Hochauflösende Chromatographie und Massenspektrometrie in der Charakterisierung von Pharma-Wirkstoffen

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Citius, altius, fortius
Faster separation, higher pressure, higher sample throughput

Analysis of synthetic pharmaceuticals

**Background**
- Thyroxin acts as a prohormone of its active form Liothyronine.
- Hypothyroidism requires a lifelong treatment with Thyroxin.
- Synthetic Thyroxin needs to be checked for synthesis by-products or stress-induced degradation products.

**Aims**
- Modification of a validated RP/phosphate buffer/UV-method to MS-compatible conditions.
- Optimization of the total analysis time from 60 to less than 5 minutes.
- Structure elucidation of impurities by high-resolution mass spectrometry.
Thermal stressing as a model for long term storage

Model compounds for thermal stressing:
- T2/T3/T4
- r-T2/r-T3
- TriProp
- DiAc/TriAc/TetraAc
- TetraFA

Thyroxin was kept at 40 °C for 6 months and finally stressed for 16 h at 60 °C.

UHPLC and the Gradient-Volume-Principle

\[ V_G = F \cdot t_G = \text{const.} \]

- \( V_G \), gradient volume
- \( F \), volumetric Flow
- \( t_G \), gradient time

If
- gradient is linear
- Starting and finishing eluents are the same

then
- Separation changes mostly in separation time

In practice: small particles enable separations at high flow rates with-out significant loss in efficiency.

In practice: doubling of the flow rate requires halving of the gradient time!
The price: column backpressure

\[ \Delta p[\text{bar}] = \frac{0.056}{d_p^2[\mu \text{m}]} \cdot N \]

Halving of particle diameter while maintaining plate number entails:
- halving of column length L,
- doubling of flow velocity,
- quadrupling back pressure \( \Delta p \),
- Reduction in run time by factor 4.

In practice: smaller particles require higher pressure in order to work at the optimum flow velocity.

Instrumental requirements

Accela 1000 bar UHPLC system:
- 1000 bar pressure limit pump
- 1000 \( \mu \)L/min maximal flow rate
- Hypersil GOLD column, 100 x 2.1 mm, 1.9 \( \mu \)m particles
- 20 Hz UV Detector

Accela 1250 bar UHPLC system:
- 1250 bar pressure limit pump
- 2000 \( \mu \)L/min maximal flow rate
- Hypersil GOLD column, 100 x 2.1 mm, 1.9 \( \mu \)m particles
- 80 Hz UV Detector
High-throughput UHPLC-ESI-Orbitrap-MS method

Conventional method
- YMC C18 Pro, 150 x 4,6 mm,
- 38 min acetonitrile gradient,
- 4.5 mM phosphate buffer,
- 900 µL/min, UV @225 nm.

Speed method
- 2.1 min acetonitrile gradient,
- 0.1 % formic acid
- 20 Hz UV detection
- Orbitrap MS detection

Optimized UHPLC-MS method

20-fold reduction of total analysis time from 60 to 3 min!

- Hypersil GOLD, 1.9 µm,
  100 x 2.1 mm,
- 30-80 % ACN + 0.1 % FA in 2.1 min,
- 1000 µL/min; 60°C.
Reproducibility of ultra-fast separation

Overlay of 20 UV-measurements

Rel. Std. Dev. of peak area

<table>
<thead>
<tr>
<th></th>
<th>UV</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00%</td>
<td>1.26 %</td>
<td>2.97 %</td>
</tr>
</tbody>
</table>

Mass accuracy of negative-ESI MS-detection

0.34 ppm

Std. Dev. $t_R$: 18 hundredth seconds!

Reproducibility of retention time and peak area

![Bar chart showing reproducibility of retention time and peak area](chart.png)
2 Higher information content: interfacing to high-resolution mass spectrometry

Instrumental platforms

Accela-Exactive:
- max. resolution of 100,000
- 1 scan per s @ R=100,000
- 10 scans per s @ R=10,000
- < 1 ppm mass accuracy
- No MS^n possibility

Accela-LTQ Orbitrap:
- max. resolution of 100,000
- 4 scans/s @ R=15,000
- 0.5 scans/s @ R=100,000
- < 1 ppm mass accuracy
- MS^n possibility
Inter-laboratory & instrument method transfer

a) Orbitrap, Salzburg September 2009

- S/N = 493
- Scan rate: 4 scans/s
- Mass accuracy: -0.2 ppm (R = 15,000)

b) Exactive, Basel, November 2009

- S/N = 109
- Scan rate: 12 scans/s
- Mass accuracy: 0.95 ppm (R = 10,000)

Variation of injection time and resolution

<table>
<thead>
<tr>
<th>Orbitrap</th>
<th>Injection Time [ms]</th>
<th>10</th>
<th>100</th>
<th>250</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resolution</td>
<td>10</td>
<td>100</td>
<td>250</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>S/N</td>
<td>10</td>
<td>100</td>
<td>250</td>
<td>500</td>
</tr>
<tr>
<td>Exactive</td>
<td>Scan rate [scans/s]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exactive</td>
<td>Mass accuracy [Δ ppm]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exactive</td>
<td>Injection Time [ms]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

V. Neu
Ultra-fast separation of thermally stressed T4

- More than 120 substances detected in a 3 min run!
- High-resolution fragmentation of the top 50 impurities (up to MS6)!

Base peak chromatogramm

Hypersil GOLD, 1.9 µm, 100 x 2.1 mm, 30-70 % ACN in 2.1 min, 0.1 % formic acid, 1000 µl/min; 60°C.

V. Neu

From accurate mass to structure

**Step 1:**
Computer-aided ranking

<table>
<thead>
<tr>
<th>Molecular formula</th>
<th>RDB</th>
<th>Δ ppm</th>
<th>e⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₁₄H₈O₃NI₄</td>
<td>9.5</td>
<td>- 0.320</td>
<td>even</td>
</tr>
<tr>
<td>C₁₂H₆O₂N₄I₄</td>
<td>10.0</td>
<td>1.481</td>
<td>odd</td>
</tr>
<tr>
<td>CHO₁₅N₈I₃</td>
<td>4.0</td>
<td>- 1.996</td>
<td>odd</td>
</tr>
</tbody>
</table>

**Step 2:**
Comparison of isotope pattern

**Step 3:**
Structure suggestion

“Core-structure”: C₁₂H₆O₂I₄

TetraAcAmide

V. Neu
Time course of T4-degradation

The thermal degradation at 100°C leads both to a massive decomposition of T4 and to an accumulation of major impurities.

Reproducibility of 20 mg-Fractionation

Good reproducibility of fractionation
Fine structure of the T3 spectrum

\[ \begin{align*}
3J_{1,2a} &= 4.8 \text{ Hz} \\
3J_{1,2b} &= 8.4 \text{ Hz} \\
3J_{2a,2b} &= 4.8 \text{ Hz} \\
3J_{2a,2b} &= 14.4 \text{ Hz} \\
3J_{2b,1} &= 8.4 \text{ Hz} \\
3J_{2b,2a} &= 14.4 \text{ Hz}
\end{align*} \]

\[ \begin{align*}
\text{T3}
\end{align*} \]

\[ \begin{align*}
\text{HO} & & \text{I} & & \text{NH} \\
\text{COOH} & & \text{I} & & \text{I}
\end{align*} \]

\[ \begin{align*}
1H-NMR \text{ spectrum of Fraction No. 40}
\end{align*} \]

\[ \begin{align*}
\text{HO} & & \text{I} & & \text{NH} \\
\text{COOH} & & \text{I} & & \text{I}
\end{align*} \]

\[ \begin{align*}
\text{TetraAcAmide}
\end{align*} \]

NMR spectrometer: 600 MHz Bruker
Solvent: MeOD
MS/MS identification of isomers

EIC: m/z 649.78

The different isomers can be distinguished in their fragment spectra.

1H-NMR spectrum of Fraction No. 12/13
Moving to higher molecular mass: intact protein analysis

Biopharmaceuticals

- Biopharmaceuticals are drugs that mimic compounds found within the body and which are produced using biotechnology.
- Examples include: human growth hormone (Somatropin), recombinant antibodies (Rituximab, MabThera), recombinant allergen (Bet v 1)
- It is estimated that by 2014 the top six best selling drugs will be biotech products.
- Characterization of biopharmaceutical compounds is essential at each stage of the biopharma pipeline.
- Essential parameters: correct folding, correct sequence, post-translational modifications.
- The latter two parameters can be readily addressed by HPLC-MS analysis.
Instrumental setups for micro- and nano-HPLC

Micro-HPLC system:
• 60 µL/min
• 50 x 1 mm i.d. monolithic column
• Acetonitrile gradients in 0.05% TFA
• Conventional ESI source with metal needle
• Very robust and easy to handle

Nano-HPLC system:
• 600 nL/min
• 150 x 0.1 mm i.d. monolithic column
• Acetonitrile gradients in 0.05% TFA
• Nano-ESI source with 90 µm o.d. 20 µm i.d. fused silica capillary
• Highest separation efficiency, lower mass detection limit

Instrument validation system

PS-DVB monoliths; 200 x 0.1 mm i.d. and 10 x 0.2 mm i.d. trap column, 3 min trapping with 0.1 % heptafluorobutyric acid (10 µl min⁻¹); gradient: 0-50 % acetonitrile in 0.050 % in 30 min; 1.5 µL min⁻¹; FT-MS detection, R= 100000; high mass range (600-3000 m/z).

Mohr et al., Proteomics 10 (2010) 3598
Robustness of protein separation

1 Ribonuclease A (790 fmol)
2 Cytochrome C (177)
3 Lysozyme (239)
4 Transferrin (130.3)
5 Myoglobin (162)
6 β-Lactoglobulin B (151)
7 β-Lactoglobulin A (104)
8 Carbonic anhydrase (166).

Average relative standard deviation of retention times in 40 runs:
0.4% (=1.4 s)

Monolithic PS-DVB column, 60 x 0.2 mm i.d., 20–50% acetonitrile in 0.05% aqueous trifluoroacetic acid in 15 min, 2 µL/min, full scan ESI-TOF-MS 50-3000 m/z.

M. Hügel

Protein recovery from polymer vs. silica

Recovery from 50 x 1 mm PS-DVB monolith, acetonitrile gradient in 0.05% TFA, 60 °C.

Recovery from 350 x 0.05 mm column packed with 1.5 µm C18-modified ethyl-bridged silica particles, acetonitrile gradient in 0.2 % formic acid, RT.

Mohr et al., Proteomics 10 (2010) 3598
Eschebach et al., Anal Chem. 78 (2006) 1697
Study of protein carryover in monoliths

Mohr et al., Proteomics 10 (2010) 3598

4 Orbitrap mass spectrometry of intact proteins
Measures to improve protein detection in the Orbitrap

- Proteins need to be carefully desalted
- For very large proteins, choose resolution 7500 or 15000 to increase scan rate
- Target value MS full scans of min. $10^6$
- Turn off HCD collision gas to lower pressure in the Orbitrap in order to minimize ion scattering on residual gas
- Set transient delay time to 0 s
- Average at least 5 microscans
- Use increased column temperature to aid defolding and removal of adducts
HPLC-FT-MS LOD determination

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amount injected [ng]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribonuclease A</td>
<td>1.16</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>0.97</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>1.17</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>1.06</td>
</tr>
<tr>
<td>β-Lactoglobulin A</td>
<td>1.01</td>
</tr>
</tbody>
</table>

Limits of detection for different setups

<table>
<thead>
<tr>
<th>Protein</th>
<th>LOD, 50 x 1.0 mm id column, LTQ-MS [fmol]</th>
<th>LOD, 50 x 0.2 mm id column, Orbitrap-MS [fmol]</th>
<th>LOD, 150 x 0.10 mm id column, Orbitrap-MS [fmol]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribonuclease A</td>
<td>244</td>
<td>24</td>
<td>3.8</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>133</td>
<td>206</td>
<td>8.1</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>50</td>
<td>7</td>
<td>2.8</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>45</td>
<td>38</td>
<td>3.2</td>
</tr>
<tr>
<td>β-Lactoglobulin A</td>
<td>49</td>
<td>26</td>
<td>2.1</td>
</tr>
</tbody>
</table>

50 x 1.0 mm id or 150 x 0.10 mm id monolithic columns; 20-50 % acetonitrile in 0.050 % aqueous trifluoroacetic acid in 40 min, 60 or 1.0 µL min⁻¹, 60 °C; Orbitrap-MS, R=100,000 or LTQ-MS; mass range, m/z 700-3,000; sample volume, 1.0 µL

Mohr et al., Proteomics (2010)
Quality of protein mass spectra

1: Ubiquitin
- 9+, *m/z* = 952.51734
- Intact mass/Mass deviation: 8,559.58 Da
- -4.67 ppm

2: Cytochrome C
- 16+, *m/z* = 773.46281
- 1,000 1,400 1,800 m/z
- 600
- 773.6 773.9
- 771.2 771.5 771.8
- -4.47 ppm

3: Myoglobin
- 22+, *m/z* = 771.55012
- 1,000 1,400 1,800 m/z
- 600
- 771.8 771.1 771.2
- -1.77 ppm

4: Carbonic anhydrase
- 37+, *m/z* = 785.37664
- 1,000 1,400 1,800 m/z
- 600
- 785.7 785.4 785.1
- -0.69 ppm

200 ng each protein
Mohr et al., Proteomics (2010)

Resolving power vs. cycle time

RP 7500
- 0.2 s

RP 30000
- 0.5 s

RP 60000
- 0.9 s

RP 100000
- 1.6 s

Courtesy of ThermoFisher Scientific
Band dispersion vs. resolution

20-50% acetonitrile in 0.050% aqueous trifluoroacetic acid in 60 min; UV-detection at 10 Hz; Orbitrap-MS detection at 2.5 Hz or 0.6 Hz

Mohr et al., Proteomics (2010)

Quality of protein mass spectra

Acetonitrile gradients in 0.050% TFA; 60 µL min\(^{-1}\); 60 °C. A: 100 ng Carbonic anhydrase, 20-50% acetonitrile in 45 min, R= 100000; B: 100 ng human serum albumin, 20-50% acetonitrile in 30 min, R= 15000; C: 100 ng human Transferrin, 20-50% acetonitrile in 30 min, R= 15000.

J. Mohr
HPLC-MS of monoclonal antibody Bet v 1 5.1

- Bet v 1: major allergen from birch pollen
- Monoclonal antibodies are utilized to characterize the binding affinity of the allergenic protein in an affinity capillary electrophoresis experiment

Detection of mAB Bet v 1 5.1 (strongly binding); 50 x 1 mm I.D. ProSwift PS-DVB monolith gradient 20-90% acetonitrile in 0.050% TFA in 20 min; 60 µL min⁻¹; 60 °C, R=7500.

J. Mohr
Bet v 1 a pollen allergen

- Bet v 1: major allergen from birch (*Betula verrucosa*) pollen.
- 159 amino acids, M, 17.5 kDa, usually present as dimer.
- Clinically most relevant allergen in industrialized countries.
- Homologues found in apple (Mal d 1), hazelnut (Cor a 1), and celery (Api g 1), causing allergic cross-reactions.
- Unknown biological function.

HPLC-Orbitrap-MS/MS analysis of Bet v 1a

Monolithic PSDVB, 150 x 0.20 mm I.D; gradient, 20-50% acetonitrile in 0.050% TFA in 10 min, 1 µl/min; 55 °C, sample 1 µl, Bet v 1-A; 8.4 ng/µl.
Conclusions

✓ Small particles enable ultra-fast separations at elevated flow rate (UHPLC) and temperature.

✓ High-resolution mass spectrometry (HRMS) is a powerful tool with high information content for structure elucidation.

✓ The coupling of both techniques opens up new perspectives in pharmaceutical quality control (productivity, cost & resource control).

✓ Mass range covers small drug compounds to large biomolecules.
Acknowledgements

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