The importance of sample preparation for protein quantification by LC-MS

Nico van de Merbel
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Sample preparation strategies

protein

peptide

Internal standard
Sample preparation strategies

Questions:
• What does digestion do to method sensitivity, accuracy and precision?
• What is best approach for internal standardization?
• How can biological samples be pretreated?
Digestion

Necessary for quantification by MS/MS, but:
• Increases complexity of sample
• May decrease method sensitivity, accuracy and precision

• To be studied systematically
• Test compound: salmon calcitonin which can be quantified with and without (tryptic) digestion
Several approaches possible:

**Intact protein** – added at start sample preparation:
- SIL form of protein analyte
- analogue protein

**Peptide** – added just before or after digestion
- SIL form of signature peptide
- idem, with cleavable group
- analogue peptide

**Differential derivatization**
Digestion/internal standards

CSNLSTCVLG KLSQELHKLQ TYPRTNTGSG TP

Test compound: salmon calcitonin

32 amino acids
MW: 3432
Digested into 4 peptides by trypsin

Signature peptide: [1-11]
11 amino acids
Digestion/internal standards

CSNLSTCVLG KLSQELHLKLQ TYPRTNTGSG TP

internal standards for intact salmon calcitonin:

CSNLSTCVLG K*LSQELHLKLQ TYPRTNTGSG TP (SIL form)
K*: $^{13}$C$_6$$^{15}$N$_2$-K

CSNLSTCVLG KLSQELHKLQ TYPRTDVGAG TP (close analogue)
eel calcitonin

CGNLSTCMLG TYTQDFNKFH TFPQTAIGVG AP (analogue)human calcitonin
Digestion/internal standards

CSNLSTCVLG **K**LSQELH**K**LQ TYP**R**TNTGS**G** TP

“protein” internal standards for digested salmon calcitonin:

CSNLSTCVLG **K**∗LSQELH**K**LQ TYP**R**TNTGS**G** TP (SIL form)

**K**∗:^{13}C_{6}^{15}N_{2} - K

CGNLSTCMLG **T**YTQDFNKFH **T**FPQTAIGVG **A**P (analogue)

human calcitonin
Digestion/internal standards

CSNLSTCVLG KLSQELHKLQ TYPRTNTGSG TP

“peptide” internal standards for digested salmon calcitonin

CSNLSTCVLG $K^*$ (SIL form)

CSNLSTCVLG $K^*X$ (extended SIL form)

$K^*:^{13}C_6^{15}N_2-K$

CSNLSTCVLG $K^{**}$ (home-made SIL form)

$K^{**}:^{18}O_2-K$

CGNLSTCMLG TYTQDFNKFH TFPQTAIGVG AP (analogue)
Digestion/internal standards

**CSNLSTCVLG KLSQELHKLQ TYPRTNTGSG TP**

**Differential derivatization:**

**CSNLSTCVLG K + O=CH₂ \rightarrow (CH₃)₂-CSNLSTCVLG K-(CH₃)₂**
for analyte (in plasma)

**CSNLSTCVLG K + O=CD₂ \rightarrow (CHD₂)₂-CSNLSTCVLG K-(CHD₂)₂**
for internal standard (in separate standard solution)

Mix both samples just before injection
Digestion/internal standards

LC-MS system

LC: Acquity I-class (Waters)
   Column: CSH C18 (100x2.1 mm, 1.7 µm particles) at 45 °C
   Mobile phase: different gradients of 0.1% aqueous formic acid and acetonitrile at 0.5 mL/min (run time: 9-12 min)

MS: Xevo TQS (Waters)
   Positive electrospray ionization
   Intact compounds: Q1: [M+4H]^{4+} and Q3: b31 fragment [M+3H]^{3+}
   Signature peptides: Q1 [M+2H]^{2+} and Q3: y2 and other fragments [M+H]^{+}
Impact on sensitivity

Human plasma digested:

- 0.5 mL plasma precipitated with methanol
- pellet digested in 0.5 mL 10 mg/mL trypsin / 50 mM bicarbonate buffer (pH 8)
- 37°C, 2 hours

Plasma digest diluted with water in different proportions
Spiked with salmon calcitonin digest at 2 ng/mL
Impact on sensitivity

salmon calcitonin [1-11], m/z 561.9 → 204.0

- 0% plasma digest, LLOQ~0.2 ng/mL
- 1% plasma digest, LLOQ~1 ng/mL
- 5% plasma digest, LLOQ~2 ng/mL
Impact on sensitivity

10% plasma digest
LLOQ~5 ng/mL

20% plasma digest
LLOQ~10 ng/mL

50% plasma digest
LLOQ~20 ng/mL
Internal standards

Extraction

SPE:

0.5 mL plasma

“protein” internal standards added before SPE

ion-pair SPE (heptafluorobutyric acid) on Oasis HLB

Analyte recovery: 75-80%
Protein removal: 99.7%
Internal standards

Extraction

Digestion:
- SPE eluate evaporated and reconstituted (0.15 mL)
- “cleavable peptide” internal standard added before digestion
- Tryptic digestion at 37°C, 2 hours
- Other “peptide” internal standards added after digestion
Internal standards

Intact salmon calcitonin [1-32] after SPE
LLOQ: 10 pg/mL (3 pM)

Digested salmon calcitonin [1-11] after SPE
LLOQ: 50 pg/mL (15 pM)

Digested salmon calcitonin [1-11] after SPE and derivatization
LLOQ: 100 pg/mL (30 pM)
Internal standards

No digestion
LLOQ=10 pg/mL

Digestion
LLOQ=50 pg/mL

Dig. + derivatization
LLOQ=100 pg/mL
Conclusions

- **digestion** increases sample complexity considerably and can lead to decreased sensitivity
- even after rigorous sample clean-up
- several **internal standard** approaches give acceptable precision and accuracy results
- stable isotope labeled forms are optimal
- $^{18}$O-labeled forms can be easily prepared
- if digestion is properly controlled, protein internal standards do not seem to be necessary
Protein extraction

Universal extraction procedures for proteins would be beneficial to reduce sample complexity

- Most applications use no protein extraction or immunocapture
- Disadvantages: relatively low sensitivity and not universally applicable, respectively
- Test compounds: two biopharmaceuticals
Case 1: Nanobody
Antibody-derived therapeutic protein
Variable domain of camel/llama antibody
Supplied by Ablynx
Test compound: test Nanobody
MW: 27,874
pl: 9.4

15 potential signature peptides, 3 usable
Protein extraction

Analogue internal standard: cytochrome C (equine)
MW: 12,384
pI: 10.2

MGDVEKGKKI  FVQKCAQCHT  VEKGKHKTG  PNLHGLFGRK  TQAPGFTYT
DANKNKGITW  KEETLMZYLE  NPKKYPGTK  MIFAGIKKKT  EREDLIAYLK  KATNE

22 potential signature peptides, 3 usable
Protein extraction

Analyte: T7 selected (XXXXXXXXXXXXXXXXX)
IS: T10 selected (TGQAPGFTYTDANK)
Protein extraction

SPE
• cation-exchanger (Sep-Pak Accell plus CM, Waters)
• pore size: 300 Å

Analyte: net positive charge below pH ~9
HSA: net negative charge above pH ~5
Protein extraction

- Load at pH 6 (50 µL plasma + 1 mL 20 mM phosphate)
- Wash with 0.5 mL 100 mM NaCl
- Elute with 0.5 mL 500 mM NaCl
Protein extraction

- Recovery analyte: ~80%, IS: <10%, add after SPE step!
- Removal plasma proteins: ~80%
- S/N ratio improves ~3-fold
Protein extraction

- LLOQ: 5 ng/mL (180 pM)
Protein extraction

Case 2: TRAIL
Tumor necrosis factor Related Apoptosis-Inducing Ligand
Trimeric metalloprotein in development for cancer treatment
Protein extraction

Test compound: rhTRAIL
MW: 19,493 per monomer
pI: 8.9

VRE RGPQ RVAAHITGTR GRSNTLSSPN SKNE KALGRK INSWESSRSG HSFLSNLHLR
NGELVIHEKG FYYIYSQTYF RFQEEIKENT KNDK QMVQYI YKYSYPDPI LLVM KSARNSC
WSKDAEYGly SIYQGGIFEL KENDRIFVSV TNEHLIDMDH EASFGAFLVG

23 potential signature peptides, 8 usable

Internal standard
completely $^{15}$N-labeled
Protein extraction

Analyte and IS: T12 selected (YTSYPDPILLM(ox)K)

m/z 728.9 [M+2H]^{2+} \rightarrow 942.2 (y_8^+)

m/z 735.4 [M+2H]^{2+} \rightarrow 951.4 (y_8^+)
Protein extraction

SPE

• IMAC (Immobilized Metal Affinity Chromatography)
• Cross-linked spherical agarose, immobilized Ni\textsuperscript{2+} (GE Healthcare)
• Selectively traps proteins with histidine moieties on surface
• Elute with imidazole
Protein extraction

- Load with 15 mM imidazole (100 µL plasma + 0.9 mL buffer), 10 min
- Wash with 1 mL 15 mM imidazole
- Elute with 0.25 mL 140 mM imidazole

- Tryptic digestion of methanol precipitated eluate pellet (3 h at 37°C)
- Oxidize digest with 5% hydrogen peroxide: M -> M(ox) in signature peptide
Protein extraction

- Recovery analyte and IS: ~70%
- Removal plasma proteins: ~95%
Protein extraction

- LLOQ: 20 ng/mL (230 pM) in human serum
Protein extraction

Conclusions

- **Protein extraction** from biological samples by generic methods would be an interesting addition to immunocapture techniques
- Ion-exchange SPE has promise for proteins with high pI
- IMAC has promise for proteins with multiple histidines
- Performance may be difficult to predict
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