Video Article

A RAPID Method for Blood Processing to Increase the Yield of Plasma Peptide Levels in Human Blood

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Abstract

Research in the field of food intake regulation is gaining importance. This often includes the measurement of peptides regulating food intake. For the correct determination of a peptide's concentration, it should be stable during blood processing. However, this is not the case for several peptides which are quickly degraded by endogenous peptidases. Recently, we developed a blood processing method employing Reduced temperatures, Acidification, Protease inhibition, Isotopic exogenous controls and Dilution (RAPID) for the use in rats. Here, we have established this technique for the use in humans and investigated recovery, molecular form and circulating concentration of food intake regulatory hormones. The RAPID method significantly improved the recovery for ^{125}I-labeled somatostatin-28 (+39%), glucagon-like peptide-1 (+35%), acyl ghrelin and glucagon (+32%), insulin and kisspeptin (+29%), nesfatin-1 (+28%), leptin (+21%) and peptide YY_{3-36} (+19%) compared to standard processing (EDTA blood on ice, p <0.001). High performance liquid chromatography showed the elution of endogenous acyl ghrelin at the expected position after RAPID processing, while after standard processing 62% of acyl ghrelin were degraded resulting in an earlier peak likely representing desacyl ghrelin. After RAPID processing the acyl/desacyl ghrelin ratio in blood of normal weight subjects was 1:3 compared to 1:23 following standard processing (p = 0.03). Also endogenous kisspeptin levels were higher after RAPID compared to standard processing (+99%, p = 0.02). The RAPID blood processing method can be used in humans, yields higher peptide levels and allows for assessment of the correct molecular form.

Video Link

The video component of this article can be found at https://www.jove.com/video/53959/

Introduction

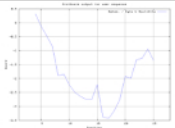
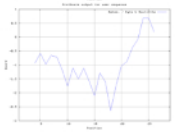
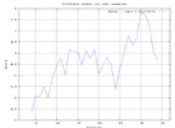
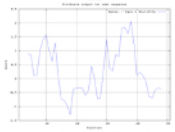
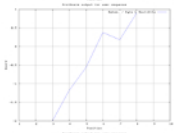
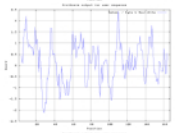
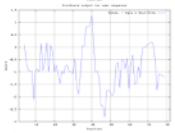
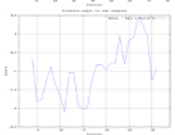
In light of the worldwide increasing prevalence of obesity[1,2], research in the field of food intake regulation is gaining importance. While so far only one peptide is known that is peripherally produced and centrally acting to stimulate food intake, namely ghrelin[3], within the past decades, a broad range of peptides has been identified that reduce food intake, *e.g.* leptin, peptide YY (PYY) and also glucagon-like peptide-1 (GLP-1) and insulin[4], Therefore, in studies investigating the regulatory mechanisms of hunger and satiety peptide levels are often assessed and at the same time, it is assumed that the peptide studied is stable and recovered at high yields during plasma formation. However, very often this is not the case due to rapid endogenous breakdown as shown before for *e.g.* ghrelin which is degraded from acyl to desacyl ghrelin[5]. Therefore, we recently described the RAPID method for blood processing in rats employing Reduced temperatures, Acidification, Protease inhibition, Isotopic exogenous controls and Dilution[6]. This method improved the recovery for 11 of 12 peptides tested and allowed for the determination of the correct circulating molecular form compared to standard blood processing (EDTA blood on ice)[6]. This method has been used in several subsequent studies[7-12] for the detection of circulating ghrelin as well as corticotropin-releasing factor[13]. Therefore, the method has proven useful for peptide research in rodents. However, since rodent studies are not always translatable to another species, the method should be established for the use in human blood as well.

The aim of the present study was to test the RAPID method for blood processing in humans compared to standard blood processing, EDTA blood on ice, which is widely recommended[14] and frequently used in the clinical as well as research setting. We tested the recovery of a selection of ^{125}I-labeled peptides involved in the regulation of food intake including established peptides as well as new candidates recently suggested to play a role in feeding regulation (effects on food intake are shown in **Table 1**) following processing with both methods. Hormones were also chosen to represent peptides of different length and charge (**Table 2**). Moreover, for ghrelin we investigated the molecular form(s) following the standard and RAPID method. Lastly, we assessed endogenous ghrelin (acyl and desacyl ghrelin) as well as kisspeptin levels,

a peptide also recently suggested to play a role in the regulation of food intake[15,16] following RAPID or standard processing. In addition, we also investigated these peptide levels in a population of subjects with a wide range of body mass index (ranging from 10.2-67.6 kg/m^2) to study possible differences related to chronically altered body weight.

Peptide	Effect	Reference
Acyl-ghrelin	Stimulation of food intake following intravenous injection in humans	17
Glucagon	Reduction of food intake following intramuscular injection in humans	18
Glucagon-like peptide 1	Reduction of energy intake following oral administration in men	19
Insulin	Stimulation of intake of high-fat foods following intravenous injection in men (*via* hypoglycemia)	20
Kisspeptin	Inhibition of food intake following intracerebroventricular injection in mice	15
Leptin	Reduction of body weight and fat mass following repeated subcutaneous injection in humans	22
Nesfatin-1	Inhibition of food intake following intraperitoneal injection in mice	23
Peptide YY_{3-36}	Reduction of caloric intake following intravenous infusion in lean and obese subjects	24
Somatostatin-28	Blunting of the corticotropin-releasing factor induced reduction of food intake following intracerebroventricular injection in rats	25

Table 1. Effects on food intake of peptides studied

Peptide	Net charge at pH 3.6 (RAPID)	Net charge at pH 7.4 (Standard)	Kyte-Doolittle hydrophobicity plot
Acyl-ghrelin 28 aa	+8.0	+4.9	
Glucagon 29 aa	+3.8	-0.1	
Glucagon-like peptide 1 37 aa	+5.3	-3.0	
Insulin 51 aa	+3.7	-2.4	
Kisspeptin-10 10 aa	+1.2	+0.8	
Leptin 167 aa	+14.3	-2.9	
Nesfatin-1 82 aa	+14.8	-4.8	
Peptide YY_{3-36} 34 aa	+5.6	-0.1	
Somatostatin-28 28 aa	+5.1	+3.6	

Kyte-Doolittle plots show the distribution of polar and apolar residues in a peptide sequence. This can predict hydrophilic or hydrophobic (values above 0) regions. These plots have a window size of 7 (for kisspeptin-10 a window size of 5 had to be chosen), indicating that seven amino acids (aa) were examined at a time to determine a point of hydrophobic character. Hydrophilic regions are likely exposed on the surface.

Table 2. Kyte–Doolittle hydrophobicity plots and net charges of tested peptides.

Diagnosis, Assessment, and Plan:

Study participants

All study participants were newly hospitalized patients (inclusion was within two days of admission to the hospital) of the Division of Psychosomatic Medicine at Charité-Universitätsmedizin Berlin and gave written informed consent. To avoid any impact of gender only female patients were included. A total of 42 subjects participated in this study and were divided into three groups: normal weight (BMI 18.5-25 kg/m^2, n = 12), anorexia nervosa (BMI <17.5 kg/m^2, n = 15) and obesity (BMI >30 kg/m^2, n = 15). Anorexic and obese patients were diagnosed according to the International Classification of Diseases-10 and hospitalized for weight gain (anorexia nervosa) or weight reduction (obesity), respectively. All normal weight patients were hospitalized exclusively due to somatoform symptoms without relevant somatic disorders. Patients with gastrointestinal somatoform symptoms or a history of gastrointestinal surgery were excluded. Exclusion criteria also encompassed an age <18 years, current pregnancy and untreated psychotic diseases. Blood collection was performed on day 2 or 3 after hospital admission before receiving dietary treatment in order to increase or reduce body weight, respectively. Anthropometric parameters were assessed on the same day.

Protocol

The protocol was approved by the local ethics committee for human research (protocol number EA1/114/10).

1. Blood Processing

1. Collect venous blood between 07:00 and 08:00 am after an overnight fast from a forearm vein and process according to standard procedure or the RAPID method. Instruct the subjects to not exercise or smoke before blood withdrawal.
2. For standard processing, collect blood in chilled EDTA-containing tubes and centrifuge within 10 min at 3,000 x g for 10 min at 4 °C. Collect the supernatant and keep at -80 °C until further processing by radioimmunoassay.
3. For RAPID processing, immediately dilute blood (within 1 min after blood withdrawal) 1:10 in ice-cold buffer (pH 3.6) containing 0.1 M ammonium acetate, 0.5 M NaCl and enzyme inhibitors (diprotin A, E-64-d, antipain, leupeptin, chymostatin, 1 µg/ml). Then, centrifuge within 10 min at 3,000 x g for 10 min at 4 °C and collect supernatant using a pipette in polypropylene tubes as detailed before in rats[6].
    1. Charge chromatography cartridges (360 mg, 55-105 µm) with 100% acetonitrile (rate 10 ml/min), equilibrate with 0.1% trifluoroacetate (TFA, rate 10 ml/min) and load with the supernatant at a constant rate of 1 ml/min using a syringe pump.
    2. Thereafter, wash cartridges with 3 ml 0.1% TFA (rate 10 ml/min) and slowly elute with 2 ml 70% acetonitrile containing 0.1% TFA (2 ml/min).
    3. Dry eluted samples using vacuum centrifugation and store at -80 °C until further processing by radioimmunoassay.
    NOTE: Conduct all steps in polypropylene (RAPID processing) and borosilicate (radioimmunoassay) tubes that exhibit significantly lower surface binding properties and thus minimize peptide loss for most of the peptides studied before[26].

2. Measurements

NOTE: Steps in this section should be performed in a laboratory certified for work with radioactive material. Standard precautions for the work with ^{125}I should be taken.

1. Recovery of Radiolabeled Peptides
    1. Obtain ^{125}I-radiolabeled human peptides (*e.g.* acyl-ghrelin, GLP-1, glucagon, insulin, kisspeptin, leptin, nesfatin-1, PYY_{3-36} and somatostatin-28).
    2. Keep peptides in powder form until the experiment, then freshly dilute in 0.1% acetic acid (~100,000 cpm per ml).
    3. For standard blood processing, directly after blood withdrawal into chilled EDTA containing tubes, transfer 1 ml of blood into a tube with 50 µl of radiolabel containing 3,000-6,000 cpm (counted directly before the experiment starts).
    4. For RAPID processing, transfer 1 ml blood of EDTA containing blood into a tube containing 9 ml RAPID buffer (for composition see 1.3) and 500 µl radiolabel containing 30,000-60,000 cpm. Due to the 1:10 dilution use 10-times higher volume of radiolabel for RAPID processing.
    5. Afterwards, process samples as described in steps 1.2 to 1.3.2.
    6. For the recovery experiments, do not dry samples by vacuum centrifugation and do not store at -80 °C. Instead, assess recovery of radioactively labeled peptides directly afterwards using a gamma counter.
    7. Measure the whole supernatant in standard samples, while in RAPID samples analyze 1/10 of the total volume to obtain comparability of the amount of radiolabel used. For measurement, transfer the supernatant in tubes fitting into the gamma counter and assess the counts per minute
    8. As a 100% standard, use two samples with 50 µl of ^{125}I-radiolabeled peptide that do not undergo processing. Measure at the same time with other samples as described in 2.1.7.
    9. Perform the experiment five to six times for each peptide.

2. High Performance Liquid Chromatography of Radiolabeled Ghrelin
    1. Withdraw blood in chilled EDTA containing tubes and transfer 1 ml to tubes containing 200 µl radiolabeled-acyl ghrelin containing 15,000-20,000 cpm (counted directly before the experiment starts).
    2. For RAPID processing, transfer 1 ml blood into a tube containing 9 ml RAPID buffer (for composition see 1.3) and 200 µl radiolabeled acyl ghrelin containing 15,000-20,000 cpm.
    3. Afterwards, process samples as described in steps 1.2 to 1.3.2.
    4. For further analysis by reverse phase HPLC, directly load samples onto a stable bond C18 column (2.1 mm x 50 mm, 1.8 µm) equilibrated in 17% acetonitrile in water (both supplemented with 0.1% TFA).
    5. After 5 min equilibration, use a gradient from 17-40% acetonitrile to elute the sample in 40 min (flow rate of 1 ml/min).
    6. Collect fractions of 1 ml every minute and analyze radioactivity using a gamma counter.
    7. In a separate experiment, load 200 µl radiolabeled acyl ghrelin containing 15,000-20,000 cpm onto the column directly and perform HPLC as described in steps 2.2.4 to 2.2.6.

3. Radioimmunoassay
    1. For radioimmunoassay, thaw frozen supernatants (standard processing) and vacuum dried powder (RAPID method) at room temperature.
    2. Immediately before radioimmunoassay, re-suspend dry RAPID samples in double distilled H_2O according to the original volume of plasma (500 µl).
    3. Assess kisspeptin and total (including both desacyl and acyl ghrelin) as well as acyl ghrelin as described before[12,27] using commercial radioimmunoassays according to the manufacturers' protocols. Use borosilicate tubes that allow stable pellet formation.

1. On day one, incubate the samples with assay buffer and primary antibody (*e.g.* anti-ghrelin) in the dilution provided by the manufacturer for a period of 24 hr.
2. On day two, add the ^{125}I tracer (*e.g.* ^{125}I-ghrelin), vortex and incubate for a period of 24 hr.
3. On day three, add the precipitating reagent, vortex and incubate as recommended by the manufacturer. Then, centrifuge tubes at 3,000 x g for 20 min at 4 °C. Remove the supernatants and count radioactivity in the pellets using a gamma counter

4. Calculate desacyl ghrelin as the difference of total minus acyl ghrelin. Assess the acyl/desacyl ghrelin ratio by dividing acyl by desacyl ghrelin for each individual sample.
5. Process all samples – if possible – in one batch to avoid inter-assay variability. The intra-assay variability in the present experiment was <8% for kisspeptin, <7% for total and <9% for acyl ghrelin.

3. Statistical Analysis

1. Determine the distribution of the data using the Kolmogorov-Smirnov test. Express data as mean ± standard error of mean (SEM).
2. Assess differences between two groups by t-test. Assess differences between multiple groups by all pair-wise multiple comparison procedures (Tukey *post hoc* test) or two-way ANOVA followed by Holm-Sidak method.
3. Consider p <0.05 significant and perform analyses using a statistics program.

Representative Results

RAPID blood processing increases the yield of ^{125}I-radiolabeled peptides in human blood compared to standard blood processing.
After standard blood processing (EDTA blood on ice), the recovery of radiolabeled peptides was ~60% in 9/9 peptides (ranging from 48-68%, **Figure 1A**-**K**). RAPID processing improved the yield in all ^{125}I-labeled peptides, namely in somatostatin-28 (+39%, **Figure 1A**), glucagon-like peptide-1 (+35%, **Figure 1B**), acyl ghrelin (n-octanoyl ghrelin, +32%, **Figure 1C)**, glucagon (+32%, **Figure 1D**), insulin (+29%, **Figure 1E**), kisspeptin (+29%, **Figure 1F**), nesfatin-1 (+28%, **Figure 1G**), leptin (+21%, **Figure 1H**) and peptide YY_{3-36} (+19%, **Figure 1K**) compared to standard processing (p <0.001). In these 9 peptides, the recovery after the RAPID method was ~90% (ranging from 71-98%, **Figure 1A**-**K**).

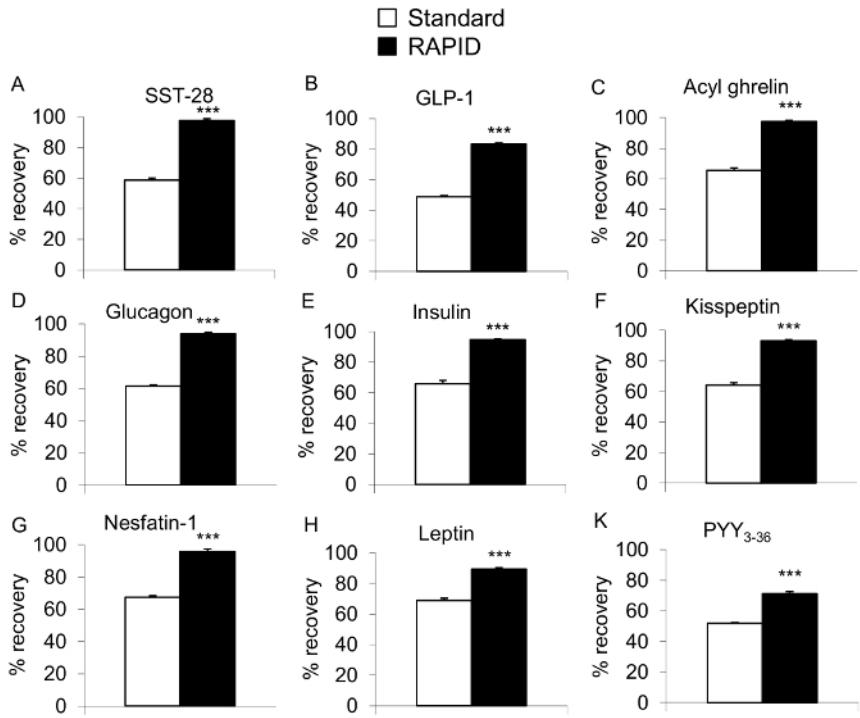


Figure 1: Recovery of ^{125}I-labeled Peptides in Human Blood following Standard or RAPID Blood Processing. Radiolabeled peptide was added to human blood and processed according to standard procedure (EDTA blood on ice) or the RAPID method (reduced temperatures, acidification, protease inhibition, isotopic exogenous controls and dilution). The supernatants were collected and counted for radioactivity. Each column represents the mean ± SEM of five to six experiments. $^{***}p$ <0.001 *vs.* standard processing. Please click here to view a larger version of this figure.

Following RAPID Processing, Ghrelin Elutes at the Expected Position.
After RAPID processing of human blood, ^{125}I-labeled ghrelin eluted at the expected position, while after standard procedure an earlier peak was observed most likely corresponding to desacyl ghrelin (**Figure 2**). This represented a 62% degradation of the peptide.

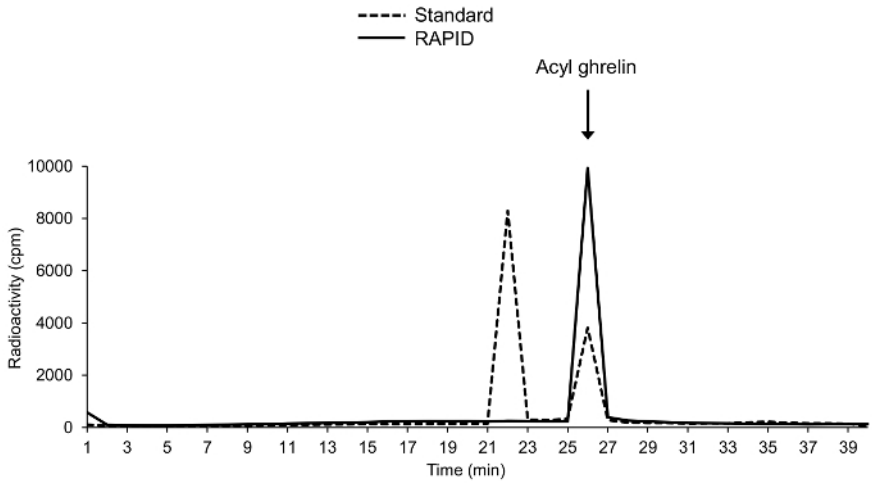


Figure 2: Elution Profile of ^{125}I-labeled Acyl Ghrelin in Human Blood following Standard or RAPID Blood Processing. Radiolabeled peptide was added to human blood and processed according to standard procedure or the RAPID method. Afterwards, the supernatant was loaded onto a high performance liquid chromatography column. The eluate was collected every minute and counted for radioactivity. After RAPID processing most of the peptide eluted at the expected time, while after standard processing a large proportion eluted earlier, most likely representing desacyl ghrelin. Abbreviation: cpm, counts per minute. Please click here to view a larger version of this figure.

RAPID Blood Processing Improves the Acyl/Desacyl Ghrelin Ratio and Results in Increased Endogenous Kisspeptin Blood Levels Compared to Standard Processing.

Blood was withdrawn from anorexic, normal weight and obese subjects and processed according to the standard or RAPID procedure. Endogenous ghrelin (total and acyl ghrelin) was assessed by radioimmunoassay. Anthropometric and comorbidities/medication of the study groups are shown in **Tables 3** and **4**. After RAPID processing the acyl/desacyl ghrelin ratio in blood of normal weight subjects was 1:3 compared to 1:23 following standard blood processing (p = 0.03; **Figure 3A**). Similar results were observed under anorexic (1:3 *vs.* 1:19, p <0.001) and obese conditions (1:3 *vs.* 1:13; p = 0.04; **Figure 3A**). No differences were observed between the three different groups for standard or RAPID processing (**Figure 3A**). Two way ANOVA indicated a significant influence of the processing method ($F_{1,64}$ = 15.3, p <0.001), while the body weight/metabolic condition had no effect ($F_{2,64}$ = 0.5, p = 0.62).

Parameter	Group		
	Anorexia nervosa (n = 15)	Normal weight (n = 12)	Obesity (n = 15)
Age (years)	26.20 ± 2.13*	35.33 ± 3.32	36.60 ± 2.11
Height (cm)	168 ± 1.32	167 ± 1.52	166 ± 1.29
Weight (kg)	40.53 ± 1.98***	61.01 ± 1.88	156.35 ± 9.45***
BMI (kg/m^2)	14.37 ± 0.56***	21.71 ± 0.72	53.00 ± 2.86***

All female. Data are expressed as mean ± SEM. *p<0.05 and ***p<0.001 *vs.* normal weight.

Table 3. Anthropometric characteristics of study groups.

Parameter	Anorexia nervosa (n = 15)	Normal weight (n = 12)	Obesity (n = 15)
Comorbidities			
Type 2 diabetes mellitus	0 (0.0%)	0 (0.0%)	5 (33.3%)
Impaired fasting glucose	n.a.	n.a.	0 (0.0%)
Insulin resistance with regular glucose control	n.a.	n.a.	4 (26.7%)
Arterial hypertension	0 (0.0%)	1 (8.3%)	8 (53.3%)
Hypercholesterinemia	5 (33.3%)	4 (33.3%)	5 (33.3%)
Hypertriglyceridemia	1 (6.7%)	0 (0.0%)	4 (26.7%)
Hyperuricemia	0 (0.0%)	0 (0.0%)	6 (40.0%)
Fatty liver disease	n.a.	n.a.	8 (53.3%)
Medication			
Oral antidiabetics	0 (0.0%)	0 (0.0%)	4 (26.7%)
Insulin	0 (0.0%)	0 (0.0%)	0 (0.0%)
Psychopharmacologicals	3 (20.0%)	4 (33.3%)	5 (33.3%)

All female. Values are given in total numbers (% in parentheses). Abbreviations: n.a., not assessed.

Table 4 Comorbidities and medication of the study groups.

Circulating endogenous kisspeptin levels were significantly higher following RAPID compared to standard processing in anorexic (+60%, p = 0.02) and normal weight subjects (+99%, p = 0.02), while the difference did not reach significance under conditions of obesity (23%, p = 0.39; **Figure 3B**). No significant differences were observed between the three different BMI groups (p >0.05; **Figure 3B**). Two way ANOVA indicated a significant influence of the processing method ($F_{1,74}$ = 10.8, p = 0.002), while the body weight/metabolic condition had no effect ($F_{2,74}$ = 0.5, p = 0.60).

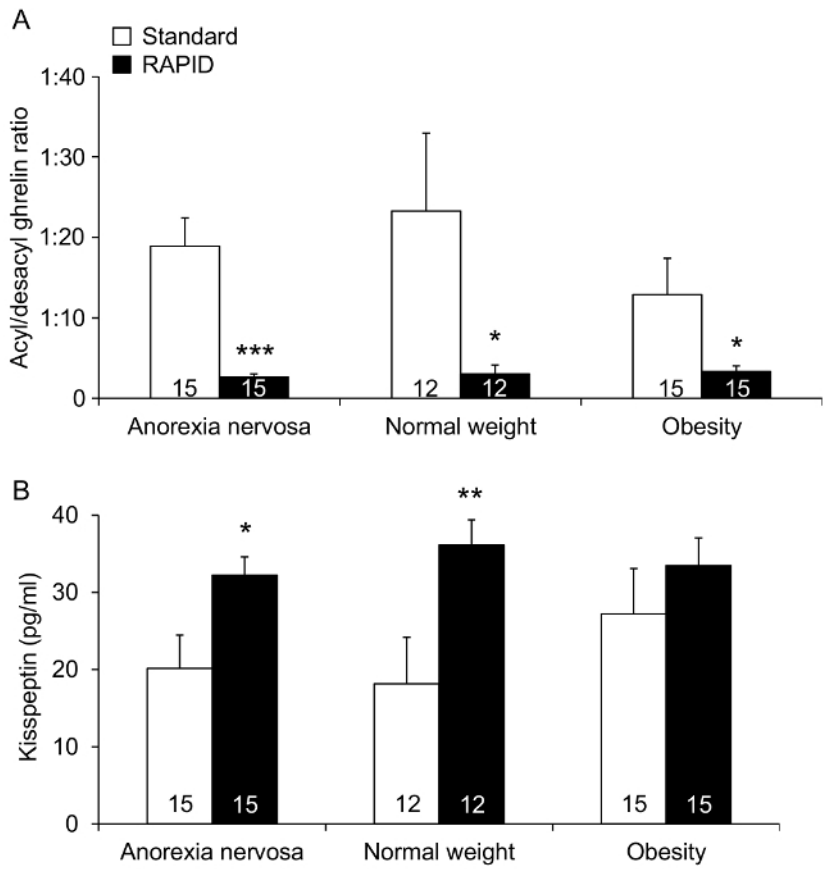


Figure 3: Circulating Acyl/Desacyl Ghrelin Ratio and Circulating Kisspeptin Levels under Different Metabolic Conditions following RAPID or Standard Blood Processing. Blood was withdrawn from anorexic, normal weight or obese subjects after an overnight fast and processed according to standard procedure or the RAPID method. The supernatant was collected and acyl as well as total ghrelin levels or kisspeptin levels (**B**) assessed by radioimmunoassay. Desacyl ghrelin was obtained by subtracting acyl from total ghrelin. The ratio was calculated by dividing acyl by desacyl ghrelin (**A**). The group size is indicated at the bottom of the columns. Data are expressed as mean ± SEM. *p <0.05, **p <0.01 and ***p <0.001 *vs.* standard processing. Please click here to view a larger version of this figure.

Discussion

We reported before that the RAPID method for blood processing improved the recovery for 11/12 peptides compared to standard blood processing in rats[6]. In the present study we have shown that this method is also suited for the use in humans. Following RAPID processing, the recovery for 9 of 9 ^{125}I-labeled peptides tested was improved compared to standard blood processing (EDTA blood on ice). The observed improvement ranged from 19-39% which is likely to be relevant, especially under conditions when only subtle differences are expected which might be masked when the yield of the peptide is low.

We also showed that after RAPID blood processing ghrelin is detected at the correct elution position after HPLC indicating the correct molecular form (acyl ghrelin), while after standard blood processing 2/3 are degraded to desacyl ghrelin. Since the acyl group is essential for binding to the ghrelin receptor[28], de-acylation is expected to greatly alter the biological function of the peptide. However, also desacyl ghrelin is increasingly recognized as a biologically active peptide[29]. Interestingly, desacyl ghrelin was suggested to counteract the effects of ghrelin as shown before for food intake[30] or colonic motility[31]. This further highlights the importance of investigating the correct molecular form.

When studying endogenous levels of kisspeptin in normal weight subjects, we showed greatly increased levels (+99%) following RAPID compared to standard blood processing. However, no improvement was observed under conditions of obesity, which may be due to the overall increased kisspeptin levels (+50% in obese *vs.* normal weight). Between BMI groups (anorexia nervosa, normal weight and obesity) in blood processed with either method, no significant differences were observed which may be due to the fact that only fasting levels were assessed. Differences might be apparent postprandially, a hypothesis that should be investigated in future studies.

Kisspeptin has been measured before in studies using only EDTA tubes on ice[32] (in the present study referred to as standard processing) or by the addition of protease inhibitors[33]. Based on the present study the chilling of the tubes is not sufficient to prevent peptide degradation. Whether acidification alone or single protease inhibitors are enough to preserve kisspeptin should be investigated in future studies.

Similarly, RAPID processing also increases the yield of endogenous acyl ghrelin as indicated by a greatly increased acyl/desacyl ghrelin ratio (1:3) compared to standard blood processing (1:23). This finding shows good concordance with rodent studies where we reported an acyl/desacyl ghrelin ratio of 1:5 following RAPID processing before (compared to 1:19 after standard processing). The broad ranges of the acyl/

desacyl ghrelin ratio of 1:15 to 1:55[34,35] reported before were therefore most likely due to a de-acylation-dependent high proportion of desacyl ghrelin. As described above for kisspeptin, no alterations of the acyl/desacyl ghrelin ratio were observed under different conditions of chronically altered body weight (anorexia nervosa *vs*. normal weight *vs*. obesity). This may also be due to the fact that only fasting levels of acyl/desacyl ghrelin were assessed and the postprandial regulation is more important. On the other hand this also indicates that despite the chronically altered body weight the proportion of acyl and desacyl ghrelin is not changed under basal conditions and may not play an important role in the adaptive changes observed under these conditions. However, future studies using standardized meal protocols and applying the RAPID method will help to further answer this question.

The RAPID method uses the following components: Since chemical reactions mostly depend on temperature[36] a reduction of temperature using an ice-cold solution slows down the degradation of the peptide. Next, also a decrease in pH (acidification) decreases the speed of the degradation[37]. Moreover, a reduction in pH decreases the adsorption of peptides to other surfaces and therefore also reduces the risk of peptide loss. The protease inhibitors used here were chosen in order to cover a broad spectrum of endogenous peptidases: diprotin A as an inhibitor of dipeptidyl peptidase IV, E-64-d as an inhibitor for thiol proteases (*e.g*. cathepsin B/H), antipain as an inhibitor for trypsin, papain and cathepsin A/B, leupeptin as an inhibitor for trypsin, plasmin, papain and cathepsin and chymostatin as an inhibitor for chymotrypsin, papain and cathepsin B/G. Moreover, isotopic peptides are useful in order to assess the improvement of recovery by the method. Lastly, as the rate of the enzyme-substrate complex formation depends on the concentration of the enzyme (peptidase) and the substrate (peptide hormone)[38] dilution of the plasma reduces the rate of formation and therefore degradation (a tenfold dilution reduces the rate a hundredfold according to the Michaelis-Menten kinetics).

Despite the advantages of the RAPID method, several limitations of the method should be acknowledged as well. The most critical step of the protocol is time. Blood samples should be diluted in the RAPID buffer immediately after blood withdrawal which may be difficult in the clinical setting. Moreover, the method is more time consuming, requires a higher level of training and is also more expensive than standard blood processing. Although it may be challenging to implement in clinical routine, it will be helpful for research purposes, especially when only subtle differences are expected and therefore a high yield of the peptide is desirable.

Although the RAPID method is described and recommended with all its components (Reduced temperatures, Acidification, Protease inhibition, Isotopic exogenous controls and Dilution) in the present paper, future studies may use modifications of this technique. The use of isotopic exogenous controls may not be mandatory and therefore restricted to pilot studies in order to assess which peptides show the greatest improvement in recovery using RAPID processing. Moreover, also the composition of the protease inhibitor cocktail may be modified when other peptides are assessed. However, this should be tested when a new peptide is measured.

In summary, the RAPID method of blood processing can be used for human blood and greatly improved the recovery of 9/9 peptides tested compared to standard blood processing. Since for some peptides only a small to moderate improvement of recovery was observed, the necessity of the method may have to be tested when a new peptide is analyzed. Moreover, the RAPID method allows for the detection of the correct molecular form as shown for acyl ghrelin and also considerably increases the yield of endogenous circulating levels of peptides such as kisspeptin compared to standard blood processing. Therefore, this method should be a useful tool in human studies assessing circulating peptide levels, *e.g*. those involved in the regulation of hunger and satiety.

Disclosures

The authors have nothing to disclose.

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