Simultaneous Quantification of Ghrelin and Desacyl-Ghrelin by Liquid Chromatography–Tandem Mass Spectrometry in Plasma, Serum, and Cell Supernatants

Manfred Rauh,* Michael Gröschl, and Wolfgang Rascher

Background: A sensitive method specific for ghrelin is needed for investigations of this gastrointestinal peptide. Our aim was to develop and validate a quantitative mass spectrometry (MS) method to measure ghrelin and desacyl-ghrelin simultaneously.

Methods: After deproteinization by precipitation, we performed reversed-phase separation with a rapid 2-column online extraction design coupled to a quadrupole mass spectrometer for electrospray ionization MS detection. Chromatography was performed on a C18 monolithic column, with ammonium acetate buffer/methanol as the mobile phase and a chromatographic run time of 6 min/sample. The 4-fold–charged ions were used for multiple reaction monitoring experiments.

Results: The method was linear with injections of 0.01–10 ng. Limits of detection and quantification were 0.02 and 0.07 µg/L for ghrelin, respectively, and 0.03 and 0.35 µg/L for desacyl-ghrelin. Intra- and interday imprecision (CVs) were 9%–4% and 12%–6% at concentrations of 0.33–5.93 µg/L for ghrelin, respectively, and 16%–6% and 15%–8% at concentrations of 1.12–10.02 µg/L for desacyl-ghrelin. The mean (SD) recoveries in plasma of added ghrelin and desacyl-ghrelin were 95.8% (12%) and 101% (1.2%), respectively. Using kinetic modeling, we determined the mean (SD) periods of half-change (t1/2) of ghrelin to be 156 (16) min in EDTA plasma and 49 (1) min in Li-heparin plasma. Bland–Altman analysis showed that the median differences between EIA and liquid chromatography–tandem mass spectrometry (MS/MS) for desacyl-ghrelin were −40% for plasma/serum samples and 85% for cell supernatants and for ghrelin were 6% for enriched plasma samples and 44% for cell supernatants.

Conclusion: Our HPLC-MS/MS procedure has excellent selectivity and sufficient limit of quantification to allow the monitoring of concentration–time profiles in biological matrices.

Peptides in biological matrices have traditionally been quantified by immunological methods such as RIA or ELISA, which have the drawback of being insufficiently selective, often not allowing differentiation between the peptide and its derivatives or degradation fragments. Peptides of similar antigenicity are often formed by posttranslational modifications essential for physiological function or consecutive peptide chain truncation by exopeptidases.

Ghrelin is the 1st bioactive peptide reported to have an essential n-octanoyl modification on the hydroxy group of Ser3. Ghrelin functions as a peripheral fasting signal and stimulates food intake (1–3). Ghrelin acylation is essential for growth hormone secretagogue receptor activation and receptor binding (4, 5). Desacyl-ghrelin, which lacks the acyl modification, does not bind to this growth hormone secretagogue receptor (6). In blood, desacyl-ghrelin is reported to circulate in much larger amounts than acylated ghrelin (7). Accumulated evidence indicates that desacyl-ghrelin, which was originally believed to be an inactive form, can counteract some of the metabolic responses of acylated ghrelin (8). Desacyl-ghrelin has its own functions, including the modulation of cell proliferation and, to a small extent, adipogenesis (9–11). Moreover, several studies suggest that an alternative receptor exists that is not dependent on octanoylation for activation (12, 13).
Very little is known about circulating ghrelin and desacyl-ghrelin catabolism (14). Compared with assays that do not differentiate between specific forms of ghrelin, assays that measure specific forms may be more useful in elucidating the physiological role of ghrelin (15) and may also be used to investigate the interconversion of ghrelin and desacyl-ghrelin in tissues and plasma.

Various immunoassays detect different ghrelin-like immunoreactivity (16), recognizing either total (acylated plus desacyl-ghrelin) (17) or acylated ghrelin (18). Other investigators have used HPLC to isolate ghrelin from desacyl-ghrelin before quantification with RIA, but the investigators have used HPLC to isolate ghrelin from desacyl-ghrelin before quantification with RIA, but the need for additional purification steps makes these methods extremely labor-intensive (18). Specific ELISAs analyzing acylated ghrelin and desacyl-ghrelin are now available commercially from Linco (19, 20) and SPI-BIO.

The improved robustness and sensitivity of LC-MS–based techniques have led to reliable alternatives for peptide quantification (21–23). We evaluated a method for the simultaneous quantification of ghrelin and desacyl-ghrelin that allows the monitoring of concentration–time profiles in biological matrices.

**Materials and Methods**

**MATERIALS AND CHEMICALS**

Human and rat ghrelin and human desacyl-ghrelin were purchased from Bachem, and rat desacyl-ghrelin was purchased from International Peptides. Ammonium acetate, formic acid, acetonitrile, and sulfosalicylic acid were supplied by Merck. All other chemicals were of the highest purity available from Sigma-Aldrich. Human EDTA (1.2–2 g/L blood) and lithium heparin (10–30 kIU/L blood) plasma were generated in-house from blood of healthy volunteers (n = 2) who gave informed consent.

**STANDARDS**

Stock solutions of ghrelin and desacyl-ghrelin were prepared by dissolving accurately weighed standard compounds in 30 mL/L acetic acid, yielding a concentration of 1.0 g/L for each compound. Different standard solutions of ghrelin and desacyl-ghrelin for a range of 0.007–70.4 and 0.007–72.6 \( \mu \)g/L were prepared with concentrations calculated based on the peptide content declared by the manufacturer. Rat ghrelin and desacyl-ghrelin were used as the internal standard solution at a final concentration of 1 mg/L. All ghrelin solutions used were dissolved in 30 mL/L acetic acid. The stock solutions, standards, and blanks were stored at −20 °C.

**SAMPLE PREPARATION**

Experiments were performed to evaluate the impact of the different precipitation solutions (mixtures [1:1 by volume] of methanol, acetonitrile, 2-propanol, and sulfosalicylic acid [100 g/L], or trichloroacetic acid [200 g/L]) on recovery. To study the recovery, we added \(^{125}\text{I}\) ghrelin to plasma samples and measured radioactive activity in supernatants and pellets after precipitation.

In the final procedure, sample preparation before injection involved protein precipitation with 100 g/L sulfosalicylic acid dissolved in water and acetonitrile. We added 10 \( \mu \)L (per 100 \( \mu \)L sample volume) of internal standard solution to plasma, serum, cell supernatants, or standards (250–1000 \( \mu \)L). Then we added 50 \( \mu \)L acetonitrile and 50 \( \mu \)L sulfosalicylic acid (100 g/L) per 200 \( \mu \)L sample volume into a low-bind protein Eppendorf tube. After vigorous shaking, the mixture was centrifuged at 4 °C at 36 000 g for 10 min. The clear supernatants were transferred to microtiter plates (polypropylene, Greiner BIO-ONE) and placed in the autosampler at 15 °C.

**CHROMATOGRAPHIC CONDITIONS**

The LC system consisted of a binary pump, a quaternary pump (Agilent 1100, Agilent Technologies), and a 12-port switching valve (VICI, Valco Instruments). The sample was injected by use of a HTC PAL autosampler (CTC Analytics) fitted with a 200 or 1000 \( \mu \)L peak sample loop, through the Valco valve onto the extraction column (Oasis HLB 2.1*20 mm, 15 \( \mu \)m, Waters). The extraction column was washed for 1.0 min (flow rate 3 mL/min) with water/acetic acid/methanol (92:3:5 by volume). The valve position was then switched to allow the bound material to be eluted from the extraction cartridge in back-flush mode. After 4 min, the Valco valve position was again switched to allow the extraction column to be purged by sulfosalicylic acid (1 g/L) and methanol and reequilibrated at a flow rate of 3 mL/min with water/acetic acid/methanol (92:3:5 by volume).

LC was performed with a Chromolith column (RP-18e, 100 · 4.6 mm, Merck). Mobile phase A consisted of ammonium acetate (10 mmol/L) and methanol (97:3) containing 1 mL/L acetic acid, mobile phase B of ammonium acetate (2 mmol/L), and methanol (5:95) containing 10 mL/L formic acid (pH 2). After 1.2 min the initial conditions (30% A) were increased to 90% A in 1.8 min via a linear gradient, followed by linear return to initial conditions within 1.0 min. Total analysis time was 6.0 min.

**MS/MS CONDITIONS**

An API 4000™ (Applied Biosystems, MDS Sciex, Analyst software, version 1.4) mass spectrometer fitted with a turbo ion spray source was operated in positive ionization mode without a split. Eluates were analyzed in the tandem mass spectrometry (MS/MS) mode, fragmenting the 4-fold–charged parent ions at \( m/z \) 812.9 for human desacyl-ghrelin, 798.8 for rat desacyl-ghrelin, 844.0 for \( ^{125}\text{I}\) ghrelin to plasma samples and measured radioactive activity in supernatants and pellets after precipitation.

1 Nonstandard abbreviation: MS/MS, tandem mass spectrometry.
human ghrelin, and 830.3 for rat ghrelin under optimized settings determined by previous flow-injection analysis. MS conditions were as follows: declustering potential of 120 V for human and rat desacyl-ghrelin and rat ghrelin and 115 V for human ghrelin and desacyl-ghrelin and 41 eV for rat desacyl-ghrelin and ghrelin, temperature of the ESI source 750 °C, ion spray voltage 5500 V, collision gas high-purity nitrogen.

FIGURE 1. ESI mass spectra of human and rat desacyl-ghrelin (A) and ghrelin (B).

**Ghrelin Degradation by Heparin and EDTA**

We analyzed enriched plasma samples from 2 different healthy volunteers to quantitatively analyze the peptide degradation in plasma. Lithium heparin and EDTA plasma were enriched with an aqueous ghrelin solution, resulting in a concentration of 50 μg/L. Incubations were carried out at 37 °C under gentle shaking for various periods (0, 10, 20, 30, 40, 50, 60, 80, 100, 120, 240, and 480 min). The incubation was stopped, and samples were centrifuged at 4 °C for 10 min at 20,000 g. After collecting the supernatants and immediately after separation we added HCl to the samples to a final concentration of 0.1 mol/L to stabilize the peptide (7). Samples were shock-frozen in liquid nitrogen and stored at −80 °C.

**Method Comparison**

For method comparison, patient samples (serum and EDTA plasma left over after clinical routine measurement) and enriched plasma pool samples (nominal concentrations of ghrelin or desacyl-ghrelin, 0.035, 0.053, 0.07, 0.175, 0.35, 0.53, 0.70, 1.05, 1.40, and 3.5 μg/L) were used. We also measured medium and cell supernatants from gastric epithelial cell cultures incubated with ghrelin (initial concentration 7 μg/L). The cells were cultured in a 5% CO₂ humidified atmosphere at 37 °C in minimal essential medium/Ham’s F-12 medium (American Type Culture Collection) containing 100,000 U/L penicillin and 100 g/L streptomycin (Sigma). The immunoassays for human acylated ghrelin and desacyl-ghrelin were purchased from SPI-BIO.

**Statistical Analysis**

Data processing and graphic presentation were performed with SigmaPlot 2001 (SPSS Corp.), and Origin software (Originlab Corp.).

**Results**

**MS Characteristics**

As demonstrated in Fig. 1A for desacyl-ghrelin and Fig. 1B for ghrelin, the peptides were detected as their 3- to 4-fold–charged ions, (M+3H)⁺ and (M+4H)⁺, showing the predominant intensity at m/z for the 4-fold–charged analyte (M, molecular mass; z, the charge on the molecule caused by the uptake of protons during ionization). The most abundant 4-fold–charged ions were fragmented by collision-induced dissociation, yielding a typical series of...
y- and b-ions assignable to the fragments indicated in Fig. 2A (desacyl-ghrelin) and Fig. 2B (ghrelin). The most intensive product ion signals were chosen for the multiple reaction with \( m/z > m/z \) parent ion (24): 844.0/918.7, 844.0/1233.3, 844.0/956.9, and 844.0/1005.7 for human ghrelin; 830.3/901 and 830.3/1206.1 for rat ghrelin; 812.9/919.4, 812.9/957.4, and 812.9/1171.3 for human desacyl-ghrelin; and 798.8/901.0 and 798.8/1114.1 for rat desacyl-ghrelin. The total signal intensity of all respective product ions was used to display the chromatogram, allowing the quantification of samples and standards.

Rat ghrelin and desacyl-ghrelin were used as internal standards because of their chemical and structural similarity to human ghrelin and desacyl-ghrelin. The amino acid sequences of human and rat differ by only 2 amino acids at positions 11 and 12 (Arg\(^{11}\) and Val\(^{12}\) in human vs Lys\(^{11}\) and Ala\(^{12}\) in rat). As a result, the molecular weight (human 3370.91, rat 3314.84 for ghrelin; human 3244.71, rat 3188.74 for desacyl-ghrelin) and isoelectric point are also quite similar.

**SAMPLE PREPARATION**

The precipitation method was used because of the good solubility and cationic properties of the peptides, allowing rapid, easy, and effective plasma preparation. The results indicated that a precipitation with acetonitrile and sulfosalicylic acid achieved the optimal extraction recovery of analytes. The mean (SD) recovery of extraction for radioiodinated ghrelin added to plasma samples (\( n = 6 \)) was 83% (1.3%).

Memory effects (carryover <0.4% for 20 \( \mu \)g/L calibrator) were minimized if a washing step of the extraction column with 1 g/L sulfosalicylic acid and methanol was performed after elution of ghrelin and desacyl-ghrelin. In addition the autoinjector was washed with 1 g/L sulfosalicylic acid/methanol (1:2 by volume) between injections to avoid adsorption of ghrelin to the tubing and stainless steel valve of the automatic injector.

**ASSAY VALIDATION**

An online extraction method with a column-switching technique was used. Representative ion chromatograms are shown in Fig. 3. At a flow rate of 0.8 mL/min, the total run time was 6 min. Under the chromatographic conditions described, the mean (SD) retention times for ghrelin and desacyl-ghrelin were 3.11 (0.01) min and 5.05 (0.01) min (repeatability <0.3% CV).

Linearity was evaluated across an injected amount of 0.01–10 ng. The method proved linear from 0.07–72 \( \mu \)g/L \( (r = 0.999, \text{ injection volume } 250 \mu \text{L}) \) for ghrelin and for desacyl-ghrelin. Calibration curves were calculated using linear least squares regression according to the equation \( y = a + bx \), where \( y \) is the peak-area ratio of substance to internal standard and \( x \) is the analyte concentration of the calibrator sample. We used 1/\( x \) weighting to ensure maximum accuracy at lower concentrations. The mean correlation coefficients ranged from 0.998 for desacyl-ghrelin to 0.999 for ghrelin \(( n = 10)\), the mean (SD) \( y \)-intercept was 0.017 (0.003) for ghrelin and 0.026 (0.008) for desacyl-ghrelin, and the mean (SD) slope was 0.146.
(0.008) for ghrelin and 0.056 (0.005) for desacyl-ghrelin. The assay acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value (except at the lower limit of quantification, 20%).

For analysis of the most intensive multiple reaction monitoring trace of calibrator samples, the limit of detection (signal-to–baseline noise ratio: \( \frac{100}{3} \)) was 0.02 \( \mu \text{g/L} \) for ghrelin and 0.03 \( \mu \text{g/L} \) for desacyl-ghrelin (injection volume 800 \( \mu \text{L} \), concentration range 0.07–0.7 \( \mu \text{g/L} \)).

The intraday imprecision and agreement with nominal concentrations were evaluated by analyzing 10 sets of standard samples (injection volume 1000 \( \mu \text{L} \)) within the same run. Intraday results are shown in Table 1. The lower limit of quantification was 0.07 \( \mu \text{g/L} \) for ghrelin and 0.35 \( \mu \text{g/L} \) for desacyl-ghrelin, defined as the lowest concentration yielding a deviation between the value of the concentration calculated by the calibration curve and the nominal concentration within 20%, and precision with a CV% <20%. Native plasma immediately processed yielded a mean concentration (n = 10) of 0.09 \( \mu \text{g/L} \) for ghrelin and 0.47 \( \mu \text{g/L} \) for desacyl-ghrelin, with an intraday imprecision of 17.4% and 18.3%. Plasma pool samples with a natural content of ghrelin <0.07 \( \mu \text{g/L} \) and 0.60 \( \mu \text{g/L} \) desacyl-ghrelin were enriched to nominal concentration of 0.35, 0.7, and 3.5 \( \mu \text{g/L} \) and showed an intraday imprecision (mean concentration; n = 10) of 8.6% (0.33 \( \mu \text{g/L} \)), 5.1% (0.71 \( \mu \text{g/L} \)), and 4.4% (3.64 \( \mu \text{g/L} \)) for ghrelin and 16.3% (1.12 \( \mu \text{g/L} \)), 10.9% (1.65 \( \mu \text{g/L} \)), and 5.6% (4.99 \( \mu \text{g/L} \)) for desacyl-ghrelin.

We assessed interday imprecision by analysis of 10 batches of 4 sets of enriched plasma pool samples (nominal concentration, 0.35, 0.7, 3.5, and 7 \( \mu \text{g/L} \)) on different days. Imprecision values for the whole procedure, including sample preparation and HPLC analysis, were 12.2% (0.35 \( \mu \text{g/L} \)), 12.2% (0.59 \( \mu \text{g/L} \)), 5.1% (0.71 \( \mu \text{g/L} \)), and 7.8% (2.90 \( \mu \text{g/L} \)) for ghrelin and 14.6% (1.97 \( \mu \text{g/L} \)), 14.6% (5.52 \( \mu \text{g/L} \)), and 8.4% (10.02 \( \mu \text{g/L} \)) for desacyl-ghrelin.

![Table 1. Intraday imprecision and agreement with nominal concentrations of ghrelin and desacyl-ghrelin in calibrator samples.](image-url)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Nominal concentration, ( \mu \text{g/L} )</th>
<th>Mean calculated concentration, ( \mu \text{g/L} )</th>
<th>Measured nominal concentration, %</th>
<th>Intraday imprecision, %</th>
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<td>0.024</td>
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<td></td>
<td>0.036</td>
<td>0.041</td>
<td>112</td>
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<td></td>
<td>0.073</td>
<td>0.077</td>
<td>106</td>
<td>19.7</td>
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<td></td>
<td>0.363</td>
<td>0.353</td>
<td>97.3</td>
<td>9.5</td>
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<td></td>
<td>0.726</td>
<td>0.725</td>
<td>99.9</td>
<td>4.9</td>
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<tr>
<td></td>
<td>3.630</td>
<td>3.637</td>
<td>100</td>
<td>3.2</td>
</tr>
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<td></td>
<td>5.64</td>
<td>5.63</td>
<td>99.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Desacyl-ghrelin</td>
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<td>0.106</td>
<td>150</td>
<td>42.2</td>
</tr>
<tr>
<td></td>
<td>0.352</td>
<td>0.373</td>
<td>106</td>
<td>19.3</td>
</tr>
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<td></td>
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<td>0.680</td>
<td>96.6</td>
<td>15.7</td>
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<td>7.20</td>
<td>7.06</td>
<td>98.1</td>
<td>1.2</td>
</tr>
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</table>

*a Injected volume, 1000 \( \mu \text{L} \).*
RECOVERY/ION SUPPRESSION

Recovery experiments were carried out by adding known amounts of ghrelin or desacyl-ghrelin to EDTA plasma samples before protein precipitation to give a nominal ghrelin and desacyl-ghrelin concentrations of 5, 10, 15, and 20 μg/L. A linear relation was observed between peak ratio and the added concentration of ghrelin ($r_{\text{mean}} = 0.998$) and desacyl-ghrelin ($r_{\text{mean}} = 0.993$), and the recoveries were calculated by linear regression. The mean (SD) recoveries (n = 4 batches) in plasma were 95.8% (12%) for ghrelin and 101% (1.2%) for desacyl-ghrelin.

Ghrelin Degradation

Typical concentration–time profiles for degradation in plasma are given in Fig. 4. The incubation of ghrelin with lithium heparin and EDTA-plasma led to the production of desacyl-ghrelin. Data points were interpolated after an exponential decay algorithm and the respective mean (SD) half-change periods ($t_{1/2}$) were calculated [EDTA, $t_{1/2} = 156 (16)$ min, Li-heparin $t_{1/2} = 49 (1)$ min]. After 480 min incubation with EDTA plasma only 11% (2.5%) of the initial ghrelin concentration was detected, whereas desacyl-ghrelin increased with time [$t_{1/2} = 121 (21)$ min] in a stoichiometric ratio. In contrast, after incubation with Li-heparin plasma the desoctanoylation of ghrelin was complete after 480 min, and the degradation of desacyl-ghrelin increased with time [Fig. 4]. The incubation of ghrelin with EDTA plasma only 11% (2.5%) of the initial ghrelin concentration was detected, whereas desacyl-ghrelin increased with time [$t_{1/2} = 121 (21)$ min] in a stoichiometric ratio. In contrast, after incubation with Li-heparin plasma the desoctanoylation of ghrelin was complete after 480 min, and the degradation of desacyl-ghrelin could be observed. The degradation profile for the formation of desacyl-ghrelin at 37 °C (Fig. 4) was fitted to a simple consecutive reaction model:

$$c_t = a_0 \cdot (k_d/(k_d - k_H)) \cdot [\exp(-k_H \cdot t) - \exp(-k_D \cdot t)] + c_0,$$

where $k_H$ is the pseudo-1st-order rate constant for the hydrolysis of ghrelin and $k_D$ is the 1st-order rate constant for the degradation of desacyl-ghrelin.

The mean (SD) half-change times calculated by nonlinear regression analysis were 45 (10) min for the hydrolysis of ghrelin and 204 (38) min for the degradation reaction. The half-change times calculated from the ghrelin profile were in good accordance with the calculated $t_{1/2}$ from desacyl-ghrelin data. In comparison, the 1st-order rate constant for the hydrolysis of ghrelin was 4 to 5 times faster than that for the degradation of desacyl-ghrelin. This reaction was not inhibited by Li-heparin but was inhibited by EDTA.

Comparison of the In-House and Commercially Supplied Ghrelin Calibrators

Calibrators were tested from different commercially available immunoassays (Phoenix Pharmaceuticals, Mediagnost, Linco Research). The RIAs from both Phoenix Pharmaceuticals and Mediaposs measure only total ghrelin. The ELISA from Linco Research specifically measures active ghrelin. The ghrelin concentration of the calibrator samples in the 3 commercial assays strongly correlated with the LC-MS/MS method ($r > 0.999$). However, the ghrelin concentrations detected depend on the supplier. The ghrelin concentrations detected for the Phoenix calibrator were 2.6-fold greater when calculated with the LC-MS/MS method. The concentrations of Mediposs were 0.67-fold lower. For Linco the ghrelin concentration was ~1.12-fold higher than those given by the supplier. Consequently, values obtained with Phoenix total ghrelin were ~4-fold higher than those obtained with Mediagnost. The results of linear regression analysis between measured ghrelin concentration and declared concentrations of the different calibrator samples were as follows (n, slope, intercept, $r$, $P$): Phoenix, 8, 2.63, -0.01, 1.000, <0.0001; Mediagnost, 6, 0.67, -0.05, 0.999, <0.0001; Linco, 2, 1.12, -0.09.

For patient and cell culture samples we compared the results of ghrelin and desacyl-ghrelin measurements obtained with LC-MS/MS with results obtained with the EIA method (Fig. 5, A and B). Method differences were assessed by Passing–Bablok regression analysis (25) independently for samples of cell culture experiments and plasma and serum samples. For desacyl-ghrelin a median difference (EIA − LC-MS/MS) of ~40% for plasma/serum ('Fig. 5).

![Fig. 4. Plasma concentration vs time profile of ghrelin (filled symbols) and desacyl-ghrelin (open symbols).](image-url)
serum and 85% for supernatants was calculated from the Bland–Altman differences plot (26).

In leftover untreated serum and plasma samples analyzed with the LC-MS/MS and the EIA methods, the concentration of ghrelin was below the lower limit of quantification. The median difference was 44% for the cell supernatants and 6% for the enriched plasma.

Discussion

As discussed previously, the current methods of measuring ghrelin and desacyl-ghrelin have involved RIA or HPLC and RIA. RIA procedures have limitations in specificity, and RIA-HPLC methods are too time-consuming. We have developed a sensitive and specific method HPLC-ESI-MS/MS for the simultaneous measurement of ghrelin and desacyl-ghrelin. The LC-MS/MS method has been successfully used to measure ghrelin and desacyl-ghrelin in different biological matrices.

The online solid-phase-extraction LC procedure provides simple and labor-saving sample cleanup and allows high sample volumes. Injection volume was studied from 100 to 1000 μL. The recorded intensities displayed a linear correlation for all ghrelin forms, with a slight saturation tendency for human ghrelin at 1000 μL. The simultaneous measurement provides an effective method for kinetic profiles. The method allows the simultaneous detection of ghrelin and desacyl-ghrelin in 1 sample aliquot, which is very advantageous because of the limited stability of the acylated peptide (7). We chose the 4-fold–charged precursor ion for maximum sensitivity. Monitoring of fragment ions at an m/z ratio greater than that of the precursor ion greatly reduces noise and improves the overall sensitivity of the assay (27).

In MS experiments, stable isotope compounds that are identical except for their masses are used as internal standards. Unfortunately, for most peptides these analogous compounds are not commercially available and must be synthesized. A promising alternative to this procedure is the use of peptides of other species, which often differ by only a few amino acids and are often commercially available. In a manner analogous to classic isotope dilution approaches, the mass difference allows the mass spectrometer to differentiate between 2 nearly identical molecules. Moreover, the method is suitable for quantification of both human samples and samples from other species, for example from rats, as shown in our study.

Comparison of ghrelin concentrations measured with different commercial assays reveals large differences, making it difficult to compare studies that use different assays. Similar results were reported by Gröschl et al. (28). Adjustment to 1 reference standard will be required to overcome this discrepancy.

The results of the method comparison for samples from cell supernatants and plasma/serum samples are clearly separated. Comparison of our values with those obtained by the immunoassay showed that the EIA generally overestimates peptide concentrations in cell supernatants, possibly caused by cross-reactivity. The difference between plasma/serum and cell culture samples indicates matrix effects and different cross-reactivity of the antibodies (29).

The limited ex vivo stability of the analytes is a drawback for precise and accurate analysis, often demanding the inactivation of enzymes to prevent degradation during sample handling, shipping, or preparation (30). Approximately 40%–60% of the total ghrelin measured by RIA is likely to be fragmented (16). The rapid degradation of ghrelin in plasma and especially in serum makes it difficult to accurately determine the bioactive serum and plasma concentrations of this hormone and

Fig. 5. Method comparison between LC-MS/MS and EIA for desacyl-ghrelin (A) and ghrelin (B; ○, plasma and serum samples; ●, cell culture supernatant samples). (A), for desacyl-ghrelin a mean slope of 0.958 (0.705–1.192) and a mean intercept of −0.249 (−0.450 to −0.032) with a standard deviation of the residuals of 0.285 was obtained in plasma and serum (n = 37, r = 0.973). A mean slope of 2.660 (2.472–2.929) and a mean intercept of −0.392 (−0.713–0.052) with a standard deviation of the residuals of 0.382 was obtained for desacyl-ghrelin in cell supernatants (n = 37, r = 0.965). (B), for the cell supernatants the regression line for the LC-MS/MS (x) and the immunoassay (y) for ghrelin was y = 1.501(1.386–1.601)x + 0.075 (−0.180–0.404) μg/L (r = 0.972, n = 37, SE = 0.374). For the enriched plasma samples (n = 19, r = 0.966) a mean slope of 0.996 (0.814–1.429) and a mean intercept of 0.036 (−0.084–0.104) with a standard deviation of the residuals of 0.165 was obtained for ghrelin.
consequently to assess its physiological and pathophysiological roles.

In preliminary experiments we used plasma as a partly predictive in vitro system to characterize the expected metabolism. The desoctanoylation reaction in Li-heparin plasma was estimated to be 3 times as fast as the degradation of desacyl-ghrelin in EDTA plasma. At variance with EDTA plasma, in heparin plasma ghrelin degradation of desacyl-ghrelin in EDTA plasma. At variance with EDTA plasma, in heparin plasma ghrelin degradation resulted from both N-terminal proteolysis and desoctanoylation. Because ghrelin desoctanoylation was not inhibited by EDTA, the present study supports the hypothesis of De Vriese et al. (14) that B-esterases, rather than A- and C-esterases, contribute to that reaction. Further plasma incubation experiments in the presence of different enzyme inhibitors would be appropriate to identify the degrading enzymes. Of particular interest is identification of the pathways regulating the production and release of ghrelin from the stomach and of the enzyme that catalyzes ghrelin’s effects.

In conclusion, we developed and validated an HPLC-MS/MS procedure to measure ghrelin and desacyl-ghrelin simultaneously. The method has excellent selectivity as well as sufficient sensitivity and limit of quantification to allow the monitoring of concentration–time profiles in biological matrices. Our study indicates the importance of highly specific methods for peptide quantification.

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