A Novel Trap-and-Elute LC-MS/MS Method to Quantify Acyl Ghrelin and Des-Acyl Ghrelin in Human Plasma

S. Menta, A. Roemmelt, M. Sury and P. Struwe
Celerion Switzerland AG, 8320 Fehraltorf, Switzerland

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Introduction

The "hunger hormone" Ghrelin is a biomarker that attracts increasing attention as a potential anti-obesity therapeutic target, since it stimulates food intake and is involved in long-term body weight regulation. Ghrelin can be detected in its active acylated (octanoyl modification on Ser3) and its inactive des-acylated form.

Nowadays, ELISA or RIA assays are commonly used for quantification of Ghrelin, but the development of specific antibodies that can distinguish active and inactive Ghrelin is challenging. Here we present an LC-MS/MS method that allows for selective quantification of acyl Ghrelin and des-acyl Ghrelin in human plasma in the range of 50.0 - 2500 pg/mL.

Sample Collection, Standard (STD) / Quality Control (QC) Sample Preparation and Extraction Procedure

Acyl Ghrelin can rapidly get enzymatically degraded in plasma. Therefore, blood collection was done in the presence of protease inhibitors (P800 tubes from BD) and plasma samples were acidified with 1% formic acid.

Unknown samples, QC Low, Mid, High

- Blood collection in EDTA/P800 tubes
- Centrifugation
- Plasma acidification
- Storage at -80 °C

STD, Blank, STD0, QC LLOQ, QC S

Surrogate matrix: water, 0.1% human serum albumin, 0.1% formic acid

Extraction procedure

- 600 µL of sample
- 60 µL of internal standard (IS)
- Precipitation with MeOH
- Centrifugation and transfer of supernatant
- Evaporation until dryness
- Reconstitution in water, 0.1% formic acid
- Centrifugation and transfer of supernatant
- Storage at 5 °C until injection

Figure 1: Workflow of clinical sample collection, standard (STD), quality control (QC) sample preparation and extraction procedure. STD0 samples contain IS only, QC LLOQ samples are spiked with both analytes at 50.0 pg/mL and QC S samples are spiked with both analytes at 150 pg/mL in surrogate matrix.

LC-MS/MS Conditions

Samples were loaded on a C4 trapping column and were eluted using in-line dilution on a C18 analytical column for chromatographic separation. As a mass detector, a TQ 6500 instrument operating in the MRM mode with positive electrospray ionization was used.

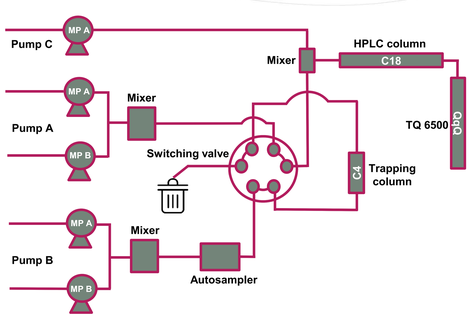

Figure 2: Schematic representation of the chromatographic trap-and-elute system (loading position).

Chromatographic Conditions	
UHPLC	AB Sciex Exion LC system
Analytical column **Trapping column**	Waters XSelect CSH C18, 100 × 2.1mm, 2.5 µm Thermo Hypersil GOLD C4, 10 × 2.1mm, 5 µm
Mobile phase A (MP A) **Mobile phase B (MP B)**	Water/Acetic acid 98:2 (v/v) Acetonitrile/Acetic acid 98:2 (v/v)
Flow rate **Column temperature** **Injection volume**	0.4 (analytical column) 1.0 (trapping column) mL/min 50 °C 100 µL
Total run time	6 min
MS/MS Parameters	
Mass spectrometer	SCIEX TQ 6500
Source/Polarity	ESI / Positive
Followed MRM transitions (Charge state)	Human acyl Ghrelin: m/z 482.8 (+7) → m/z 535.8 Human des-acyl Ghrelin: m/z 464.5 (+7) → m/z 517.7 Rat acyl Ghrelin (IS): m/z 474.7 (+7) → m/z 523.2 Rat des-acyl Ghrelin (IS): m/z 399.7 (+8) → m/z 450.5

Table 1: Chromatographic conditions and MS/MS parameters.

Results

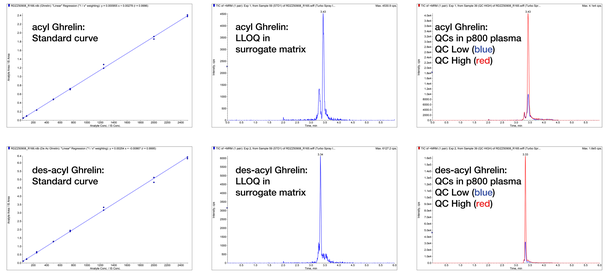

Figure 3: Calibration curves from 50.0 to 2500 pg/mL, linear regression, 1/x weighting factor (left panels), typical chromatograms of the LLOQ in surrogate matrix (central panels), typical chromatograms of QC Low (blue) and QC High (red) in P800 plasma (right panels). Acyl Ghrelin (top), des-acyl Ghrelin (bottom).

acyl Ghrelin			
Sample Concentration	**19-0245 [pg/mL]**	**19-0246 [pg/mL]**	**19-0247 [pg/mL]**
Mean	125	74.3	111
SD	7.40	13.9	13.8
CV [%]	5.9	18.7	12.4
n	9	9	9

des-acyl Ghrelin			
Sample Concentration	**19-0245 [pg/mL]**	**19-0246 [pg/mL]**	**19-0247 [pg/mL]**
Mean	104	76.5	105
SD	6.58	6.50	5.21
CV [%]	6.3	8.5	5.0
n	9	9	9

Table 2: Quantification of acyl Ghrelin and des-acyl Ghrelin in human plasma samples from an in-house blood donation. Samples were measured in triplicate in three independent runs.

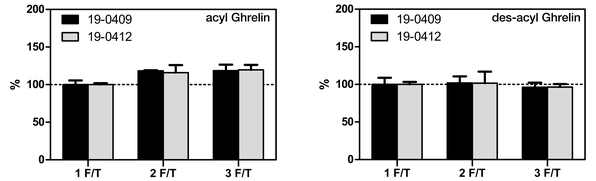

Figure 4: Freeze/thaw (F/T) stability assessment of acyl Ghrelin (left panel) and des-acyl Ghrelin (right panel) in two human plasma samples (black and grey bars) from an in-house blood donation. Samples were stored at -80 °C and were thawed 1-3 times on ice for 3 hours between each cycle. Data are expressed relative to 1 F/T cycle in percent (Mean, SD).

Intra-batch and Inter-batch Precision and Accuracy Results for acyl Ghrelin						
		QC LLOQ 50.0 pg/mL	**QC S 150 pg/mL**	**QC Low 500 pg/mL**	**QC Mid 1000 pg/mL**	**QC High 1800 pg/mL**
Run 1	**Accuracy (%)**	99.8	99.3	90.4	83.0	78.3
	CV (%)	7.8	3.5	3.6	4.2	2.8
	N	6	6	6	6	6
Run 2	**Accuracy (%)**	96.2	95.3	87.0	80.1	76.7
	CV (%)	3.2	4.1	5.6	3.0	3.0
	N	6	6	6	6	6
Run 3	**Accuracy (%)**	102.2	94.0	79.8	72.2	69.4
	CV (%)	6.8	1.9	5.1	4.4	3.0
	N	6	6	6	6	6
Inter-batch Precision and Accuracy	**Accuracy (%)**	**99.4**	**96.0**	**85.8**	**78.4**	**75.0**
	CV (%)	**6.5**	**4.0**	**7.0**	**7.0**	**6.2**
	N	**18**	**18**	**18**	**18**	**18**

Table 3: Intra- and inter-run precision and accuracy of three independent runs for acyl Ghrelin.

Intra-batch and Inter-batch Precision and Accuracy Results for des-acyl Ghrelin						
		QC LLOQ 50.0 pg/mL	**QC S 150 pg/mL**	**QC Low 500 pg/mL**	**QC Mid 1000 pg/mL**	**QC High 1800 pg/mL**
Run 1	**Accuracy (%)**	109.6	104.0	81.8	88.6	87.2
	CV (%)	8.1	7.1	3.3	4.5	4.0
	N	6	6	6	6	6
Run 2	**Accuracy (%)**	103.4	100.0	78.8	83.5	85.6
	CV (%)	4.7	4.6	5.1	3.3	3.0
	N	6	6	6	6	6
Run 3	**Accuracy (%)**	108.2	104.0	77.6	90.3	89.4
	CV (%)	12.1	3.3	3.4	4.4	5.6
	N	6	6	6	6	6
Inter-batch Precision and Accuracy	**Accuracy (%)**	**107.0**	**102.7**	**79.4**	**87.5**	**87.2**
	CV (%)	**8.8**	**5.3**	**4.4**	**5.2**	**4.5**
	N	**18**	**18**	**18**	**18**	**18**

Table 4: Intra- and inter-run precision and accuracy of three independent runs for des-acyl Ghrelin.

Conclusions

To our knowledge this is the first LC-MS/MS method which permits to selectively quantify acyl and des-acyl Ghrelin in human plasma in the range of 50.0 - 2500 pg/mL for both analytes. Using this method we were able to quantify endogenous active and inactive Ghrelin levels in human plasma samples with a high precision. Furthermore, stability of acyl and des-acyl Ghrelin in acidified human plasma containing protease inhibitors could be shown. This allows for getting more insights into the complex role of Ghrelin in the regulation of hunger and metabolism.

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