Stability of Pro-Gastrin-Releasing Peptide in Serum versus Plasma

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Key Words

Pro-gastrin-releasing peptide • Small cell lung cancer • Thrombin

Abstract

Background/Aims: Although serum assays for pro-gastrin-releasing peptide (ProGRP) assays have been commercially available in Japan for several years, the stability of ProGRP in serum and plasma has not been well documented. We investigated the stability of ProGRP in serum and plasma with fresh and stored (frozen) specimens, as well as the cause of the observed instability in serum. **Methods/Results:** ProGRP concentrations in fresh serum were decreased by 6–28% after room temperature storage for 2 h and by 8–32% after 2–8°C storage for 24 h. The average change in ProGRP concentrations in fresh plasma was within $\pm 10\%$ of baseline for more than 4 h at room temperature and for more than 24 h at 2–8°C. The incubation of a serine protease, thrombin (activated blood coagulation factor II), in a buffer solution containing ProGRP caused decreases in ProGRP concentrations. Following the addition of phenylmethylsulfonyl fluoride, a serine protease inhibitor, to serum, the serum stability for ProGRP was similar to that in plasma. ProGRP is significantly

more stable in plasma than in serum. We speculate that thrombin in serum is one of the factors that inactivate ProGRP in serum by proteolysis of the ProGRP antigen. **Conclusion:** The use of plasma samples for ProGRP may improve the clinical reliability of this marker by minimizing preanalytical changes in ProGRP concentrations.

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Introduction

Pro-gastrin-releasing peptide (ProGRP) is a biomarker of small cell lung cancer (SCLC). ProGRP is used to aid in the diagnosis and monitoring of SCLC. The relationship between increased GRP and SCLC was originally reported [1]; however, GRP was not used for clinical diagnosis due to its very poor stability in specimens. In 1994, an assay to detect ProGRP (residues 31–98) in serum specimens was developed to have a more stable molecule for clinical use that correlated to SCLC [2–4]. The clinical usefulness of the ProGRP 31–98 measurement has been widely reported for use in the diagnosis [2, 4, 5], the prediction of prognosis [6] and the monitoring of treatment in patients with SCLC [7–9]. Although the sta-

bility of ProGRP was of significantly longer duration than that of GRP, its stability was still worse than the stability of other clinically used cancer markers, such as carcinoembryonic antigen, carbohydrate antigen 19-9 [10, 11]. Furthermore, the cause of the ProGRP instability was not well defined.

The objectives of this study were (1) to evaluate the stability of ProGRP in serum and plasma with fresh and stored specimens, (2) to investigate the cause of ProGRP instability, and (3) to evaluate methods to increase the stability of ProGRP in blood specimens.

Materials and Methods

Materials

Synthetic ProGRP (residues 31–98) peptide obtained from Abbott Laboratories (Lake County, Ill., USA) was prepared with standard solid-phase peptide synthesis using 4× Fmoc amino acid on a 1-mmol scale, with extended cycle for coupling step, following the previously reported amino acid sequence [3]. The peptide was purified twice to obtain high-purity peptide (>98% purity). Recombinant ProGRP was obtained from Advanced Life Science Institute, Inc. (Wako, Japan). The antigen was prepared by the method reported [3]. Serum from a patient with SCLC containing high levels of ProGRP was obtained from ProMedDx, LLC (Norton, Mass., USA), as were the stored matched normal serum and plasma specimens from apparently healthy individuals. These specimens were stored frozen at –20°C or colder until use. The specimens were collected under an institutional review board-approved protocol. Phenylmethylsulfonyl fluoride (PMSF) and thrombin (bovine) were obtained from Sigma Aldrich (St. Louis, Mo., USA).

Measurement of ProGRP

Three different ProGRP immunoassay kits, Seramurabo ProGRP from Fuji-rebio (Tokyo, Japan), Imucheck ELSIA F-ProGRP from Sysmex (Kobe, Japan) and Architect ProGRP (in development) from Abbott Japan (Tokyo, Japan)/Abbott Laboratories were used to determine the ProGRP concentration. The Seramurabo ProGRP and the Imucheck ELSIA ProGRP assays use a monoclonal antibody capture and a polyclonal antibody for detection. The Architect ProGRP assay is an automated, 2-step sandwich chemiluminescent immunoassay that uses paramagnetic microparticles coated with anti-ProGRP (residues 31–98) monoclonal antibody and acridinium-labeled anti-ProGRP (residues 31–98) monoclonal antibody conjugate. The assay calibration range is from 0 to 5,000 pg/ml [12]. The minimum limit of detection of the assay determined as 2 standard deviations above the mean for a calibrator that is free of ProGRP is better than 2 pg/ml [12]. The functional sensitivity demonstrating a coefficient of variation of 20% is better than 5 pg/ml [12]. The correlation with the Seramurabo ProGRP on-market assay was excellent: y (Architect ProGRP) = $0.914 \times$ (Seramurabo ProGRP) – 1.490; $r = 0.994$ with 109 specimens.

Measurement of ProGRP Stability in Serum and Plasma Samples

200 pg/ml of the synthetic ProGRP (residues 31–98) peptide or recombinant ProGRP (residues 31–98) was added to matched serum, ethylenediaminetetraacetic acid (EDTA) plasma, lithium heparin plasma, sodium heparin plasma or citrate plasma. The spiked specimens were held from 0 to 7 days at 2–8°C or for 0–24 h at room temperature. ProGRP concentrations were determined with three diagnostic assay kits: Seramurabo ProGRP, Imucheck ELSIA F-ProGRP and Architect ProGRP.

Measurement of ProGRP Stability of Fresh Specimens

The matched serum and plasma specimens were obtained from volunteers at Abbott Laboratories under written informed consent with approval by the institutional review board. Vacuum blood collection tubes for serum (plain tube) or for EDTA plasma were used for specimen collection. The serum and plasma specimens were tested within 1 h of collection for baseline determinations. The specimens were held from 0 to 24 h at 2–8°C or for 0–4 h at room temperature. ProGRP concentrations were determined with Architect ProGRP assay.

Spike Recovery

60 pg/ml (donors 1–3) or 180 pg/ml (donors 4 and 5) of ProGRP from a high titer serum of a patient with SCLC was spiked into matched serum, EDTA plasma and heparin plasma. The ProGRP values were measured with the Architect ProGRP assay and then percent recovery was calculated by the following formula: % recovery = [(observed concentration of sample spiked ProGRP serum) – (concentration of the unspiked sample)]/ProGRP concentration added. Note that the calibrator buffer was added to the unspiked sample to maintain the same volume as the spiked sample.

ProGRP Stability with Addition of Thrombin or PMSF

Synthetic ProGRP (residues 31–98) antigen was added to these diluents: (1) 1% bovine serum albumin (BSA) and 2 mM calcium chloride in phosphate-buffered saline (PBS), (2) 1% BSA and 2 mM calcium chloride in PBS including thrombin (activation form of blood coagulation factor II, a serine protease), (3) a serum specimen, and (4) the matched EDTA plasma. The solutions were stored for 0, 3 and 24 h at room temperature. Synthetic ProGRP (residues 31–98) antigen was also added to (1) a serum specimen, to (2) the serum specimen including 1 mM PMSF, a serine protease inhibitor, and to (3) the matched EDTA plasma. The solutions were stored for 0 and 24 h at room temperature. The ProGRP concentrations were measured with the Architect ProGRP assay.

Results

A comparison of the ProGRP stability in matched sera and plasmas following storage at 2–8°C and at room temperature measured using the Architect ProGRP assay is presented in figure 1. The ProGRP concentrations decreased in serum by 33–60% after 2–8°C storage for 7 days and by 38–50% after room temperature storage for

Fig. 1. ProGRP stability in serum and plasma specimens stored at 2–8°C for 7 days (**a**) or at room temperature for 24 h (**b**) measured using the Architect ProGRP assay. Li heparin = lithium heparin; Na heparin = sodium heparin.

Fig. 2. ProGRP stability in serum and plasma specimens stored at 2–8°C for 7 days (**a**) or at room temperature for 24 h (**b**). ProGRP was determined with 3 assays: Seramurabo ProGRP (kit A), Imucheck ELSIA F-ProGRP (kit B) and Architect ProGRP (kit C).

24 h. The average remaining ProGRP concentrations were 103, 98, 103 and 90% in EDTA plasma, lithium heparin plasma, citrate plasma and sodium heparin plasma, respectively, after 2–8°C storage for 7 days, and 94, 84, 87 and 83% in EDTA plasma, lithium heparin plasma, citrate plasma and sodium heparin plasma, respectively, after room temperature storage for 24 h.

The time course of the ProGRP degradation using three immunoassay kits (Seramurabo ProGRP, Imucheck ELSIA F-ProGRP and Architect ProGRP) is presented in figure 2a for 2–8°C storage and in figure 2b for room

temperature storage using stored (frozen) matched serum or EDTA plasma. The ProGRP concentrations decreased by 7–10% after 1 day (24 h) and by 50–56% after 7 days in serum at 2–8°C storage. The ProGRP concentrations decreased by 0–2% after 1 day (24 h) and by 10–14% after 7 days in EDTA plasma at 2–8°C storage. The ProGRP concentrations decreased by 38–46% in serum, and by 8–9% in EDTA plasma after storage at room temperature for 24 h. There were no significant differences in the stability of ProGRP as measured among three immunoassay kits.

Fig. 3. a–f ProGRP stability at 2–8°C or room temperature storage in fresh serum and plasma specimens that were tested within 1 h from blood draw. Serum, 2–8°C (**a**); EDTA plasma, 2–8°C (**b**); serum, room temperature (**c**); EDTA plasma, room temperature (**d**); serum, room temperature storage by 30 min (**e**); EDTA plasma, room temperature storage up to 4 h (**f**). **g** Comparison of ProGRP stability in fresh serum (average of 10 donors) and stored frozen serum (average of 10 donors) at 2–8°C. ProGRP concentrations were measured with the Architect ProGRP assay.

Fig. 4. Comparison of ProGRP stability for the diluent, the diluent including thrombin (activation form of blood coagulation factor II, a serine protease), serum and EDTA plasma (a), and serum, serum with PMSF added and EDTA plasma (b) at room temperature using the Architect ProGRP assay.

Table 1. Recovery of ProGRP in serum and plasma

	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Average
Serum	106	111	100	106	101	105
EDTA plasma	106	105	99	105	106	104
Heparin plasma	108	105	101	106	106	105

Data are percentages.

The time course of the ProGRP concentration decrease in freshly drawn matched serum and plasma specimens was also evaluated. ProGRP was tested within 1 h from blood draw, and stabilities up to 24 h at 2–8°C storage are presented in figures 3a and b and stabilities up to 2 h at room temperature storage in figures 3c and d. The average remaining concentration of ProGRP was 82% in serum after 24 h of storage at 2–8°C. The average remaining concentration of ProGRP was more than 90% in EDTA plasma after 24 h of storage at 2–8°C. The degradation rate in serum was more variable than in plasma. The remaining percentage of baseline ProGRP in fresh serum ranged from 72 to 94% after room temperature storage for 2 h and from 68 to 92% after 2–8°C storage for 24 h. The remaining percentage of baseline ProGRP in fresh plasma ranged from 96 to 100% after room temperature storage for 2 h and from 87 to 99% after 2–8°C storage for 24 h. Figure 3e presents the time course of the change in ProGRP concentration every 30 min with fresh serum specimens stored at room temperature. Figure 3f presents the time course of the ProGRP concentration for

up to 4 h with fresh EDTA plasma specimens at room temperature. The baseline ProGRP values for the specimens presented in figure 3a–d averaged 26% higher in EDTA plasma than the value in serum when testing freshly drawn specimens. The decrease in ProGRP level is faster in freshly drawn serum than in previously stored (frozen) samples as shown in figure 3g.

The calculated recovery in the Architect ProGRP assay after spiking a high-titer ProGRP specimen into matched serum, EDTA plasma and heparin plasma specimens is presented in table 1. The average recovery was 105, 104 and 105% with serum, EDTA plasma and heparin plasma, respectively. No significant differences in recovery between serum and plasma were observed.

A comparison of the stability of ProGRP in 1% BSA solution with and without the addition of thrombin (activated blood coagulation factor II, a serine protease) measured using the Architect ProGRP assay is presented in figure 4a. Significant degradation of ProGRP was observed in the BSA solution with thrombin as compared with the control. The effect of adding PMSF, a serine pro-

tease inhibitor, to serum on ProGRP is shown in figure 4b. The addition of PMSF to serum improved the stability of ProGRP in serum. The percentage of baseline ProGRP remaining after 24 h improved from 51% in the control to 87% after addition of PMSF.

Discussion

Stability of ProGRP in Serum

We investigated the stability of ProGRP in serum, which is the recommended sample type of the existing ProGRP assays, and also the stability in plasma. Since ProGRP stability was much better in plasma than in serum, we investigated the cause of ProGRP instability in serum.

ProGRP concentrations decreased by more than 10% in serum specimens when stored at 2–8°C for more than 24 h using previously stored (frozen) specimens. Although the Seramurabo ProGRP and the Imucheck ELSIA F-ProGRP assays use a monoclonal-polyclonal format and the Architect ProGRP assay uses a monoclonal-monoclonal format, there were no significant differences in stability among the 3 immunoassay kits. ProGRP concentrations decreased more rapidly when freshly drawn specimens were assayed. The freshly drawn samples might have a higher proportion of intact ProGRP than the stored samples, which have already undergone some degradation of ProGRP during their initial handling. Also, the fresh samples might have higher activities of the enzyme that degrades ProGRP than the stored samples. The average ProGRP level in freshly drawn serum was within 10% of the baseline level for up to 4 h after storage at 2–8°C and for up to 1 h after storage at room temperature. The rate of degradation of ProGRP was not consistent among individuals. The variation in change by individual specimens increased as storage time increased. The percentage of baseline of ProGRP ranged from 68 to 92% for 24 h storage at 2–8°C with fresh specimens. Serum specimens should not be stored for over 4 h at 2–8°C or for over 1 h at room temperature. To minimize variation in the degradation by individual specimens, serum specimens should be tested as soon as possible after the serum is separated from blood.

Since ProGRP was stable in a non-serum-based diluent (fig. 4), the cause of the instability of ProGRP in serum was due to substances in serum. The addition of PMSF, a serine protease inhibitor, improved the poor stability of ProGRP in serum. The addition of thrombin, a serine protease, caused the loss of ProGRP stability in the

diluent. These results indicated that the activity of the activated blood coagulation factor, thrombin, which is generated during the clotting process, is a probable cause for the instability of ProGRP in serum.

Stability of ProGRP in Plasma

The stability of ProGRP was significantly improved by the use of plasma specimens. The magnitude of this effect was equivalent with all three of the immunoassays tested: Seramurabo ProGRP, Imucheck ELSIA F-ProGRP and Architect ProGRP. The decrease in ProGRP concentration was less than 10% following storage for 3–7 days at 2–8°C and for 24 h at room temperature with previously stored specimens. The decrease in ProGRP concentration was less than 10% after 24 h of storage at 2–8°C and for at least 4 h of storage at room temperature for fresh specimens. The variation in the ProGRP concentration decrease among individuals was smaller in plasma than in serum. When fresh specimens are used for the assay, plasma can be used for up to 24 h at 2–8°C and up to at least 4 h at room temperature.

The baseline ProGRP concentrations for the specimens presented in figure 3a–d averaged 26% higher in EDTA plasma (average ProGRP concentration of 46.0 pg/ml) than the concentrations in serum (36.3 pg/ml) when testing freshly drawn specimens. Since the spiked recovery with serum and plasma matrices was equivalent and accurate (table 1), the difference is probably caused by the degradation of ProGRP during the clotting process. The cutoff value used for plasma ProGRP and the normal reference range will be higher than those currently used for serum.

In conclusion, ProGRP is more stable in plasma than in serum. Since ProGRP concentrations in SCLC patients are frequently very high when compared with the cutoff level [2, 4, 5], this marker has shown strong clinical utility even in serum samples. However, the use of plasma samples for the clinical evaluation of ProGRP concentrations would result in more accurate, reproducible and robust results than when using serum and should improve the clinical reliability of this marker in the evaluation of patients with SCLC. It is recommended to begin using plasma to measure ProGRP in the future, but while additional clinical information about the use of plasma ProGRP is being generated, these results highlight the need for careful control during the handling of serum samples. These specimen matrix stability differences for ProGRP highlight the importance of understanding the preanalytical conditions affecting tumor markers to obtain the greatest clinical reliability.

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