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Validation of a method for the quantitation of ghrelin and unacylated ghrelin by HPLC

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ABSTRACT

An HPLC/UV method was first optimized for the separation and quantitation of human acylated and unacylated (or des-acyl) ghrelin from aqueous solutions. This method was validated by an original approach using accuracy profiles based on tolerance intervals for the total error measurement. The concentration range that achieved adequate accuracy extended from 1.85 to 59.30 μM and 1.93 to 61.60 μM for acylated and unacylated ghrelin, respectively. Then, optimal temperature, pH and buffer for sample storage were determined. Unacylated ghrelin was found to be stable in all conditions tested. At 37 °C acylated ghrelin was stable at pH 4 but unstable at pH 7.4, the main degradation product was unacylated ghrelin. Finally, this validated HPLC/UV method was used to evaluate the binding of acylated and unacylated ghrelin to liposomes.

1. Introduction

Peptides offer a great therapeutic interest. Nevertheless, their administration is often limited not only due to their high molecular weight, but also owing to their hydrophilicity and their metabolic instability [1]. In 1999, Kojima et al. [2] isolated and characterized an unusual endogenous peptide of 28 amino acids, which was modified by a n-octanoic acid on the hydroxyl group of its serine 3 residue and called it ghrelin. The physiological roles of ghrelin are numerous, among which growth hormone (GH) secretion, stimulation of food intake and gastrointestinal motility, glucose and energy homeostasis, cardiovascular protective effects, cell proliferation and differentiation as well as immune regulation [3]. Ghrelin is mainly produced by the stomach in endocrine X/A-like cells and by the pancreas. Another naturally occurring variant of ghrelin is unacylated ghrelin also called des-acyl ghrelin. This form is the most abundant in plasma [3]. The acylation of ghrelin is required for the binding and the activation of the GHS-R1a that mediates most of its endocrinological activities [2]. However, some other biological activities have been attributed to the binding of acylated and/or unacylated ghrelin to a yet unidentified receptor [4]. Acylated and unacylated ghrelin present similar and opposite actions (see [3,4] for review). As both these peptides offer a great therapeutic potential, their physiological and pathological roles have been widely investigated. Since ghrelin is a pleiotropic hormone, it has several potential clinical applications, among which the diagnostic of GH deficiency, but also the treatment of hypomotility syndrome, cachexia–anorexia syndrome and aging [3,5]. If we want to formulate ghrelin as a drug, the stability of the formulation is essential. Indeed, the octanoyl–serine ester bond is both chemically and enzymatically unstable. Therefore, deoctanoylation is likely to occur during manufacturing, storage, handling and *in vitro* testing. Ghrelin storage stability has been assessed but only in blood samples [6]. As ghrelin is degraded by esterases [7,8], it has been recommended to collect blood samples in EDTA-aprotinin tubes and then to acidify samples to a final pH of 3–4 to avoid deacylation of ghrelin [6]. Even though the ester bond is meant to be more stable in solution devoid of enzymes, it is important to know whether ghrelin stability is affected in water or in commonly used buffers for *in vitro* tests or parenteral formulations. Selection of optimal conditions, including temperature, pH and buffer is therefore required.

Acylated and unacylated human ghrelin are generally quantitated by immunological methods such as RIA [6,9], ELISA [10,11], EIA [12,13]. An HPLC/MS/MS method has also been developed [13]. However, all these methods are expensive, time consuming and quantitate samples in the pico- or nanomolar range. Therefore, dosing micromolar samples with these techniques require high dilutions and are thus subject to errors. Even though RP-HPLC has been widely used in purification processes [14–16] to separate acy-

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lated from unacylated human ghrelin, UV was sometimes used as a detection method [2,16] but RIA was typically used as the quantification method [17,18]. Here we provide the first validated RP-HPLC/UV method to separate and reliably quantify both forms of human ghrelin in the micromolar range.

The objectives of this paper were first to adapt the described HLPC/UV method for rat ghrelin [2,19,20] in order to separate and quantify human acylated (hAG) and unacylated (hUAG) ghrelin in the micromolar range adapted to ghrelin injectable formulations and *in vitro* tests [21,22]. The stability and storage conditions of the acylated and unacylated ghrelin in media that are commonly used for formulations or *in vitro* studies were also evaluated. Secondly, a thorough validation of this HPLC/UV method was performed to provide guarantees about the accuracy and thus the reliability of the results. Finally, this validated HPLC/UV method was used to quantify the ghrelin binding to liposomes to illustrate its applicability.

2. Experimental procedures

2.1. Chemicals

Synthetic human acylated ghrelin (hAG) and human unacylated ghrelin (hUAG) were purchased from NeoMPS (Strasbourg, France). Peptide HPLC purity was >97%, according to the manufacturer.

Acetonitrile, HPLC grade for far UV, was purchased from Acros Organics (Thermo Fisher Scientific Inc., Waltham, MA, USA). Trifluoroacetic acid (TFA), HPLC grade, was obtained from Fisher Scientific (Thermo Fisher Scientific Inc.). Hanks' balanced salt solution (HBSS) and phosphate-buffered saline pH 7.4 (PBS) were purchased from Invitrogen (Paisley, United Kingdom). Bovine brain sphingomyelin (SM) and cholesterol (Chol) were obtained from Sigma–Aldrich (St. Louis, MO, USA), whereas phosphatidylcholine (PC) (Grade 1, from egg yolk), phosphatidylinositol (PI) (Grade 1) and phosphatidylethanolamine (PE) (Grade 1) were obtained from Lipid Products (Redhill Surrey, United Kingdom). All other reagents were of ACS or analytical grade.

2.2. Quantitation of ghrelin by RP-HPLC

The concentrations of human acylated and unacylated ghrelin were determined by an HPLC method adapted from Date et al. [20]. The HPLC Waters system (Waters Associates Inc., Milford, MA) consisted of a refrigerated autosampler (717plus), a binary pump (1525), a column heater and a dual wavelength absorbance UV detector (2487). 50 μL of samples were injected in a TSK-gel ODS 120 column (150 mm × 4.6 mm I.D.; 5 μm particle size: Tosoh Bioscience, Stuttgart, Germany) protected by a Hypersil Gold guard column (10 mm × 4 mm I.D.: 5 μm particle size: Thermo Fisher Scientific), both thermostatised at 37 °C. The mobile phase A was water with 0.1% (v/v) trifluoroacetic acid, and B was acetonitrile with 0.1% trifluoroacetic acid. The samples were eluted with a linear gradient of A and B, from 12% B (0 min) to 52% B (32 min). The flow rate was 1 mL/min. The acylated and unacylated ghrelin UV absorbances were recorded at 210 nm.

2.3. Validation of the HPLC/UV method

In each experiment the stocks of acylated and unacylated ghrelin were reconstituted at 1 mg/mL with water or with 12.5 mM citrate 14 mM NaCl pH 6 from pre-weighted 100 μg or 1 mg lyophilized peptide (NeoMPS). A calibration curve was established for each analyte from the reconstituted stock by twofold serial dilutions (7 dilutions). The concentrations of the calibration curve ranged from 0.93 to 118.67 μM and 0.96 to 123.28 μM for human acylated and unacylated ghrelin, respectively. The validation standards, prepared from similarly reconstituted stock solutions, were set at three concentrations ranging from 1.85 to 59.30 μM and from 1.93 to 61.60 μM, for human acylated and unacylated ghrelin, respectively. The number of replicates per concentration level and the number of runs were all ≥3. Enoval software v2.0 (Arlenda, Liège, Belgium) was used to compute accuracy profiles and validation results.

2.4. Preparation of stability samples

Lyophilized human acylated and human unacylated ghrelin were reconstituted in water or in buffer at a final concentration of 200 μg/mL. Buffers tested were: (1) 12.5 mM citrate 14 mM NaCl pH 6 (I_c = 0.06); (2) 12.5 mM citrate 46.2 mM NaCl pH 4 (I_c = 0.06); (3) HBSS pH 6; (4) HBSS pH 7.4 and (5) PBS pH 7.4. Peptide stability was evaluated at 37 °C for 4 h in all solutions. Stability of the peptides was also assessed at 4 °C in PBS pH 7.4 and in 12.5 mM citrate 14 mM NaCl pH 6. 200 μg/mL stock solutions of each buffer were used as controls. Controls were immediately frozen and stored at −80 °C upon preparation whereas samples were incubated prior to freezing and storage in the same conditions. Samples and controls were thawed at room temperature 5–10 min before injection into the HPLC system.

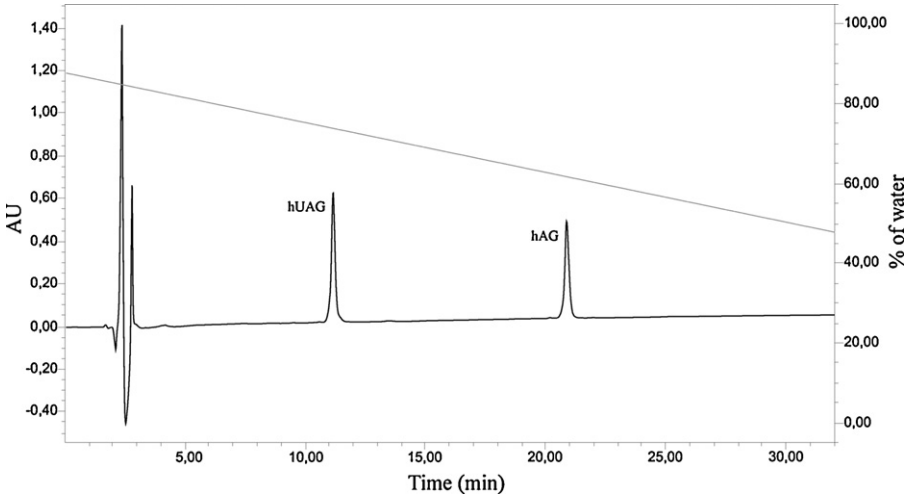

Fig. 1. HPLC chromatogram of 33.9 μM of human acylated ghrelin (hAG) and 35.5 μM of human unacylated ghrelin (hUAG) in 12.5 mM citrate 14 mM NaCl pH 6 detected at 210 nm.

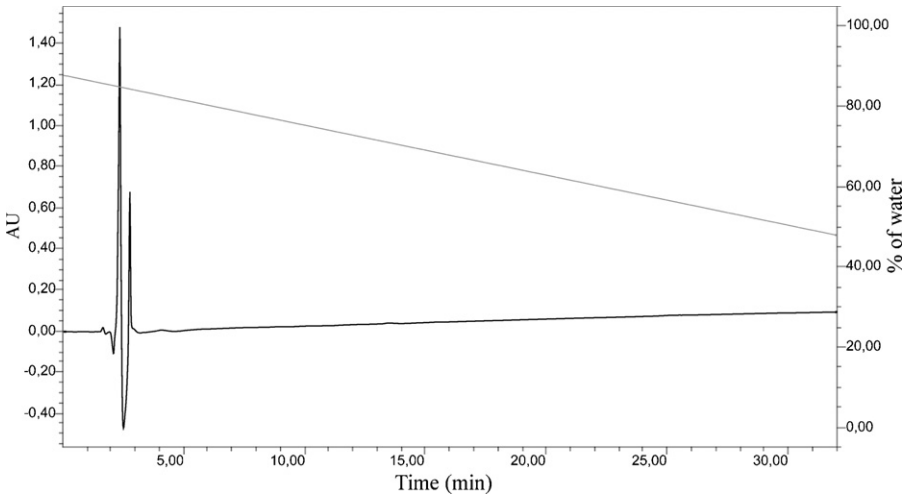

Fig. 2. HPLC chromatogram of 12.5 mM citrate 14 mM NaCl pH 6 detected at 210 nm.

2.5. Ghrelin binding to liposomes

The binding of human acylated and unacylated ghrelin to large unilamellar liposomes made of Chol/PC/SM/PI/PE (5.5:4.0:1.7:3.0:2.3, M/M) in 12.5 mM citrate 14 mM NaCl pH 6 buffer was assessed. These liposomes were prepared by extrusion under nitrogen through two 100 nm pore superposed Nucleopore® Track-Etched polycarbonate membranes (Whatman, Maidstone, UK) using a Thermobarrel extruder (Lipex Biomembranes Inc., Vancouver, Canada), as previously described [23]. The phospholipids concentration was determined by phosphorus assay [24] and the total lipid concentration was calculated assuming similar recovery of phospholipids and cholesterol. Liposomes were stored under nitrogen at 4 °C and used within 3 days. The *z*-average diameter of these liposomes was 107 ± 5 nm as measured using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). The ghrelin binding to liposomes was assessed at lipid/peptide molar ratios from 13:1 to 40:1 by ultracentrifugation. The liposomes/peptide (either acylated or unacylated ghrelin at 100 μg/mL final concentration) mixture (160 μL) was incubated for 3 h at 37 °C and then ultracentrifuged at 4 °C (250,000 × *g*) for 1 h. In controls, liposomes were replaced by the equivalent volume of buffer. The supernatants that contained the free peptide were then collected for HPLC/UV quantitation. The binding percentage was calculated as follows: ((control concentration – supernatant concentration)/control concentration) × 100.

3. Results and discussion

3.1. HPLC method

As shown in Fig. 1 the HPLC method developed allowed the separation of acylated and unacylated ghrelin. Retention times were 11.4 and 21.0 min for unacylated and acylated ghrelin, respectively.

3.2. Validation of the HPLC method

An original approach using accuracy profiles based on tolerance intervals for the total error measurement, including both bias and standard deviation for intermediate precision, was applied to demonstrate the method validity [25–27]. It allows to evaluate the calibration curve, trueness, precision, accuracy, linearity, dosing range, limits of quantitation and of detection [28,29]. The method is declared as valid within the range for which the accuracy profile computed by means of β-expectation tolerance intervals is fully included inside the accuracy acceptance limits set at ±30% [30]. The

Table 1
Validation results for the human acylated ghrelin using a weighted (1/*X*) linear regression as calibration curve.

True concentration (μM)	Bias (μM)	Relative bias (%)
Trueness		
1.85	0.12	6.7
7.42	−0.42	−5.6
59.30	1.69	2.9
True concentration (μM)	**Repeatability (RSD %)**	**Intermediate precision (RSD %)**
Precision		
1.85	9.9	14.3
7.42	5.2	13.9
59.30	2.6	6.1
True concentration (μM)	**Lower relative tolerance interval (%)**	**Upper relative tolerance interval (%)**
Accuracy		
1.85	−14.6	28.0
7.42	−29.3	18.0
59.30	−5.4	11.1

Table 2
Validation results for the human unacylated ghrelin (hUAG) using a weighted (1/*X*) linear regression as calibration curve.

True concentration (μM)	Bias (μM)	Relative bias (%)
Trueness		
1.93	0.02	1.2
7.71	0.30	3.9
61.60	0.98	1.6
True concentration (μM)	**Repeatability (RSD %)**	**Intermediate precision (RSD %)**
Precision		
1.93	4.0	5.9
7.71	4.3	4.6
61.60	2.7	4.1
True concentration (μM)	**Lower relative tolerance interval (%)**	**Upper relative tolerance interval (%)**
Accuracy		
1.93	−8.3	10.6
7.71	−2.4	10.2
61.60	−3.9	7.1

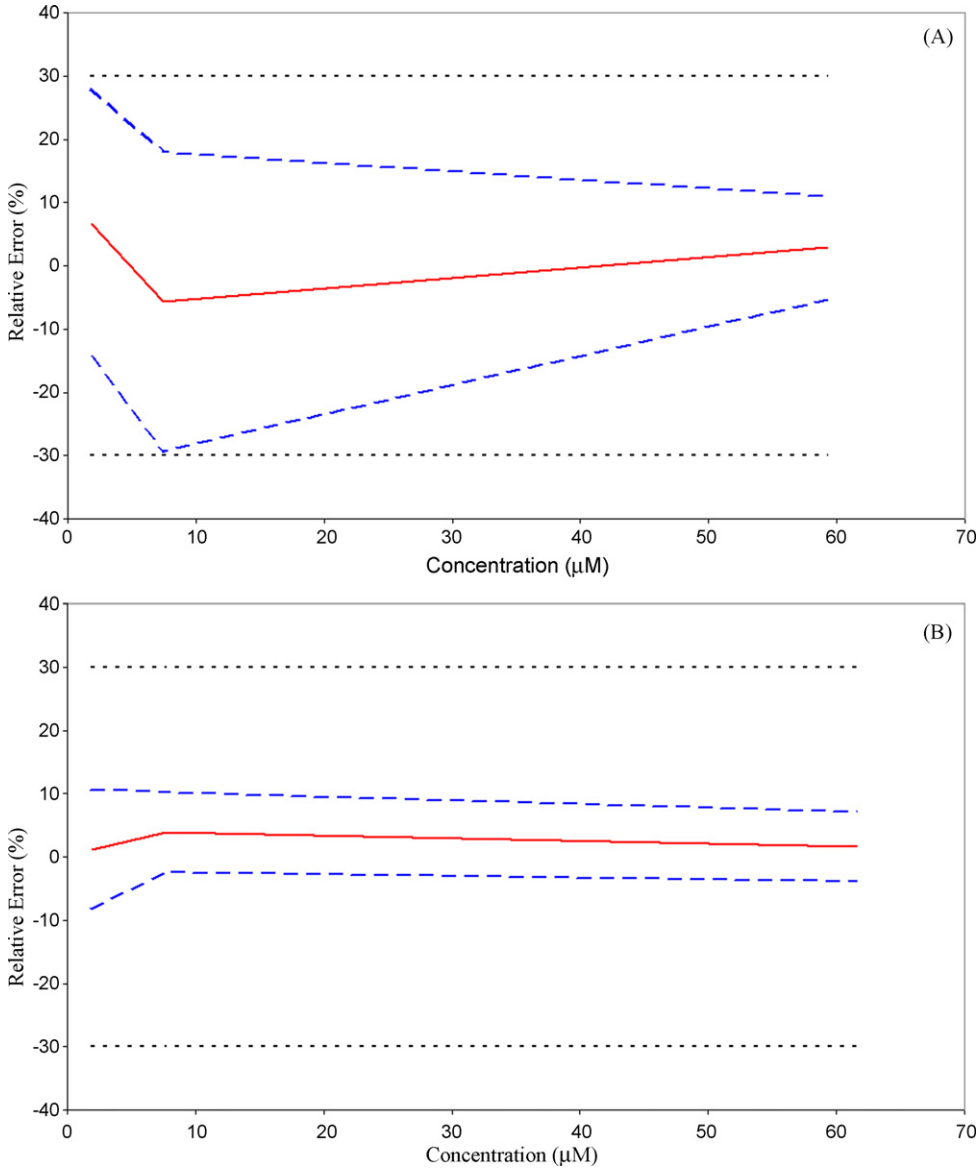

Fig. 3. Accuracy profiles obtained for the validation of the HPLC/UV analytical method for the quantitation of human acylated ghrelin (A) and human unacylated ghrelin (B) by considering a weighted (1/*X*) linear regression as calibration curves; plain line: relative bias, dashed lines: β-expectation tolerance limits (β = 80%), dotted lines: ±30% acceptance limit.

β-expectation tolerance interval that describes a region where, on average, a proportion β of future measurements will fall, was fixed at 80% [31]. In this way, the guarantee that each further measurement of unknown samples will be included within the acceptance limits is fixed.

3.2.1. Selectivity

The selectivity of the developed method is acceptable since no interfering peak was observed at the retention times of the well separated acylated and unacylated ghrelin for all the buffers tested (Figs. 1 and 2).

Table 3
Evaluation of the results linearity for human acylated ghrelin (hAG) and human unacylated ghrelin (hUAG).

	Equation	R^2
hAG	$y = 1.0326x - 0.2792$	0.999
hUAG	$y = 1.0147x - 0.0854$	0.999

3.2.2. Trueness and precision

Trueness refers to the closeness of agreement between a conventionally accepted value and a mean experimental one [28,29,32]. It gives information on systematic error. Tables 1 and 2 report trueness expressed as absolute bias and relative bias for the different level of validation standards for both analytes. The maximum relative biases were 7% and 4% for human acylated and unacylated ghrelin, respectively.

Precision is the closeness of agreement among measurements from multiple sampling of a homogeneous sample under the recommended conditions [28,29,32]. It gives information on random errors and it can be evaluated at two levels: repeatability and intermediate precision. Results are presented in Tables 1 and 2. Relative standard deviation for repeatability and intermediate precision seemed relatively high, with maximum values of about 14% and 6% for acylated and unacylated ghrelin, respectively.

3.2.3. Accuracy, limits of quantitation and range

Accuracy refers to the closeness of agreement between the test result and the accepted reference value, namely the convention-

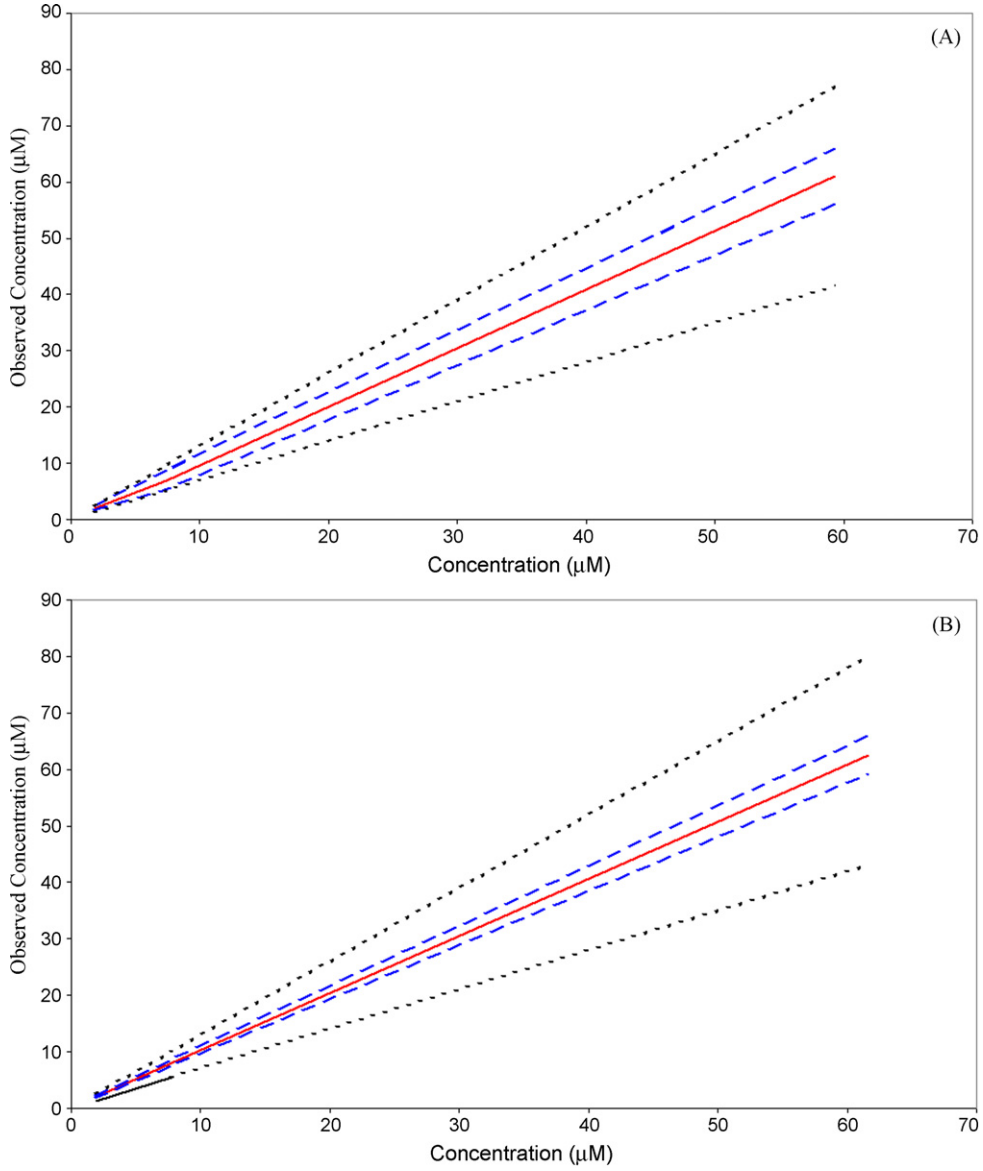

Fig. 4. Linearity obtained for the validation of the HPLC/UV analytical method for the quantitation of human acylated ghrelin (A) and human unacylated ghrelin (B) by considering a weighted (1/*X*) linear regression as calibration curves; plain line: relative bias, dashed lines: β-expectation tolerance limits (β = 80%), dotted lines: ±30% acceptance limit expressed in μM.

ally true value [28,29,32]. The accuracy takes into account the total error, i.e. systematic and random errors, related to the test result (Tables 1 and 2). It is assessed from the accuracy profile illustrated in Fig. 3A and B for acylated and unacylated ghrelin, respectively. The method is considered as valid in the range for which the accuracy profile is within the accuracy acceptance limits set at ±30%. This approach gives the guarantee that each further measurement of unknown samples will be included within the tolerance limits with a probability of 80%.

The lower limit of quantitation (LOQ) is the smallest quantity of the targeted substance in the sample that can be assayed under experimental conditions with well defined accuracy [28,29,32]. The definition can also be applicable to the upper LOQ, which is the highest quantity of the targeted substance in the sample that can be assayed under experimental conditions with well defined accuracy. The limits of quantitation are obtained by calculating the smallest and highest concentration beyond which the accuracy limits or β-expectation limits step outside the acceptance limits. Experimentally, the lower LOQs were evaluated at 1.85 and 1.93 μM for acylated and unacylated ghrelin, respectively and the upper LOQs at 59.30 and 61.60 μM for acylated and unacylated ghrelin, respectively. The concentration range extends then to the interval between the lower and upper limits where the procedure achieves adequate accuracy.

3.2.4. Results linearity

In order to demonstrate results linearity [28,29], a regression line was fitted on the calculated concentrations of the validation standards as a function of the introduced concentrations by applying a linear regression model. The equations obtained for each ghrelin with their coefficient of determination are presented in Table 3. The slopes values obtained for the two analytes were between 0.85 and 1.15. The linearity of the method is demonstrated using the β-expectation tolerance interval approach. Indeed, as illustrated in Fig. 4 the upper and lower β-expectation tolerance limits were included inside the absolute acceptance limits irrespective of the concentration levels for the two analytes studied.

Table 4
Effect of temperature and pH on human acylated (hAG) and unacylated ghrelin (hUAG) stability.

Medium	pH	Temp (°C)	Time (h)	Mean (SD) percentage of controls[a]		
				hAG		**hUAG**
				hAG	hUAG	
H_2O	~4.7[b]	37	4	99.7 (2.0)	99.2 (2.7)	101.3 (2.4)
Citrate buffer	4	37	4	98.9 (2.0)	100.5 (2.4)	100.3 (0.2)
Citrate buffer	6	37	4	96.2 (1.3)	109.7 (0.5)[*]	99.3 (1.5)
Citrate buffer	6	4	4	99.3 (0.6)	103.4 (7.8)	99.5 (1.6)
HBSS	6	37	4	98.5 (3.3)	103.0 (4.9)	99.4 (0.9)
HBSS	7.4	37	4	91.9 (2.2)[£]	178.5 (7.7)[**]	99.2 (1.8)
PBS	7.4	37	4	95.1 (1.8)[£]	126.2 (1.7)[***]	98.6 (0.2)
PBS	7.4	37	24	85.3 (1.0)[***]	269.9 (9.9)[***]	98.7 (1.3)
PBS	7.4	4	24	98.2 (0.7)	104.5 (3.1)	100.0 (1.6)

No statistical differences unless otherwise stated.
[a] hUAG control concentrations were ~202 μg/mL, whereas hAG controls contained ~198 μg/mL hAG and ~8 μg/mL hUAG.
[b] As determined using a pH-meter.
[£] $p < 0.05$ (statistical analysis by unpaired t-test within each condition tested vs control).
[*] $p < 0.01$ (statistical analysis by unpaired t-test within each condition tested vs control).
[**] $p < 0.0005$ (statistical analysis by unpaired t-test within each condition tested vs control).
[***] $p < 0.0001$ (statistical analysis by unpaired t-test within each condition tested vs control).

3.3. Stability of ghrelin in aqueous solutions

To determine the optimal storage conditions for ghrelin samples, the stabilities of acylated ghrelin and unacylated ghrelin were tested in different types of buffers, at different temperatures and different times of storage. The stability of each analyte was determined as a percentage of the stock solutions concentrations (=controls). As acylated ghrelin stock solutions (~202 μg/mL = 100% hAG) already contained some unacylated ghrelin (~8 μg/mL = 100% hUAG), the degradation of acylated ghrelin into unacylated ghrelin implies that unacylated ghrelin mean percentage of controls exceeds 100%. As shown in Table 4 unacylated ghrelin was stable under all conditions tested while the stability of acylated ghrelin was impaired at 37 °C at pH 7.4. The main degradation product of acylated ghrelin was unacylated ghrelin, probably due to the saponification of the ester bound in basic conditions. The higher stability of acylated ghrelin at acidic pH is also consistent with the optimal storage conditions of ghrelin, i.e. pH 4, that were determined for blood samples by Hosoda et al. [6]. Consequently, ghrelin samples can be kept at 4 °C for at least 24 h before analysis. If formulated at pH 7.4, acylated ghrelin should be kept at 4 °C or used and analysed immediately.

3.4. Ghrelin binding to liposomes

To check if acylation could affect the binding of ghrelin to lipid membranes, the validated method was used to assess the binding of acylated and unacylated ghrelin to negatively charged liposomes made of Chol/SM/PC/PE/PI (5.5:4.0:1.7:3.0:2.3, M/M) at different lipid/peptide molar ratios. Indeed, peptide-membrane interactions are mainly due to electrostatic and/or hydrophobic interactions [33,34]. As shown in Fig. 5, the binding of acylated ghrelin to Chol/SM/PC/PE/PI liposomes was higher than the binding of unacylated ghrelin at all the lipid/peptide ratios. It thus indicates that acylation promotes the interaction of the peptide with the lipid membrane and hence that hydrophobic interactions seem to play a role in ghrelin-membrane binding as both peptides carry the same net positive charge. The binding of acylated ghrelin increased with the lipid content.

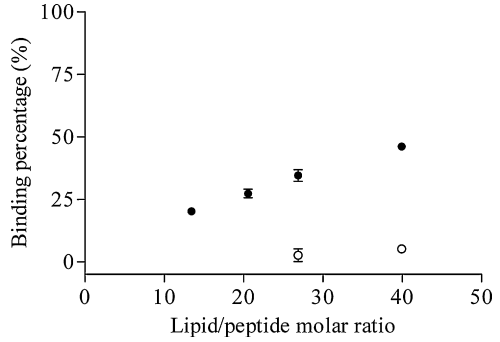

Fig. 5. Binding of human acylated ghrelin (hAG) (●) and human unacylated ghrelin (hUAG) (○) to Chol/PC/SM/PI/PE (5.5:4.0:1.7:3.0:2.3, M/M) liposomes at increasing lipid/peptide molar ratios, $T = 37$ °C ($n = 3$). Circles represent the mean values ± SD.

4. Conclusions

The described HPLC/UV method allows the separation and the quantitation of the human acylated and unacylated ghrelin. An original validation approach using accuracy profiles based on β-expectation tolerance intervals for the total measurement error demonstrated the reliability of the results generated. The concept of accuracy profile was also used to select the most appropriate regression model for calibration, to determine the lower limit of quantitation and the range over which the method can be considered as valid. The described method is therefore reliable for its intended use, the accurate quantitation of human acylated and unacylated ghrelin in the micromolar concentration range. In addition, this method was used to assess the stability of acylated and unacylated ghrelin in various media. Unacylated ghrelin was found to be stable in all conditions tested. Acylated ghrelin solutions, however, required preparation or storage at acidic pH and/or at low temperature to avoid deacetylation. Finally, this validated HPLC/UV method was successfully applied to evaluate the binding of acylated and unacylated ghrelin to liposomes.

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