The RAPID Method for Blood Processing Yields New Insight in Plasma Concentrations and Molecular Forms of Circulating Gut Peptides

Andreas Stengel, David Keire, Miriam Goebel, Lena Evilevitch, Brian Wiggins, Yvette Taché, and Joseph R. Reeve, Jr.

CURE Digestive Diseases Research Center, University of California, Los Angeles, and Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, California 90073

The correct identification of circulating molecular forms and measurement of peptide levels in blood entails that the endocrine peptide being studied is stable and recovered in good yields during blood processing. However, it is not clear whether this is achieved in studies using standard blood processing. Therefore, we compared peptide concentration and form of 12 125I-labeled peptides using the standard procedure (EDTA-blood on ice) and a new method employing Reduced temperatures, Acidification, Protease inhibition, Isotopic exogenous controls, and Dilution (RAPID). During standard processing there was at least 80% loss for calcitonin-gene-related peptide and cholecystokinin-58 (CCK-58) and more than 35% loss for amylin, insulin, peptide YY forms (PYY(1–36) and PYY(3–36)), and somatostatin-28. In contrast, the RAPID method significantly improved the recovery for 11 of 12 peptides (P < 0.05) and eliminated the breakdown of endocrine peptides occurring after standard processing as reflected in radically changed molecular forms for CCK-58, gastrin-releasing peptide, somatostatin-28, and ghrelin. For endogenous ghrelin, this led to an acyl/total ghrelin ratio of 1:5 instead of 1:19 by the standard method. These results show that the RAPID method enables accurate assessment of circulating gut peptide concentrations and forms such as CCK-58, acylated ghrelin, and somatostatin-28. Therefore, the RAPID method represents an efficacious means to detect circulating variations in peptide concentrations and form relevant to the understanding of physiological function of endocrine peptides. (Endocrinology 150: 5113–5118, 2009)

Endocrine peptides have been studied for over 100 yr (1). Analytical methods were developed that could sequence linear peptides purified from bioassays that quickly led to the determination of the sequences of insulin, secretin, gastrin, and cholecystokinin (CCK) (2). The bioassays to purify these peptides were expensive and time-consuming, and peptide levels were often difficult to accurately measure.

The study of peptides took a gigantic leap forward when Berson and Yalow (3) developed a sensitive and specific detection method for blood and tissue extracts now known as RIA. For accurate determination of peptide concentration by RIA, the endocrine peptide being studied should be stable and recovered in good yields during blood processing. An often unstated hypothesis underlying reports on forms and concentrations of circulating peptides is that they are stable and well recovered during plasma formation.

However, we showed that both aspects of this hypothesis are false for CCK when plasma is formed (4). Several endocrine forms of CCK have been reported: CCK-58 (4, 5), CCK-33/39 (6), CCK-22 (7), and CCK-8 (8, 9). We hypothesized that this diversity of observations was due to differences in blood processing and not to actual differ-
enches of circulating forms. To test this, CCK-58 was radio
dilabeled to produce $[^{125}\text{I}]$CCK-58 and added to blood.
After plasma was formed as commonly done by others
when evaluating the molecular forms of CCK (6–9), the
recovery was determined by counting supernatant and pel-
let and the stability evaluated by the elution position of
radioactivity during HPLC. Surprisingly, during plasma
formation, most of $[^{125}\text{I}]$CCK-58 was associated with the
pellet and only 20% observed in the plasma, indicating
that recovery could be improved if methods were devel-
oped that prevented peptides from associating with
plasma. Furthermore, most of the radiolabeled peptide
present in the plasma eluted as several peaks in earlier
positions than the one observed for intact $[^{125}\text{I}]$CCK-58.
These data indicated that the differences in the observed
CCK forms in previous studies (4–9) may involve vari-
dous amounts of ex vivo degradation and are probably
not due to actual differences in circulating forms of
CCK.

The hypothesis that loss or degradation of endocrine
peptides can occur during plasma formation was tested for
twelve bioactive peptides: amylin, calcitonin, calcitonin-
genre-related peptide (CGRP), CCK, gastrin, ghrelin (Ghr),
glucagon-like peptide (GLP)-1, gastrin-releasing peptide
(GRP), insulin, peptide YY forms (PYY$_{(1–36)}$, PYY$_{(3–36)}$),
and somatostatin-28. Processing according to standard
plasma formation was compared with new processing
combining Reduced temperatures, Acidification, Protease
inhibition, Isotopic exogenous controls, and Dilution that
we termed the RAPID method.

Materials and Methods

Animals

Adult male Sprague Dawley rats (Harlan, San Diego, CA),
weighing 280–350 g, were housed four per cage under controlled
illumination (0600–1800 h) and temperature (21–23 C). Rats
had free access to rodent chow (Prolab RMH 2500; LabDiet,
PMI Nutrition, Brentwood, MO) and tap water. Protocols were
approved by the Animal Research Committee at Veterans Affairs
Greater Los Angeles Healthcare System (no. 99-059-04).

Radiolabeling CCK-58, PYY$_{(1–36)}$, or PYY$_{(3–36)}$

CCK-58, PYY$_{(1–36)}$, and PYY$_{(3–36)}$ were synthesized in the
University of California, Los Angeles, Peptide Synthesis Facility,
purified by reverse-phase HPLC and characterized as previously
described (5). All peptides had the correct mass, CCK-58 had a
purity greater than 90% and PYY peptides greater than 97%.
Ten micrograms of CCK-58, PYY$_{(1–36)}$, or PYY$_{(3–36)}$ were
dissolved in 20 μl PBS (pH 7.4). Radiolabel Na$^{125}$I (500 μCi) in 5
μl PBS was added to the peptide solution. Chloramine-T (10
μg/10 μl PBS) was added to the mixture. After 20 sec, the
reaction was quenched by adding an equal volume of 50% acetic
acid. The labeled peptide was separated from free $^{125}$I by G-10
gel-permeation chromatography. Tubes containing the first peak
of radioactivity from the G-10 column were pooled, diluted
3-fold with 0.1% trifluoroaceticate (TFA) and loaded onto a C-18
reverse-phase column. The $^{125}$I-labeled peptides were eluted
with a gradient of 22.5–37.5% acetonitrile. Other iodinated pep-
tides were purchased (Bachem, Inc., Torrance, CA). All radio-
active peptides eluted as a single major radioactive peak during
reverse-phase HPLC of the label before it was added to blood.
Calcitonin, CGRP, and GLP-1 needed to be purified to a single
radioactive peak before use in the experiments. The specific ac-
tivity was 500-2000 cpm/fmol peptide.

Recovery after standard and RAPID method for
blood processing

For each of the 12 peptides, blood from six naive rats was used
to compare recovery by the two processing methods. Rats were
anesthetized with pentobarbital (10 mg/kg, ip) between 0800
and 0900 h, and blood (10 ml) was collected by cardiac puncture
into EDTA-rinsed syringes, and 1-ml aliquots added into tubes
containing the respective radiolabeled analog (20,000–50,000
cpm in 10–20 μl 0.1% acetic acid). Thereafter, the two methods
were performed separately to compare recovery and forms.

For the standard method, blood was kept on ice (10 min
maximum) until centrifugation. The RAPID method used blood
diluted 1:10 in ice-cold buffer (pH 3.6) containing radiolabel, 0.1M
ammonium acetate, 0.5 M NaCl, and enzyme inhibitors diprotin
A, E-64-d, antipain, leupeptin, chymostatin (1 μg/ml; Peptide
International, Louisville, KY). All samples were centrifuged at
3000 × g for 10 min at 4 C.

The supernatants were collected, counted for radioactivity,
and frozen at −80 C for 24–72 h. The pellets were also counted
for radioactivity and then discarded. Frozen supernatants were
thawed at room temperature for 30 min, chromatographed by
Sep-Pak C18 cartridges (360 mg, 55–105 μm, product no.
WAT051910; Waters Corp., Milford, MA) and characterized by
reverse-phase HPLC.

Stepwise Sep-Pak chromatography of standard or
RAPID samples

Sep-Pak cartridges were charged with 2 ml 100% acetonitrile
and equilibrated with 4 ml 0.1% TFA. The equilibrated columns
were loaded with sample, rinsed with 5 ml 0.1% TFA, and eluted
with 70% acetonitrile containing 0.1% TFA. Fractions (2 ml)
were collected starting with sample loading. All fractions were
collected. Those containing radioactivity were pooled for
HPLC analysis of peptide stability. For RIA, samples were di-
luted, centrifuged, Sep-Pak chromatographed, and dried by vac-
cuum centrifugation. Peptide powder was stored at −80 C until
further processing (Fig. 1).

Reverse-phase HPLC evaluation of peptide stability
during standard and RAPID methods

For all 12 peptides, all radioactive fractions from the Sep-Pak
chromatography were pooled, diluted 1:3, and chromato-
graphed on an analytical Vydac C-18 HPLC column (10 μm,
4.6 × 250 mm; Western Analytical, Hesperia, CA) equilibrated
in 0.1% TFA. The diluted sample was injected in 7-ml aliquots
with a 10-ml injection loop. The sample was eluted with a 10 min
gradient to 20% acetonitrile, then 120 min gradient to 35%
acetonitrile at a flow rate of 1 ml/min. Fractions (2 ml) were
collected from the start of loading and counted to determine recovery of radioactivity. No radioactivity was observed before the 120-min gradient.

**RIA for acyl Ghr (A-Ghr) and total Ghr (T-Ghr)**

Rats received a jugular vein catheter and were allowed to recover for 4 days in previous studies (10). Blood (1 ml) was withdrawn from conscious lightly hand-restrained ad libitum-fed and 24-h fasted rats between 0800 and 0900 h and processed according to the standard or RAPID method. Samples were resuspended in ddH$_2$O before use. Plasma T- and A-Ghr was measured using separate RIA kits (Linco Research, St. Charles, MO).

**Data analysis**

Data are expressed as mean ± SD and were analyzed by ANOVA followed by Tukey’s post hoc test, and $P < 0.05$ was considered significant.

**Results**

The recovery of 12 radiolabeled peptides (supplemental Fig. 1, published as supplemental data on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org) was studied, selected for diversity in net ionic charge ($-6$ to $+6$), size (17-58 amino acids), end groups (NH$_3$ and pyroglutamyl amino termini and free acid and amide carboxyl termini), and posttranslational modification (acylation and sulfation). Only three peptides, [$^{125}$I]calcitonin, [$^{125}$I]gastrin, and [$^{125}$I]Ghr (T-Ghr), were recovered with higher than 75% yields after standard processing, but still significantly less compared with the RAPID method ($P < 0.05$, Fig. 2). Importantly, [$^{125}$I]CGRP and [$^{125}$I]CCK-58 showed a recovery in plasma of only 14 and 20%, respectively (Fig. 2). More than half of the peptides, namely amylin, CGRP, CCK-58, insulin, PYY(1–36), PYY(3–36), and somatostatin-28 were recovered at 60% yield or less, indicating a loss of 40% by standard processing (Fig. 2). Plasma acidification alone did not prevent the breakdown of CCK-58 (supplemental Fig. 2). The RAPID method improved the yields to 75% or above for all 12 endocrine peptides (Fig. 2).

During reverse-phase HPLC, degraded peptides elute earlier than the intact peptide. After standard processing, radiolabel eluted before intact label for CCK-58, Ghr, GRP, and somatostatin-28, indicating substantial degradation (>80% for three peptides) resulting in radically changed molecular forms (Fig. 3, A–D). The RAPID method eliminated this degradation (Fig. 3, A–D). No degradation was observed for the other eight peptides when blood was processed by either method.

Changes in total T-Ghr and A-Ghr plasma levels assessed after RAPID method showed fasting-induced increases of T-Ghr and A-Ghr compared with freely fed rats ($P < 0.001$; Fig. 4) with an A/T-Ghr ratio remaining 1:5 [A-Ghr to des-acyl Ghr (D-Ghr) ratio 1:4] under both met-
abolic conditions. Standard processing also showed a fasting-induced increase in T-Ghr and A-Ghr compared with ad libitum-fed rats \( (P < 0.05; \text{Fig. 4}) \); however, the A/T-Ghr ratio was 1:41 and differed from freely fed conditions, which was 1:19. T-Ghr did not differ between the two methods \( (P > 0.05, \text{Fig. 4A}) \). With the standard method, about 80% of the endogenous A-Ghr was degraded \( (\text{Fig. 4, B and C}) \), which was inhibited by the RAPID method \( (P < 0.001, \text{Fig. 4B}) \).

### Discussion

Our study provides converging evidence that standard blood processing can hinder accurate measurement of amylin, calcitonin, gastrin, Ghr, GRP, insulin, PYY\(^{(1-36)}\), PYY\(^{(3-36)}\), and somatostatin-28 and is unsuitable for estimating blood levels of CGRP and CCK-58. It also leads to misidentification of molecular forms of CCK-58, Ghr, GRP, and somatostatin-28 due to their ex vivo degradation. The improvement in determination of levels and molecular forms is likely to have a major impact on endocrine peptide physiology.

In clinical routines, and often in research, hematological analysis commonly uses blood collected in EDTA tubes to inhibit peptide degradation occurring via proteolytic enzymes with metal cofactors. For investigating plasma proteins and peptides, EDTA sampling and storage at 4°C is widely recommended \( (11) \). However, this procedure shows significantly inferior results for 11 of 12 peptides compared with the RAPID method. The rates of most chemical reactions strongly depend on temperature \( (12, 13) \) and pH \( (14, 15) \). Reducing pH also protonates aspartate, glutamate, histidine, and C-terminal carboxylic

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**FIG. 3.** Elution profile of radioactive peptides from reverse-phase HPLC after the standard (○) or RAPID (■) method for blood processing. Degraded peptides elute earlier than the intact peptide. The degradation seen in the plasma sample can be avoided by using the RAPID method for CCK-58 (A), A-Ghr (B), GRP (C), and somatostatin-28 (D). The arrows show the elution position of labeled peptides not added to blood and chromatographed separately. These elution positions correspond to the elution position of peptides added to blood and processed by the RAPID method. The right panel shows the percentage of degradation observed after the standard and the RAPID method, respectively. cpm, Counts per minute.

**FIG. 4.** Circulating A- and T-Ghr levels after the standard method (white bars) compared with the RAPID method (black bars). Blood was withdrawn from ad libitum-fed and 24-h fasted rats and processed according to the standard or the RAPID method, and plasma T-Ghr (A) and A-Ghr (B) levels were measured by RIA. Standard blood processing results in degradation of 75 and 87% of A-Ghr under ad libitum and fasting conditions, respectively \( (C) \). n.s., Not significant; *, \( P < 0.05 \); **, \( P < 0.001 \) vs. standard method; #, \( P < 0.05 \); ##, \( P < 0.001 \) vs. ad libitum feeding.
acids present in plasma (26, 27) or that the circulating form of bombesin-like immunoreactivity compared with untreated plasma samples. Moreover, our data show most of the GRP is degraded during standard processing, but no degradation occurred using the RAPID method. Therefore, acidification of blood may be required for accurate GRP measurements.

Proper blood processing will be critical to understand how PYY, GLP-1, CCK, Ghr, and GRP regulate food intake. This is even more important in light of the increasing prevalence of human obesity accompanied by increasing research in this field and concomitantly higher frequency of hunger and satiety peptide measurements. Because ex vivo degradation was shown for circulating peptides in human blood samples (25, 29, 30), the RAPID method may also have relevance to human blood processing.

The RAPID method is an important advance over the standard method because 1) it can improve recovery over 5-fold and 2) it yields correct identification of endocrine molecular peptide forms. The RAPID method should facilitate the study of various endocrine peptides not included here. Our results demonstrate that the RAPID method is essential for correct assessment of CGRP, CCK, Ghr, GRP, and somatostatin.

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Address all correspondence and requests for reprints to: Joseph Reeve, Jr., Ph.D., University of California and CURE Digestive Disease Research Center, Building 115, Room 117, Veterans Affairs Greater Los Angeles Healthcare System, 11301 Wilshire Boulevard, Los Angeles, California 90073. E-mail: J.Reeve@mednet.ucla.edu.

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