Solid-phase extraction treatment is required for measurement of active glucagon-like peptide-1 by enzyme-linked immunosorbent assay kit affected by heterophilic antibodies

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Keywords
Active glucagon-like peptide-1, Immunoassay, Solid phase extraction

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J Diabetes Investig 2019; 10: 302–308
doi: 10.1111/jdi.12896

INTRODUCTION
Incretin hormones, namely, glucose-dependent insulinitropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), are secreted from enteroendocrine cells on intake of nutrients and enhance insulin secretion in response to glucose⁴⁻³. Among the incretins, GLP-1 shows an insulin secretion enhancing effect even in the diabetic state, and has inhibitory actions on glucagon concentrations⁴. After it is secreted from intestinal enteroendocrine cells as an active peptide, GLP-1 has a very short half-life, as is rapidly inactivated by dipeptidyl peptidase-4, which is present in the intestinal tract and blood⁵. Recently, dipeptidyl peptidase-4 inhibitors, which increase the concentration of active incretin in the blood, were developed and are widely used clinically⁶. In this context, the measurement of total incretin in the blood, including active and inactivated forms, is important for evaluating incretin secretion from enteroendocrine cells after nutrient ingestion. In contrast, to evaluate the incretin effect, it seems to be more useful to measure active incretin concentrations. Therefore, it is important to measure active GLP-1 when evaluating the effect of incretin-based drugs.

Previous reports showed differences in active GLP-1 concentrations in the human blood, suggesting the presence of substances that might affect the value based on the measurement system used⁷,⁸. Subsequently, solid phase extraction (SPE) or ethanol extraction was recommended to eliminate this interference⁹⁻¹¹. Furthermore, the active GLP-1 values in the literature are comparable after extraction pretreatment¹²⁻¹⁴. In contrast, SPE is time-consuming and expensive, which makes measuring...
active GLP-1 less desirable. Few reports have evaluated SPE in terms of its influence on interfering substances.

In the present study, we confirmed that active GLP-1 values in the plasma affected by interfering substances differ widely among individuals. Under different pretreatment conditions, we showed that these differences can be eliminated by SPE or by absorption of heterophilic antibodies. We therefore propose that these pretreatments are not required if using an immunoassay kit that is not affected greatly by heterophilic antibodies.

**METHODS**

**Selection of extraction column and elution solvent**

Iodine-labeled active GLP-1; that is, [125-I]GLP-1 (7-36 amide; catalog no. H-6804; Peninsular Laboratories, LLC, Bachem Group, San Carlos, California, USA) with non-labeled GLP-1 (7-36 amide; catalog no. 4344-S; Peptide Institute, Inc., Osaka, Japan) adjusted to 20,000–30,000 c.p.m. was added to three types of extraction columns to select the appropriate column for SPE: (i) a C18 column (VARIAN Bond elute 200 mg; Agilent Technologies Japan Ltd., Tokyo, Japan); (ii) a C18 particle column (C18 125A; Wako, Osaka, Japan); and (iii) a silica particle column (Silica GelODS-Q3; Waters Corporation, Milford, Massachusetts, USA). The radioactivity of each sample eluted using the same eluent, namely, 0.5% ammonia (v/v) and 75% ethanol (v/v), from the three columns was measured using a gamma counter (1470WIZARD Gamma Counter; Perkin Elmer Co., Massachusetts, USA). The study was designed in compliance with the considerations of the Helsinki Declaration and ethics committee affiliated with Kyoto University (registry no. C362). All participants provided informed consent.

**Measurement of active GLP-1**

Active GLP-1 was measured using two active GLP-1 enzyme-linked immunosorbent assay (ELISA) kits: (i) the Millipore ELISA kit (catalog no. EGLP-35K; Millipore, Billerica, Massachusetts, USA) that has 100% cross-reactivity with GLP-1 (7–36) amide, 99.5% cross-reactivity with GLP-1 (7–37), 0.2% cross-reactivity with GLP-1 (1–36) amide and 0.2% cross-reactivity with GLP-1 (1–37), and does not detect GLP-1 (9–36) amide and GLP-1 (9–39), intra-assay coefficient of variation (CV) 6–9%, interassay CV <1–13%, and percentage recovery in human plasma 78–94%; and (b) the IBL ELISA kit (catalog no. 27784; IBL, Gunma, Japan) that recognizes GLP-1 isoforms, has 0.04% cross-reactivity with GLP-1 (1–37), 100% cross-reactivity with GLP-1 (7–37) and GLP-1 (7–36) amide, and 0.02% cross-reactivity with GLP-1 (9–36) amide, and does not detect GLP-1 (9–39), intra-assay CV 4.5–5.5%; interassay CV 7.1–10.9%, and percentage recovery in human plasma 82.0–88.4%.

**Comparison SPE and heterophilic antibody blocking**

To investigate the effect of removing the heterophilic antibody, heterophilic blocking tubes (HBT; catalog no. 3IX762; Scantibodies Laboratory Inc., Santee, California, USA) were used. To compare the effects of SPE and HBT, sample pretreatments were carried out as follows: both pretreatments (SPE[+]+HBT[+]), SPE alone (SPE[+]+HBT[−]), HBT alone (SPE[−]+HBT[+]) and no pretreatment (SPE[−]+HBT[−]). In each case, active GLP-1 was measured twice using each ELISA kit (Millipore ELISA kit and IBL ELISA kit). Thus, from the combinations of four pretreatments and two ELISA kits, eight different values were obtained for each sample (Figure 1).

**Figure 1** Comparison study of solid phase extraction and heterophilic antibody blocking. In each pretreatment condition, two kinds of active glucagon-like peptide-1 enzyme-linked immunosorbent assay (ELISA) kits were used. (1), Millipore ELISA Kit; (2), IBL ELISA kit. HBT, heterophilic blocking tube.
Statistical analysis
Pearson’s correlation test was used to test for correlations between the values. A $P$-value of $<0.05$ was considered statistically significant.

RESULTS
SPE and value of plasma active GLP-1 concentration
We examined differences in the values of the active GLP-1 concentration in the plasma obtained using the Millipore ELISA kit with or without SPE. Figure 2 shows a comparison of values obtained for the active GLP-1 concentration in the plasma of each individual (Figure 2). Although the values after the extraction were generally lower compared with those without extraction, the difference was large between individuals, and the ratio of the measured value after extraction to that before extraction ranged widely from 0.27 to 1.19.

HBT pretreatment eliminates the difference of active GLP-1 values by SPE
The Millipore ELISA kit includes a mouse monoclonal antibody, and examined the effect of HBT and ELISA kits using antibodies other than mouse monoclonal antibody. Table 1 shows the correlation of active GLP-1 values measured with the two types of ELISA kits (Millipore and IBL) under various pretreatment conditions. Using the Millipore ELISA kit, active GLP-1 values after SPE correlated significantly with those without extraction ($r = 0.594, P < 0.0001$; Figure 3a). A similar correlation was observed between active GLP-1 values with and without HBT pretreatment ($r = 0.628, P < 0.0001$; Figure 3b).

It should be noted that the correlation in the cross-study of the pretreatments (SPE and HBT) was greatly increased between SPE(+)/HBT(−) and SPE(−)/HBT(+) ($r = 0.940, P < 0.0001$; Figure 3c).

ELISA kit that did not use a mouse monoclonal antibody eliminates the differences of active GLP-1 values by SPE
In comparison among measurements with the IBL ELISA kit, which includes rabbit immunoglobulin G as the capture antibody, the correlations between active GLP-1 values with and without the use of pretreatments increased dramatically; that is, SPE(+) vs SPE(−) ($r = 0.911, P < 0.0001$), HBT(+) vs HBT(−) ($r = 0.980, P < 0.0001$; Figure 4a,b). Similarly to the results obtained using the Millipore ELISA kit, the cross-study of two pretreatments with the IBL ELISA kit showed a good correlation between SPE(+)/HBT(−) vs SPE(−)/HBT(+) ($r = 0.898, P < 0.0001$; Figure 4c).

DISCUSSION
In the present study, we confirmed the following: (i) by comparing extraction carriers and extraction solutions, we established an SPE method for measuring active GLP-1 concentrations in the plasma; and (ii) active GLP-1 values are underestimated because of the influence of interfering substances, and SPE pretreatment eliminates this interference. Additionally, pretreatment produces a large difference in active GLP-1 values; and, (iii) by comparing values obtained by various combinations of pretreatments and ELISA kits, we showed that pretreatment is not required when using an ELISA kit that is not greatly affected by heterophilic antibodies. The reproducibility...
of these two kits in the present study is important as one of prerequisites for these observations. Considering the measured values of active GLP-1 in this study, the performance of these kits can be considered to adequately withstand comparison of measurement values between various conditions. Moreover, the use of an ELISA kit that is not greatly affected by heterophilic antibodies reduces the apparent difference between active GLP-1 values with or without pre-treatments. A relatively higher value of active GLP-1 was detected, which appeared to be due to interfering substances, and this was eliminated in the present study by SPE. This finding confirms that a pretreatment, such as SPE, to remove interfering substances is necessary for reliable measurement of active GLP-1.

Table 1 | Correlation of active glucagon-like peptide-1 values measured with the two types of enzyme-linked immunosorbent assay kits (Millipore and IBL) under various pretreatment conditions

| Kit          | Extraction (+) | Extraction (-) | IBL kit   |
|--------------|----------------|---------------|-----------|
|              | HBT (+)        | HBT (-)       | HBT (+)   | HBT (-) |
| Millipore kit| 1.000          | 0.991         | 0.591     | 0.531   |
|              | (P < 0.0001)   | (P < 0.0001)  | (P < 0.0001) | (P < 0.0001) |
|              | 1.000          | 0.501         | 0.91      | 0.498   |
|              | (P < 0.0001)   | (P < 0.0001)  | (P < 0.0001) | (P < 0.0001) |
|              | 0.941          | 1.000         | 0.643     | 0.606   |
|              | (P < 0.0001)   | (P < 0.0001)  | (P < 0.0001) | (P < 0.0001) |
|              | 1.000          | 0.628         | 0.661     | 0.628   |
|              | (P < 0.0001)   | (P < 0.0001)  | (P < 0.0001) | (P < 0.0001) |
|              | 1.000          | 0.373         | 0.373     | 0.414   |
|              | (P < 0.0001)   | (P < 0.0001)  | (P < 0.0001) | (P < 0.0001) |
|              | 0.503          | 0.630         | 0.373     | 0.414   |
|              | (P < 0.0001)   | (P < 0.0001)  | (P < 0.0001) | (P < 0.0001) |
|              | 0.506          | 0.628         | 0.373     | 0.414   |
|              | (P < 0.0001)   | (P < 0.0001)  | (P < 0.0001) | (P < 0.0001) |

SPE, solid phase extraction; HBT, heterophilic blocking tube.
without SPE and HBT treatment. This suggests that heterophilic antibodies, especially human anti-mouse antibodies (HAMA), are responsible, at least in part, for the interference. In the Millipore ELISA kit, where mouse monoclonal antibody is used as a capture antibody, the measured values are affected depending on the HAMA in each individual. In contrast, when we used the IBL kit, in which a rabbit polyclonal antibody is used as the capture antibody, active GLP-1 values were not affected by HAMA. Based on these characteristics of the two ELISA kits used in the present study, a difference in the active GLP-1 values for each individual was observed with the Millipore kit, but not with the IBL kit, with and without HBT pretreatment. Therefore, HAMA is considered to be a strong candidate as a responsible interfering substance. Furthermore, active GLP-1 measurement values obtained with HBT pretreatment, which results in the removal of mouse immunoglobulin G, mouse serum, non-specific monoclonal antibodies and agglutinated immunoglobulin G, were similar to those with SPE pretreatment. This finding supports the possibility that the interfering substance is a heterophilic antibody, such as HAMA.

HAMA is a human antibody that recognizes mouse antibodies as an antigen and has long been known to be an interfering substance that affects measurement values in assay systems that use immunological techniques. In the thyroid-stimulating hormone immunoassays in which the mouse monoclonal antibody is used, it is well known that the measurement value is affected by HAMA. The mechanism that elicits pseudo high values is not different from the active GLP-1 immunoassay. However, the clinical problem frequency in thyroid-stimulating hormone measurement is quite low. One of the reasons is that the balance between plasma HAMA concentration and the amount of the antigen (hormone) to be measured is related to the interferences with the measurement value. The amount of active GLP-1 that we try to see is very small, therefore, interference by HAMA with the measurement system could be particularly conspicuous. HAMA production is induced by various stimuli, including administration of mouse-derived vaccines, which was a common practice when purification techniques were insufficient. HAMA is also produced after administration of molecular targeted drugs. In the present study, we found a difference in measured values in many individuals, possibly due to heterophilic antibodies, even without any history of administration of molecular targeted drugs. It is difficult to prove that the interfering substance is HAMA, as it is necessary to determine the epitope of the interfering substance and to show that it is a common antigen in the measurement system. Nevertheless, HAMA is a strong candidate.

Notably, the results of the present study showed that the difference in the measured values before and after pretreatment for removal of interfering substances, carried out for the measurement of activated GLP-1, were dramatically reduced by using an ELISA kit that is not greatly affected by heterophilic antibodies. This means that by using an ELISA kit that is not greatly affected by heterophilic antibodies, plasma active GLP-1 values can be measured more reliably, without requiring pretreatment, such as SPE or HBT. SPE requires extensive equipment, is time-consuming and expensive. In addition, SPE removes the impurities non-specifically, which can be a disadvantage from the viewpoint of measurement stability. Taking these points into consideration, this finding is very important.

The present study had the following limitations. First, comparison of plasma active GLP-1 values was carried out using two ELISA kits, but the specificity of the active GLP-1 measurement of these kits themselves was not investigated. We also did not clarify which kit provides a more accurate measurement.
The purpose of the present study, however, was to evaluate the SPE method and to identify substances that affect the measurement. Therefore, if the reproducibility in each kit is stable, the findings remain valid despite this limitation. Second, the samples were derived from a single facility. Further investigations with a larger number of participants from multiple facilities are required.

In studies of the physiology of glucose metabolism and the pathology of diabetes, there are many issues to be clarified regarding the secretion and actions of incretins. Stable, easy and inexpensive measurements of active GLP-1 should be useful for studies of incretins.

ACKNOWLEDGMENTS
This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology (MEXT); the Japan Society for the Promotion of Science (JSPS); Ministry of Health, Labor and Welfare; the Ministry of Agriculture, Forestry, and Fisheries; and the Japan Diabetes Foundation.

DISCLOSURE
Nobuya Inagaki receives fees from Takeda Pharmaceutical Co., Ltd., Mitsubishi Tanabe Pharma Corporation, Merck Sharp & Dohme (MSD), Sanofi, Novartis Pharma, Dainippon Sumitomo Pharma, Kyowa Hakko Kirin Co., Ltd., Eli Lilly Japan, Shiratori Pharmaceutical, Roche Diagnostics, Japan Tobacco (JT), Nippon Boehringer Ingelheim Co., Ltd., Astellas Pharma Inc., Daiichi Sankyo Company, Ltd., Ono Pharmaceutical Co., Ltd. and Taisho Toyama Pharmaceutical Co., Ltd. outside the submitted work. Tomonori Hasegawa and Miho Komagata are employees of SRL Inc. The other authors declare no conflict of interest.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** | Procedure of solid phase extraction. A C18 column was used as the extraction column. Samples were dissolved in the assay buffer for each enzyme-linked immunosorbent assay kit.

**Table S1** | Comparison of relative ratios of active glucagon-like peptide-1 values after treatment with extraction carriers.

**Table S2** | Extraction rates of iodine-labeled active glucagon-like peptide-1 when using various extract solutions.