

Do different assays for human acylated ghrelin concentrations provide comparable results?

Günes KADAS¹, Markus KLOTZ², Karl-Herbert SCHÄFER², Klaus FASSBENDER¹, Daniel THOMI³, Wolfgang H. OERTEL³, Marcus M. UNGER¹

¹ Saarland University, Department of Neurology, Homburg, Germany

² University of Applied Sciences Kaiserslautern, Department of Biotechnology, Zweibrücken, Germany

³ Philipps-University Marburg, Department of Neurology, Marburg, Germany

Correspondence to: Marcus M. Unger, MD.
Department of Neurology, Saarland University
Kirrberger Strasse, 66421 Homburg, Germany.
TEL: +49-6841-16-24100; FAX: +49-6841-16-24107; E-MAIL: marcus.unger@uks.eu

Submitted: 2014-01-21 Accepted: 2014-01-30 Published online: 2014-02-27

Key words: **ghrelin; enzyme immunoassay; comparison of methods; Bland-Altman plot; multiplex analysis; validation**

Neuroendocrinol Lett 2014;35(1):37-41 PMID: 24625914 NEL350114A04 © 2014 Neuroendocrinology Letters • www.nel.edu

Abstract

BACKGROUND: Different assays have been used in investigations on human ghrelin blood concentrations. The range of human ghrelin blood concentrations varies markedly between different studies. The variance of reported ghrelin concentrations might be due to patient specific factors, differences in sample processing, different analytical methods and different manufacturers of the assays. It is unknown how well ghrelin concentrations measured by different analytical methods are comparable and few data exist on the validity (for external consistency) of ghrelin assays.

METHODS: We analyzed 256 human plasma samples for acylated ghrelin concentrations with a commercially available enzyme-linked immunoassay (ELISA) and a multiplex analysis kit using Luminex® technology.

RESULTS: Both methods yielded ghrelin concentration within the same range. Concentrations measured by ELISA were systematically higher (median 1.4-fold). The measured concentrations of both methods correlated well as shown by a high Pearson's correlation coefficient (0.753, $p < 0.01$). Bland-Altman plotting revealed complementary aspects concerning the agreement of the two tested methods at low and high concentrations.

CONCLUSIONS: We conclude that the two investigated techniques yield results with an acceptable agreement. The agreement of both measurements indicates a good external consistency and reliability of both analytical methods. In the absence of a gold standard for ghrelin measurement, our data are a cross-validation for both methods.

INTRODUCTION

Research interest in the neuropeptide ghrelin is constantly increasing during the last years due to ghrelin's various biological functions (Steiger *et al.* 2011; Kluge *et al.* 2011; Li *et al.* 2013; Andrews *et al.* 2009; Perboni & Inui 2010) and due to its thera-

peutical potential (Garin *et al.* 2013). Clinical studies that investigated ghrelin blood concentrations applied different analytical methods for quantitative measurement of ghrelin. Most studies used enzyme-linked immunoassays (ELISA) or radio immunoassays (RIA) to measure ghrelin concentrations in human biosamples. An innovative and

straightforward approach for quantitative measurement of ghrelin concentrations (and simultaneously multiple other analytes in the same biosample) is multiplex analysis using Luminex® technology. This technology uses capture antibodies coupled to color-coded (magnetic) microspheres and tagged detection antibodies. The readout is the fluorescence from the bead (coding for one specific analyte in the multiplex approach) and the fluorescence from the detection antibody (reflecting concentration).

The range of human acylated ghrelin concentrations varies between different studies. The divergence in reported ghrelin concentrations might be due to patient specific factors, differences in sample processing (there is rapid degradation of acylated ghrelin if no protease / esterase inhibitor is added to the sample (Loo *et al.* 2011)), different analytical methods and different manufacturers of the assays.

It is unclear how well ghrelin concentrations obtained by different analytical methods are comparable. In the absence of a gold standard for the measurement of ghrelin, analytical methods should be compared to a reference method to evaluate the reliability with respect to external consistency. This all led us to investigate the agreement of human acylated ghrelin concentrations measured by a commercially available ELISA and a commercially available multiplex analysis kit from two different manufacturers.

METHODS

Samples

Biosamples analyzed in this work represent a subset of samples collected in two studies that investigate ghrelin concentrations in healthy controls and patients with Parkinson's disease. The two studies were approved by the Ethical Committee of the Saarland Medical Chamber and the Ethical Committee of the Philipps-University Marburg, Germany. Sampling tubes (EDTA 2.6 mL, Sarstedt, Germany) were supplemented with 10 µL p-hydroxymercuribenzoic acid (PHMB) per mL blood to prevent pre-analytic degradation of acylated ghrelin by esterases. Samples were stored on ice during processing and plasma was separated immediately after collection by centrifugation (10 minutes with an acceleration of 2,383 g at 4 °C). The plasma was treated with 100 µL of 1 N hydrochloric acid per mL plasma and centrifuged again (5 minutes with an acceleration of 2,383 g at 4 °C). The supernatant was stored at -20 °C until analysis.

Enzyme-linked immunoassay

Samples were analyzed with a commercially available enzyme-linked immunoassay for human acylated ghrelin from SPI-Bio (Cayman Chemical, Cat. #10006306) according to the manufacturer's manual. Samples were measured in duplicate with the ELISA-Reader Dynex MRX20310.

Multiplex Luminex® assay

Samples were analyzed on a commercially available multiplex hormone magnetic bead panel (Millipore, Cat. # HMHMAG-34K) for human acylated ghrelin together with seven other analytes (GIP, PYY, PP, amylin, GLP-1, insulin, leptin; data not shown). The assay was performed according to the manufacturer's manual. Data were acquired on a Bio-plex 200 system (Bio-Rad, Germany).

Statistical analyses

IBM® SPSS® Statistics software version 19 was used for all statistical analyses. The Pearson's correlation coefficient was calculated as a measure of correlation. Correlation per se does not equate good agreement of two analytical methods. We therefore performed Bland-Altman plotting of the data (Bland & Altman 1995; Bland & Altman 1986). Bland-Altman plots allow graphical analysis of the data in order to identify fixed bias.

RESULTS

The absolute concentrations measured by both techniques were in the same range (maximum concentration measured by ELISA: 384 pg/mL, maximum concentration measured by the multiplex assay: 376 pg/mL; minimum concentration measured by ELISA: 7 pg/mL, minimum concentration measured by the multiplex assay: 6 pg/mL). Figure 1A shows a scatter plot of acylated ghrelin concentrations obtained by ELISA and the multiplex assay. Human acylated ghrelin concentrations measured by ELISA and multiplex assay correlated well (Pearson's correlation coefficient 0.753, $p < 0.01$). The mean intra-assay coefficient of variation (CV) was < 0.05 (ELISA), < 0.10 respectively (multiplex assay).

Taking into account that correlation analyses can be misleading and might miss a systematic bias, we used Bland-Altman plots as complementary graphical analysis tool to evaluate the agreement of the two methods. For Bland-Altman plots, the difference between two measurements is plotted against the average of the two measurements for each sample. Bland-Altman plots show that the ELISA yields systematically higher concentrations (median 1.4-fold) compared to the multiplex assay (average difference + 26.2 pg/mL). The absolute difference between the two methods increased with increasing concentrations (Figure 1B). The values were distributed equally on both sides of the midline on the Bland-Altman plot, i.e. there was no additional, concentration-dependent shift with one method. As recommended (Dewitte *et al.* 2002) for situations when differences increase with increasing concentrations, we also made a Bland-Altman plot that visualizes the percentage of difference (Figure 1C). This plot shows that the relative difference (expressed as percentage of average) between the methods is more pronounced at lower concentrations.

DISCUSSION

Different techniques (ELISA (Akamizu *et al.* 2005), RIA (Uehara *et al.* 2005), HPLC (Staes *et al.* 2010), multiplex analysis (Loo *et al.* 2011)) and assays from different manufacturers are available for quantitative measurement of ghrelin. Many of the available assays are single-site competitive assays. The two assays used in this study are both two-site sandwich assays. Compared to single-site competitive assays, two-site sandwich assays generally have a higher specificity (Prudom *et al.* 2010). The assays compared in this study were chosen because the reported ELISA is available not only for acyl ghrelin but also for deacyl ghrelin from the same manufacturer. The multiplex assay was chosen because multiplex analysis is a relatively new and upcoming technique with the advantage of analyzing several analytes at the same time.

In order to prevent degradation of acyl ghrelin we took several precautions (see *Methods*, e.g. addition of PHMB to inhibit proteases and esterases). Yet, the amount of hydrochloric acid added to the samples (in accordance with the optimized and validated protocol of the manufacturer) to inhibit butyrylcholinesterase activity might be suboptimal according to the data reported by Liu *et al.* (Liu *et al.* 2008). On the other hand, strong acidification of biosamples (below pH 2) is also associated with decreased stability of acyl ghrelin (Hosoda *et al.* 2004).

As stated in the *Methods* section, the samples investigated in this study are a subset of samples of other studies. One of these studies investigated the pre- and postprandial secretion pattern of ghrelin after a standardized test meal. In this study, a physiological secretion pattern of ghrelin was observed (high fasting ghrelin concentrations that decreased in the early postprandial phase and recovered after a few hours, data not shown). These data indicate that the physiological action of ghrelin is reflected by the assays.

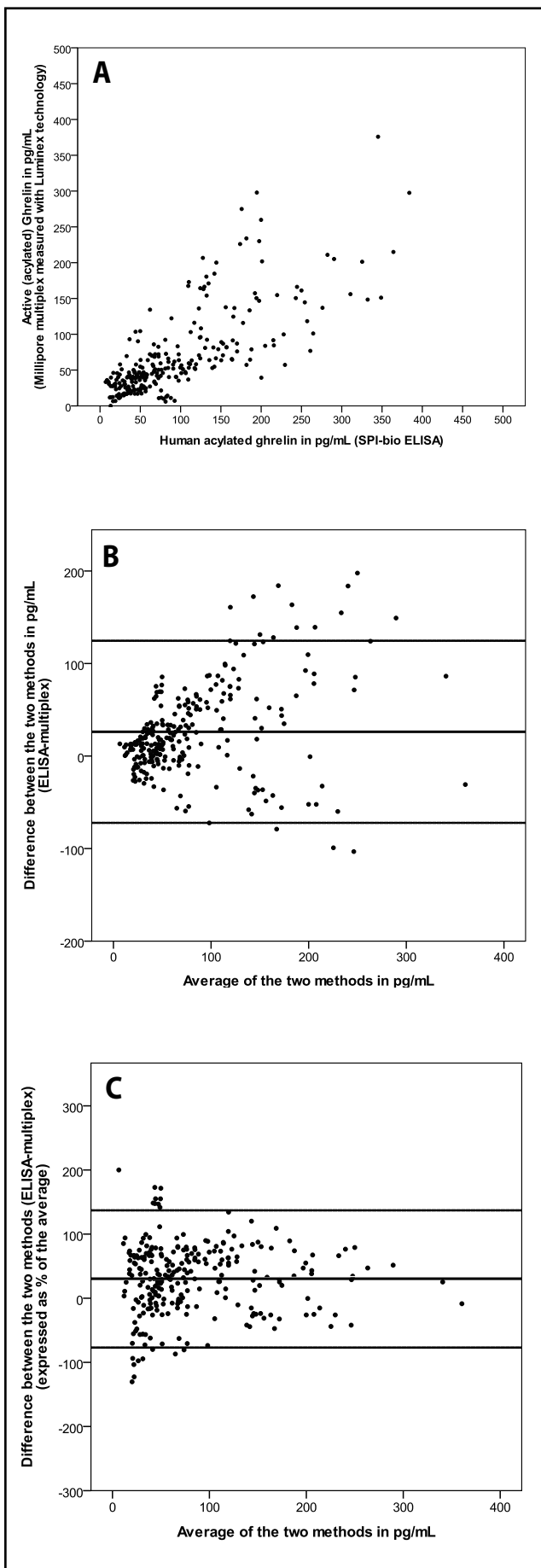


Fig. 1. A: A scatter plot of the measured acylated ghrelin concentrations. Each dot represents the acylated ghrelin concentration measured by the multiplex assay (y-axis) with respect to the concentration measured by ELISA (x-axis) in pg/mL. **B:** Each dot shows the absolute difference between ELISA and the multiplex assay (ELISA - multiplex) on the y-axis with respect to the average concentration $([ELISA + multiplex] / 2)$ given on the x-axis. The bold black line shows the average difference (26.2 pg/mL) between ELISA and the multiplex assay. The two dotted lines represent ± 1.96 standard deviations of the two measurements difference. **C:** Each dot shows the relative difference between ELISA and multiplex assay expressed as percentage on the y-axis with respect to the average concentration $([ELISA + multiplex] / 2)$ given on the x-axis. The bold black line shows the average difference expressed as percentage of average between the two measurements. The two dotted lines show the average difference ± 1.96 standard deviations of the measurements differences.

The lack of reference values and the lack of a gold standard method for quantitative measurement of ghrelin make it difficult to compare results reported in different studies. Most studies report the reproducibility (test-retest reliability) and intra-assay coefficient of variation of the applied method, but only few studies tested the validity (for external consistency) of the applied method by comparing the agreement of two different analytical techniques (Loo *et al.* 2011; Prudom *et al.* 2010; Akamizu *et al.* 2005).

Loo and colleagues reported a good agreement between ghrelin concentrations measured by a *non-magnetic* multiplex assay analyzed by Luminex® technology and an ELISA of the same manufacturer (Millipore) (Loo *et al.* 2011). Loo *et al.* used the same pairs of antibodies for both assays in their study. Here we report the agreement of data obtained by a *magnetic* multiplex assay with an ELISA of a different manufacturer. The comparison of different techniques (provided by different manufacturers and using different antibodies) reflects the situation when results of different studies are to be compared. In addition, the comparison of two different methods allows drawing conclusions about the external consistency validity of each analytic method.

Similar to the report by Loo *et al.* (2011), we observed a good correlation of the *magnetic* multiplex assay with the ELISA of a different manufacturer.

As correlation does not equate good agreement of two methods and might miss bias, we performed Bland-Altman plotting (Bland & Altman 1986; 1995). Bland-Altman plots disclose complementary aspects (average difference between the two methods, direction of the difference, variation of measurements with respect to the magnitude of the measured concentrations, etc.). We identified a systematic shift towards higher concentrations measured by ELISA. We identified no additional concentration-dependent systematic shift. There was a trend towards a higher absolute difference between the two measurements with increasing concentrations. Yet, this finding does not mean that agreement between the two methods is worse at higher concentration. Indeed, the difference expressed as percentage (Figure 1C) shows that the relative difference is even higher at lower concentrations.

We conclude that the two analytical methods yield acylated ghrelin concentrations within a similar absolute range. Nevertheless, absolute acylated ghrelin concentrations obtained by one of the two methods are not directly interchangeable. The ELISA yields systematically higher concentrations compared to the multiplex assay. Even though data obtained by the two techniques are not directly interchangeable (i.e. comparison of absolute values), the agreement of both methods is sufficient to compare the dynamic secretion pattern of ghrelin between different studies. This agreement is remarkable as we compared different techniques (using different antibodies) provided by different manufacturers. In the absence of a reference

method for ghrelin measurements our data indicate a good external consistency reliability of both techniques for quantitative measurement of acylated human ghrelin concentrations.

ACKNOWLEDGMENT

The study was supported by funds from the Michael J. Fox Foundation for Parkinson's Research and the International Parkinson Funds Germany.

Ethical standards and Conflict of interest

The authors declare that the study complies with the current laws of the country in which the study was performed. The authors declare that they have no conflict of interest.

REFERENCES

- 1 Akamizu T, Shinomiya T, Irako T, Fukunaga M, Nakai Y, Kangawa K (2005). Separate measurement of plasma levels of acylated and desacyl ghrelin in healthy subjects using a new direct ELISA assay. *J Clin Endocrinol Metab.* **90**: 6–9.
- 2 Andrews Zb, Erion D, Beiler R, Liu Zw, Abizaid A, Zigman J, Elsworth Jd, Savitt Jm, *et al.* (2009). Ghrelin promotes and protects nigrostriatal dopamine function via a UCP2-dependent mitochondrial mechanism. *J Neurosci.* **29**: 14057–14065.
- 3 Bland Jm, Altman Dg (1986). Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet.* **1**: 307–310.
- 4 Bland Jm, Altman Dg (1995). Comparing methods of measurement: why plotting difference against standard method is misleading. *Lancet.* **346**: 1085–1087.
- 5 Dewitte K, Fierens C, Stockl D, Thienpont Lm (2002). Application of the Bland-Altman plot for interpretation of method-comparison studies: a critical investigation of its practice. *Clin Chem.* **48**: 799–801; author reply 801–792.
- 6 Garin Mc, Burns Cm, Kaul S, Cappola Ar (2013). Clinical review: The human experience with ghrelin administration. *J Clin Endocrinol Metab.* **98**: 1826–1837.
- 7 Hosoda H, Doi K, Nagaya N, Okumura H, Nakagawa E, Enomoto M, Ono F, Kangawa K (2004). Optimum collection and storage conditions for ghrelin measurements: octanoyl modification of ghrelin is rapidly hydrolyzed to desacyl ghrelin in blood samples. *Clin Chem.* **50**: 1077–1080.
- 8 Kluge M, Schussler P, Dresler M, Schmidt D, Yassouridis A, Uhr M, Steiger A (2011). Effects of ghrelin on psychopathology, sleep and secretion of cortisol and growth hormone in patients with major depression. *J Psychiatr Res.* **45**: 421–426.
- 9 Li E, Chung H, Kim Y, Kim Dh, Ryu Jh, Sato T, Kojima M, Park S (2013). Ghrelin directly stimulates adult hippocampal neurogenesis: implications for learning and memory. *Endocr J.* **60**: 781–789.
- 10 Liu J, Prudom Ce, Nass R, Pezzoli Ss, Oliveri Mc, Johnson Ml, Veldhuis P, Gordon Da, *et al.* (2008). Novel ghrelin assays provide evidence for independent regulation of ghrelin acylation and secretion in healthy young men. *J Clin Endocrinol Metab.* **93**: 1980–1987.
- 11 Loo Bm, Marniemi J, Jula A (2011). Evaluation of multiplex immunoassays, used for determination of adiponectin, resistin, leptin, and ghrelin from human blood samples, in comparison to ELISA assays. *Scand J Clin Lab Invest.* **71**: 221–226.
- 12 Perboni S, Inui A (2010). Appetite and gastrointestinal motility: role of ghrelin-family peptides. *Clin Nutr.* **29**: 227–234.

- 13 Prudom C, Liu J, Patrie J, Gaylinn Bd, Foster-Schubert Ke, Cummings De, Thorner Mo, Geysen Hm (2010). Comparison of competitive radioimmunoassays and two-site sandwich assays for the measurement and interpretation of plasma ghrelin levels. *J Clin Endocrinol Metab.* **95**: 2351–2358.
- 14 Staes E, Rozet E, Ucakar B, Hubert P, Preat V (2010). Validation of a method for the quantitation of ghrelin and unacylated ghrelin by HPLC. *J Pharm Biomed Anal.* **51**: 633–639.
- 15 Steiger A, Dresler M, Schussler P, Kluge M (2011). Ghrelin in mental health, sleep, memory. *Mol Cell Endocrinol* **340**: 88–96.
- 16 Uehara T, Omori I, Nakamura K, Suda M, Hosoda Y, Minegishi T, Mikuni M, Kangawa K (2005). Plasma des-acyl and acyl ghrelin in patients with eating disorders. *Eat Weight Disord.* **10**: 264–266.