
27 during the cell lysis applying a mixture of 0.1% triton X and 2% CH₃COOH. Very few studies
28 have reported on speciation of cell lysates. Holm *et. al.* investigated the intracellular
29 degradation of CF-penetratin in yeast cells by RP-LC-fluorescence detection (FLD) after one
30 hour of incubation. They showed that a high total uptake of the fluorophore was accompanied
31 by extensive intracellular degradation of the peptide.¹¹ A similar pattern was reported by Palm
32 *et. al.* for CF-penetratin uptake in non-mammalian cells upon incubation for one hour.⁴² These
33 results are in contrast to the results of the present study. The percentage of intact peptides
34 observed in HeLa WT cell lysates were 73% and 76% for i-PenM^{Se} and N-PenM^{Se}, respectively.
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7 FI-ICP-MS and LC-ICP-MS of HeLa WT cell lysates demonstrated that cellular uptake of i-
8 PenM^{Se} and N-PenM^{Se} mainly represented internalization of intact peptides. Direct comparison
9 of these results to existing literature was not possible, as different cell models and labels were
10 applied.
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45 **Figure 2** LC-ICP-MS chromatograms of cell lysates from HeLa WT cells incubated with 10 μ M
46 of selenopeptide for 2 h.
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51 **Degradation of selenopeptides during cell uptake studies**

52 Degradation of CF-penetratin has been reported to be a critical determinant for cellular uptake
53 of penetratin.³⁷ In order to fully exploit the drug delivery potential of penetratin, consistent
54 evaluation of cellular uptake and stability is critical. Thus, degradation of the two synthetic
55 selenopeptides, during incubation with HeLa WT cells in HBSS cell medium for two hours, was
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investigated. LC-ICP-MS analysis of samples from the HBSS medium revealed considerable additional degradation of the selenopeptides upon exposure to cells as compared to results obtained in cell-free HBSS. This is shown in Figure 3. It appears that several new species appeared after exposure to cells in medium as compared to the cell-free medium in Figure 1. The presence of intact peptide decreased for i-PenM^{Se} from 81% to 55% while N-PenM^{Se} decreased from 86% to 61%. The enhanced degradation during exposure to HeLa WT cells indicated that the degradation was primarily proteolytic rather than chemical. Proteolytic degradation of CF-penetratin when exposed to various cell lines has previously been correlated to cell line-dependent variations in stability.^{10;34}

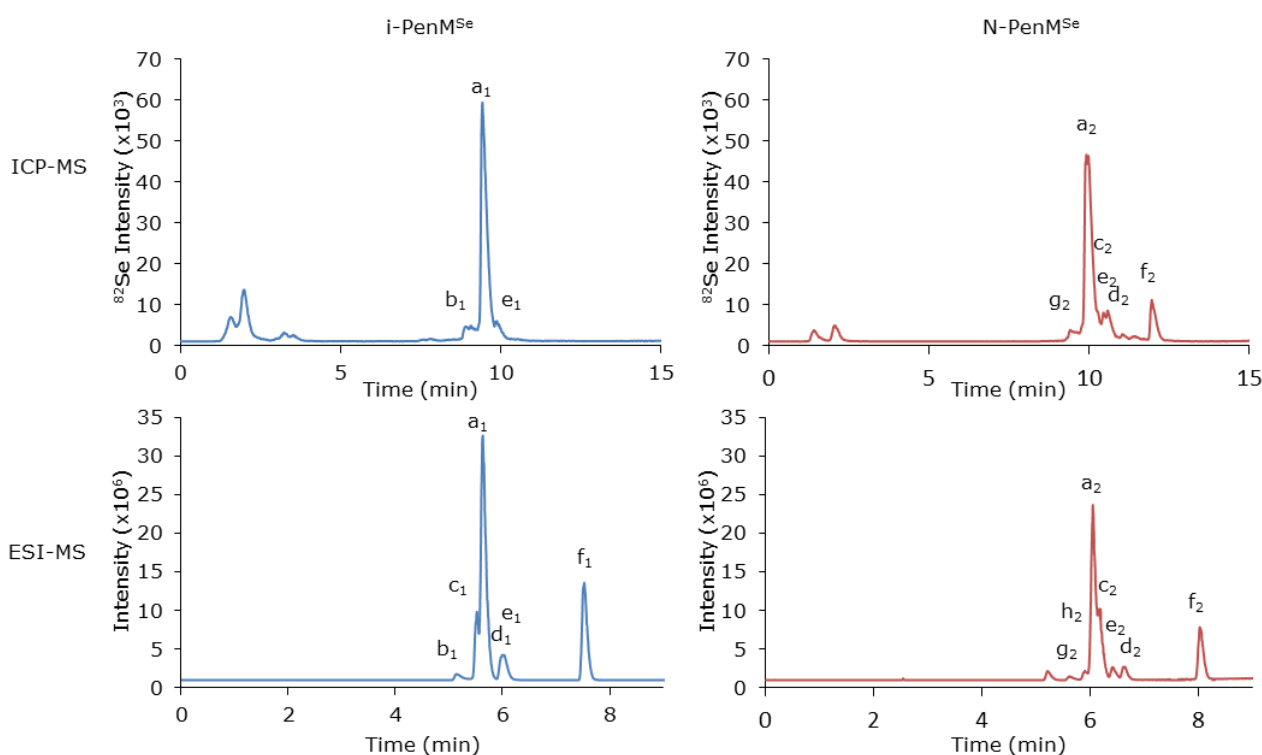


Figure 3 Chromatograms of samples of Se-peptides in HBSS medium detected by ESI-MS and ICP-MS. MS chromatograms are extracted ion chromatograms (EIC) (i-PenM^{Se} m/z : $[M+2H]^{2+}$ 495.798; $[M+3H]^{3+}$ 515.637; $[M+2H]^{2+}$ 694.899; $[M+3H]^{3+}$ 722.721; $[M+3H]^{3+}$ 765.092; $[M+3H]^{3+}$ 770.425. N-PenM^{Se} m/z : $[M+3H]^{3+}$ 523.264; $[M+3H]^{3+}$ 573.997; $[M+3H]^{3+}$ 575.998; $[M+2H]^{2+}$ 585.290; $[M+3H]^{3+}$ 697.409; $[M+3H]^{3+}$ 766.400; $[M+3H]^{3+}$ 808.100; $[M+3H]^{3+}$ 808.774). Annotation of the peaks corresponds to peptide sequences presented in Table 3.

In order to investigate the mode of degradation further, the samples from the HBSS medium from uptake studies were analyzed by LC-ESI-MS. Degradation products of i-PenM^{Se} and N-PenM^{Se} were identified via their *m/z* values, and sequences corresponding to these masses are suggested in Table 3. The main compound observed for both i-PenM^{Se} and N-PenM^{Se} was the intact peptide (Figure 3, peak a). Similar cleavage sites were observed for i-PenM^{Se} and N-PenM^{Se} as illustrated in Figure 4. Previous unpublished studies on penetratin stability by UPLC-QTOF identified cleavage sites in native penetratin when exposed to human and rat gastrointestinal fluids that correspond to those observed for selenium-labelled penetratin analogues in the present study.⁴³ Cleavage at the C-terminal side of arginine (R) and lysine (K) were confirmed to be caused by trypsin whereas cleavage at the C-terminal side of phenylalanine (F) was caused by chymotrypsin.⁴³ Thus, the degradation products c, d and e shown in Figure 3 may be due to presence of trypsin while product f may be a result of chymotrypsin. The composition of enzymes produced by in HeLa cells are not reported, but some proteolytic activity is expected, which may include trypsin and chymotrypsin.

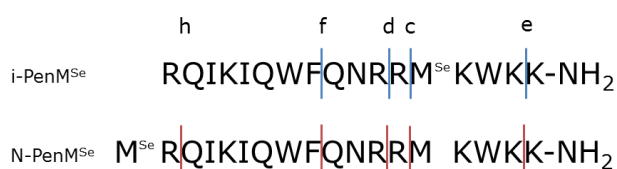


Figure 4 Cleavage sites of i-PenM^{Se} and N-PenM^{Se} after 2 h incubation at 37°C with HeLa WT-cells. Annotation corresponds to peaks in chromatograms in Figure 3.

Table 3 Sequences and masses of identified degradation products in medium samples from cell uptake experiments of i-PenM^{Se} and N-PenM^{Se} determined by LC-ESI-MS, mass accuracy \leq 1 ppm. Sequences annotated in conformity to chromatograms shown in Figure 3.

| | i-PenM ^{Se} (1) | M _{monoisotopic} | N-PenM ^{Se} (2) | M _{monoisotopic} |
|----------|---|---------------------------|---|---------------------------|
| a | RQIKIWFQNRRM ^{Se} KWKK-NH ₂ | 2292.250 | M ^{Se} RQIKIWFQNRRKWKK-NH ₂ | 2423.290 |
| b | Oxidized i-PenM ^{Se} | 2308.245 | - | - |
| c | RQIKIWFQNR-OH | 1543.885 | M ^{Se} RQIKIWFQNR-OH | 1722.870 |
| d | RQIKIWFQNR-OH | 1387.784 | M ^{Se} RQIKIWFQNR-OH | 1566.769 |
| e | RQIKIWFQNRRM ^{Se} KWK-OH | 2165.139 | M ^{Se} RQIKIWFQNRRKWK-OH | 2296.17 |
| f | RQIKIWF-OH | 989.581 | M ^{Se} RQIKIWF-OH | 1168.566 |
| g | - | - | N-PenM ^{Se} - 2 Da | 2421.275 |
| h | - | - | QIKIWFQNRRKWKK-NH ₂ | 2088.204 |

The degradation products identified from i-PenM^{Se} and N-PenM^{Se} are listed in Table 3 and differed in presence of selenium. In extracted ion chromatograms (EIC) of the LC-ESI-MS analysis, dissimilarities were at first just observed by a minor difference in retention time of the peaks in the chromatograms (Figure 3). Comparison to the LC-ICP-MS chromatograms revealed that the difference was due to the selenium labelling site. For instance peak f was present in the LC-ICP-MS chromatogram of N-PenM^{Se} in medium, but not in that of i-PenM^{Se} in medium, whereas the peak appeared in both LC-ESI-MS EICs. Zooming in on the isotope patterns of the LC-ESI-MS peaks revealed a characteristic pattern for compounds containing selenium which was easily distinguished from a carbon pattern, exemplified for peak f in Figure 5. The short peptide RQIKIWF from i-PenM^{Se} exhibited the common peptide carbon pattern (¹²C, 98.90%; ¹³C, 1.10%). The selenium-containing degradation product from N-PenM^{Se}, M^{Se}RQIKIWF displayed a combined pattern of carbon and selenium (⁷⁶Se, 9.0%; ⁷⁷Se, 7.6%; ⁷⁸Se, 23.6%; ⁸⁰Se, 49.7%; ⁸²Se, 9.2%). Hence, the characteristic isotope pattern of selenium demonstrates that selenium is also a useful molecular MS label.

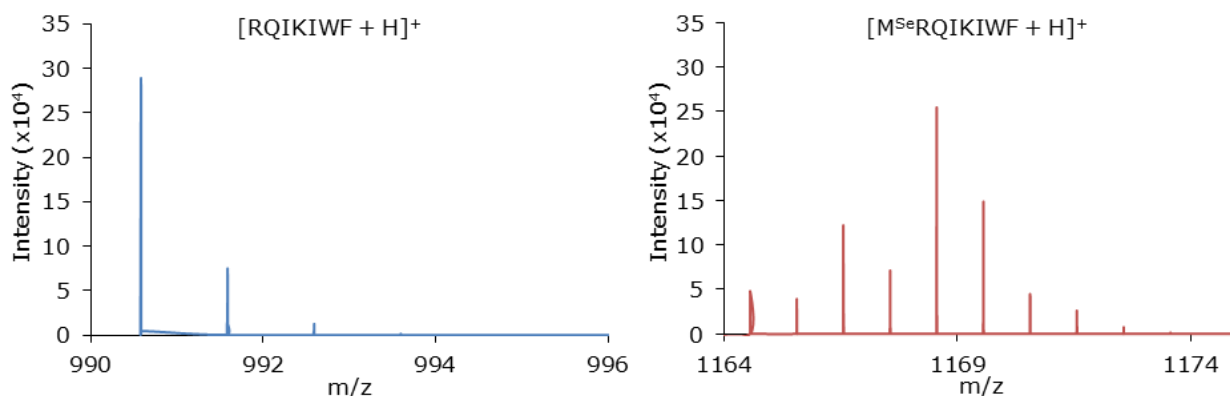


Figure 5 Simulated isotope patterns of single charged peptides of [RQIKIWF+H]⁺ and [M^{Se}RQIKIWF+H]⁺ (peak f₁ and f₂ in Figure 3). Left: degradation product from i-PenM^{Se}, isotope pattern without selenium. Right: degradation product of N-PenM^{Se} containing selenium.

Thus, differences between selenium-containing degradation products obtained from i-PenM^{Se} and N-PenM^{Se}, respectively, were revealed by LC-ICP-MS (chromatograms in Figure 3) as only selenium-containing compounds were present. In general, amino acids and short polar peptides eluted in the beginning of the LC-ICP-MS chromatograms, while larger fragments and oxidation products appeared around the intact peptide. In N-PenM^{Se}, SeMet was added in the N-terminal, and was thus retained in most of the cleaved peptides resulting in larger selenium-containing degradation products. By contrast, SeMet was incorporated towards the highly positively charged C-terminal in i-PenM^{Se}, and therefore SeMet was predominantly cleaved off the parent peptide as very polar smaller Se-containing peptides eluting in the beginning of the chromatogram. Thus, the position of the label in the peptide sequence must be considered carefully in development and optimization of the LC methods. However, these results demonstrated that selenium labelling of penetratin indeed may be used as a readily detectable probe in evaluation of peptide degradation in various matrices by LC-ICP-MS and LC-ESI-MS. The possibility of specific detection of selenium offered by the ICP-MS technique implies that degradation in even more complex sample matrices may be analyzed.

Analytical figures of merit

Total selenium uptake in HeLa cells was successfully quantified by FI-ICP-MS while uptake of intact peptide was semi-quantitatively determined by LC-ICP-MS, exploiting the high selectivity and robustness of the ICP ionization. Limit of detection (LOD) of FI-ICP-MS was estimated to 6.0 ng Se mL⁻¹ (0.76 nmol Se injected) as 3 σ of peak area of 2.4 μ M i-PenM^{Se}. FI-ICP-MS precision of three repeated injections of 2.4 μ M i-PenM^{Se}, relative standard deviation (RSD) was 1.0%. LOD for the LC-ICP-MS method was 6.5 ng Se mL⁻¹ (0.41 nmol Se on column) estimated as 3 σ of peak area of 2.4 μ M i-PenM^{Se}. Column recovery was 101.5 \pm 1.1% (n=3) and 101.9 \pm 0.3% (n=2) of i-PenM^{Se} and N-PenM^{Se} respectively. Precision of three repeated injections of 2.4 μ M i-PenM^{Se} was 1.3% RSD.

LOD of the FI-ICP-MS method in this study was higher than what has previously been reported for low molecular selenium compounds.³⁹ Peptides are known to exhibit unspecific adsorption to different materials, *e.g.* glass, metal and plastic.⁷ Adsorption to vials, injection needle or tubing, might explain the increased LOD. The flow injection method was thoroughly optimized to avoid unspecific adsorption by acidification and increase of peptide concentration in samples. However, adsorption might still contribute to increased deviation between injections, resulting in higher LOD for peptides as compared to smaller selenium compounds. Furthermore, the low-abundant selenium isotope ⁸²Se (9.2%) was measured in the present work, due to polyatomic interference of ⁴⁰Ar₂ of the most abundant isotope ⁸⁰Se (49.7%) in the ELAN 6000 quadrupole instrument. Sufficient sensitivity was achieved by the ELAN 6000 for the measurements of the present study. However, detection limits may be reduced by application of improved ICP-MS instrumentation *e.g.* applying a dynamic reaction cell (DRC) instrument.⁴⁴

Conclusion

In this study, selenium incorporated as SeMet was demonstrated to be a useful label for quantification of pharmaceutically relevant peptides exemplified by the cell-penetrating peptide, penetratin. Selenium was readily introduced during solid-phase peptide synthesis, however, the stability of SeMet at higher temperatures must be considered. The introduction of the non-toxic SeMet label is considered to have minimal effect on the biological activity of the peptide. Selenium labelling thus offers minimal disturbance of the native structure of the peptide of interest as compared to labelling with fluorophores or metal-chelating agents. The labelling technique and combination of FI-ICP-MS, LC-ICP-MS and LC-ESI-MS techniques provided detailed information of the fate of penetratin in cell uptake studies. In addition to qualitative information on degradation products obtainable from LC-ESI-MS, LC-ICP-MS allowed for quantification of the peptide. Most therapeutic peptides are synthetic analogues of endogenous peptides, and incorporation of selenium, allows for improved critical assessment of the native drug or drug delivery candidate early in the drug development process.

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