Identification and quantification of falsified peptide drugs via HILIC-DAD-MS

Steven Janvier\textsuperscript{1,2}, Evelien De Sutter\textsuperscript{2}, Evelien Wynendaele\textsuperscript{2}, Bart De Spiegeleer\textsuperscript{2}, Celine Vanhee\textsuperscript{1} and Eric Deconinck\textsuperscript{1}

\textsuperscript{1}Division of Food, Medicines and Consumer Safety, Section Medicines and Healthcare Products, Scientific Institute of Public Health (IPH), J.Wytmanstraat 14, B-1050 Brussels, Belgium

\textsuperscript{2}Laboratory for Drug Quality & Registration (DruQuar), Faculty of Pharmaceutical Sciences, University of Ghent, Ottergemsesteenweg 460, B-9000 Ghent, Belgium

Introduction

Biopharmaceuticals have established themselves as highly efficient medicines, and are still one of the fastest growing parts of the health-product industry. Unfortunately, the introduction of these promising new drugs went hand in hand with the creation of a black market for falsified biotechnology drugs. Particularly popular are the lyophilized peptides with a molecular weight of less than 5 kDa. Multiple systems based on reversed-phase LC have been developed to tackle these grievous practices. The emerging of more polar peptides however requires the introduction of other separation techniques such as Hydrophilic Liquid Interaction Chromatography (HILIC).

HILIC chromatography allows for the analysis of polar (or ionic) compounds based on a bifasic (aqueous/organic) system and is compatible with mass spectrometric detection (no ion pairing agents necessary). Therefore, we set out to develop and validate an analytical method based on HILIC to identify and quantify the most frequently encountered illegal peptides on the European market. For this objective, five HILIC columns with different types of stationary phases were tested on their chromatographic performance in terms of resolution and peak symmetry. The most suitable system was subsequently optimised and validated for the detection and quantification of these illegal preparations.

1. Testing of HILIC conditions

Set-up

Ten peptides (doping peptides, hormones, preclinical drugs)

Five HILIC columns:

\begin{itemize}
  \item Acquity BEH HILIC (100x2.1 mm, 1.7 µm)
  \item Acquity BEH HILIC amide (100x2.1 mm, 1.7 µm)
  \item Acquity Cortecs HILIC (100x2.1 mm, 1.6 µm)
  \item Merck ZIC HILIC (100x2.1 mm, 3.5 µm)
  \item Merck ZIC-c HILIC (100x2.1 mm, 3.0 µm)
\end{itemize}

Variable:

- Flow: 0.2, 0.3, 0.4, 0.5 mL/min
- Temperature: 30, 40, 50 °C

Response variables:

- Resolution
- Peak symmetry

Enhanced stability due to

1) Introduction of online cleaning step (blue line) to wash away remaining matrix constituents of falsified preparations

Full chromatographic separation

Separation of critical pare @

Flow: 0.27 mL/min
Temperature: 45 °C
Gradient time: 20 min (red line)
Linear increase gradient: 3% ACN/min

Matrix effect

Matrix constituents falsified products: 150 mM NaCl, 10 mM Na$_2$PO$_4$, 1% PEG, 5% mannitol
Recoveries at high concentrations influenced by mannitol
$\Rightarrow$ Solved by changing sample solvent to: 10 mM ammonium formate 80% ACN + 2% formic acid

2) Washing column at low flow rate with 25 mM ammonium formate 80% H$_2$O after 100 injections

2. Optimization

Identification

UHPLC-MS\textsuperscript{3}: Dionex UltiMate 3000 (Thermo Scientific, USA) hyphenated to an Amazon\textsuperscript{TM} speed ETD MS (Bruker, Germany).

MS-settings: ESI(+), mass range 300-1200 m/z

Criterias:

- Selectivity: Matrix comprised of 150 mM NaCl, 10 mM Na$_2$PO$_4$, 0.02% PEG, 5% mannitol
- Sensitivity: S/N ≥ 3
- Peptide identification: (C) Sport drug testing
- Correct MS mass (±0.3 Da)
- Minimum 2 MS\textsuperscript{2} fragments (±1.0 Da)

Acquired screening detection limit = 10 µg/mL

Quantification

Acquriy UPLC coupled to a DAD detector (Waters, USA)

Settings: detection wavelength 214 or 277 nm

Validation performed according to ISO 17025 in matrix for 6 peptides as proof of principle

3. Validation

Conclusion

In this study a ZIC HILIC system was selected in favour of four other columns based on the chromatographic performance with frequently encountered peptide drugs on the European market.

The developed ZIC HILIC system allows for the detection and quantification of a wide spectrum of falsified peptide drugs available on the internet. Furthermore, the method could also be envisaged for the detection of new emerging polar peptide drugs found in cosmetics.