Original Article

Estimation of Lactose Interference in Vaccines and a Proposal of Methodological Adjustment of Total Protein Determination by the Lowry Method

Hideki Kusunoki, Kazu Okuma, and Isao Hamaguchi*

*Department of Safety Research on Blood and Biological Products,
National Institute of Infectious Diseases, Tokyo 208-0011, Japan*

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SUMMARY: For national regulatory testing in Japan, the Lowry method is used for the determination of total protein content in vaccines. However, many substances are known to interfere with the Lowry method, rendering accurate estimation of protein content difficult. To accurately determine the total protein content in vaccines, it is necessary to identify the major interfering substances and improve the methodology for removing such substances. This study examined the effects of high levels of lactose with low levels of protein in freeze-dried, cell culture-derived Japanese encephalitis vaccine (inactivated). Lactose was selected because it is a reducing sugar that is expected to interfere with the Lowry method. Our results revealed that concentrations of ≥ 0.1 mg/mL lactose interfered with the Lowry assays and resulted in overestimation of the protein content in a lactose concentration-dependent manner. On the other hand, our results demonstrated that it is important for the residual volume to be ≤ 0.05 mL after trichloroacetic acid precipitation in order to avoid the effects of lactose. Thus, the method presented here is useful for accurate protein determination by the Lowry method, even when it is used for determining low levels of protein in vaccines containing interfering substances. In this study, we have reported a methodological adjustment that allows accurate estimation of protein content for national regulatory testing, when the vaccine contains interfering substances.

INTRODUCTION

The Lowry method is widely used for protein quantitation in biological samples, from protein assays in laboratories to national regulatory testing of biological products, such as vaccines (1). In general, this method is sensitive, detecting protein content in the approximate range of 5–100 $\mu\text{g/mL}$ (2). However, the Lowry method possesses a significant disadvantage; the assay is known to be affected by many interfering substances (3,4) such as some amino acid derivatives, certain buffers, lipids, reducing reagents (5), detergents, and thiomersal (also known as thimerosal) (6). Several modified Lowry methods, including a sodium deoxycholate (DOC)-trichloroacetic acid (TCA) precipitation method (3,7), a heated TCA precipitation method, and a sodium dodecyl sulfate method (8,9), have been devised to reduce the effects of interfering substances.

In national regulatory testing in Japan, the general test procedure for Lowry protein determination and the upper limit of allowable total protein content are defined by the Minimum Requirements for Biological Products (MRBP) (10). Currently, the Lowry method is mainly applied to the estimation of total protein of influenza HA and freeze-dried Japanese encephalitis (JE)

vaccines. A heated TCA precipitation step, defined by the MRBP in Japan, is utilized to suppress the effects of interfering substances (10).

In Japan, freeze-dried, cell culture-derived JE vaccines (inactivated) developed by two Japanese manufacturers are utilized for JE prevention (11–13). JE vaccines are prepared by growing the JE virus (Beijing strain) in Vero cells (derived from African green monkey kidney cells) and then inactivating the resulting virus with formalin. As described by the manufacturers, both vaccines contain a small amount of protein as the immunogen (approximately 5 or 8 $\mu\text{g/mL}$) and a large amount of lactose as an excipient (36 or 50 mg/mL).

As lactose is a reducing sugar and because many reducing reagents have already been shown to interfere with the Lowry assay, it is predicted that high lactose concentrations in JE vaccines may interfere with protein quantification by the Lowry method (5). In fact, it has been reported that lactose in the aluminum hydroxide-adsorbed *Neisseria meningitidis* serotype 2b protein-group B polysaccharide vaccine interferes with the Lowry assays (14). The vaccine's protein content was assessed by a modified Lowry method using a TCA precipitation step and, although the TCA precipitation was probably useful for avoiding lactose interference, detailed information was not provided (14). Thus, it remains unclear whether such a modified Lowry method is in fact useful and suitable for total protein determination in JE vaccines.

Accurate estimation of total protein is essential for quality control via national regulatory testing in order to ensure both safety and effectiveness of vaccine

Corresponding author: Mailing address: Department of Safety Research on Blood and Biological Products, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan. Tel: +81-42-848-7120, Fax: +81-42-567-2790, E-mail: 130hama@nih.go.jp

products. To achieve the above goals, we examined lactose interference in the Lowry method and proposed a methodological adjustment to overcome this interference.

MATERIALS AND METHODS

Chemicals: All chemical reagents for the Lowry method were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Bovine serum albumin (BSA) was obtained from the National Institute of Infectious Diseases (Tokyo, Japan). Alkaline copper solution was freshly prepared by adding 0.5 mL of 4% (w/v) disodium tartrate solution and 2% (w/v) copper sulfate solution to 50 mL of alkaline solution. Folin-Ciocalteu solution was diluted 1:11 (v/v) with Milli-Q purified water. Bradford reagent was purchased from Sigma-Aldrich (St. Louis, Mo., USA). Freeze-dried, cell culture-derived JE vaccine (inactivated) was purchased from the Research Foundation for Microbial Diseases of Osaka University (BIKEN, Osaka, Japan).

Lactose influence test: Three lactose concentrations (10, 25, and 50 mg/mL) were prepared and assayed by the Lowry method. In 1 mL of each sample, 2.5 mL of alkaline copper solution was added, briefly vortex mixed, and incubated at room temperature for approximately 60 min. Next, 3 mL of diluted Folin-Ciocalteu solution was added to each sample, and the samples were then briefly mixed and incubated at 37°C for ≥ 30 min. Finally, the absorbance at 750 nm (A_{750}) of each sample was measured with a Hitachi U-3310 spectrophotometer (Hitachi, Tokyo, Japan).

Nine different lactose solutions, ranging in concentration from 0–2 mg/mL, in the presence or absence of 6.2 $\mu\text{g/mL}$ BSA, were prepared. The BSA concentration mimics the total protein content in JE vaccines. Six BSA solutions, ranging in concentration from 0–99 $\mu\text{g/mL}$, were used to obtain a standard curve. Each lactose solution in the presence or absence of 6.2 $\mu\text{g/mL}$ BSA or standard BSA solution was assessed by the Lowry method, as described above. The A_{750} values obtained from each lactose concentration in the presence or absence of 6.2 $\mu\text{g/mL}$ BSA were estimated as the values corresponding to BSA samples from the BSA standard curve.

Three lactose concentrations (10, 25, and 50 mg/mL) were measured using the Bradford method, according to the manufacturer's protocol (Sigma-Aldrich), with 5 BSA solutions (concentrations ranging from 0–14.9 $\mu\text{g/mL}$) used as standards. The absorbance at 595 nm (A_{595}) of each lactose or BSA sample was measured by the spectrophotometer.

All samples were prepared to a volume of 1 mL. Three independent experiments were performed in triplicates.

Lactose interference tests in residual liquid volumes after heated TCA precipitation: The influence of residual liquid volumes after heated TCA precipitation was examined using 50 mg/mL lactose or 6.2 $\mu\text{g/mL}$ BSA in the presence or absence of 50 mg/mL lactose at 2 mL. Here, 0.11 mL of 100% (w/v) TCA solution was added to 2 mL of each sample, and the mixture was heated in boiling water for 15 min. After cooling, the BSA samples were centrifuged at $2,500 \times g$ for 20 min, and the

supernatants were removed partly or as much as possible without aspirating the precipitates. The volumes of supernatant remaining with the protein precipitates were approximately 0.05, 0.1, 0.2, and 0.4 mL. Next, 2 mL of 5% (w/v) TCA was added, and the mixture was centrifuged as above. The liquid volumes were equalized by bringing each sample to 1 mL with Milli-Q water, and then alkaline copper and diluted Folin-Ciocalteu solutions were added, as described above. The A_{750} of each sample was measured by spectrophotometer. The A_{750} values obtained from each sample were estimated as the values corresponding to BSA concentrations according to the BSA standard curve. Two independent experiments were performed in triplicates.

DOC-TCA precipitation method: A DOC-TCA precipitation method (3) was tested and compared with the heated TCA precipitation method. A solution of 6.2 $\mu\text{g/mL}$ BSA in the presence or absence of 50 mg/mL lactose was prepared in a volume of 2 mL. The heated TCA method was performed as described above. For the DOC-TCA method, 0.11 mL of 0.3% (w/v) DOC solution was added to 2 mL of each sample, and the mixture was incubated at room temperature for 10 min. Next, 0.12 mL of 100% (w/v) TCA solution was added, and the mixture was incubated at room temperature for 15 min. Thereafter, the Lowry assay was performed, as described above. The residual liquid volume after the DOC-TCA precipitation step was set to 0.05 mL. Two independent experiments were performed in triplicates.

Determination of the total protein in JE vaccine: The total protein in JE vaccine (BIKEN) was determined by the Lowry assay according to the MRBP in Japan, as described above. Two milliliters of sample vaccine was used to measure total protein content in the BSA standard range. The residual liquid volume after heated TCA precipitation was set to 0.05 mL, and the total protein in 2 lots was determined in triplicates.

Total protein content in the same 2 lots was also determined in triplicates by the Bradford method according to the manufacturer's protocol (Sigma-Aldrich), as described above.

RESULTS

Lactose interferes with the Lowry assay: The possible lactose interference in Lowry assays was first investigated using 3 lactose concentrations (10, 25, and 50 mg/mL) subjected to Lowry assessment. The results clearly showed an enhanced lactose concentration-dependent blue color development (Fig. 1A), and the A_{750} values increased in a lactose concentration-dependent manner (Fig. 1B). Data obtained from BSA samples in the range of 0–99 $\mu\text{g/mL}$ are shown in Figs. 1C and 1D. These results indicated that the blue color resulting from lactose was similar to that resulting from BSA, and thus confirmed that lactose interferes with the assay, as previously described by Frasch et al. (14). In addition, these results revealed that lactose in samples should be removed in order to accurately determine the protein content.

In contrast, even high lactose concentrations (50 mg/mL) did not interfere with the Bradford assay (Figs. 1E, F), which was in good agreement with previous results (15). BSA samples ranging in concentration from

Fig. 1. Influence of lactose on the Lowry and Bradford method. (A) Three lactose concentrations of 10, 25, and 50 mg/mL subjected to Lowry assay; deep blue color development produced in lactose concentration-dependent manner; lane 1, water only; lanes 2–4, 10, 25, and 50 mg/mL lactose, respectively. (B) Influence of lactose concentration (10, 25, and 50 mg/mL) at the absorbance of 750 nm. (C) Five BSA concentrations of 3–99 µg/mL BSA subjected to Lowry assay; lane 1, water only (blank); lanes 2–6, 3, 6, 25, 50, and 99 µg/mL BSA, respectively. (D) Standard curve of 5 concentrations of BSA with blank (Regression equation, $y = 0.0044x + 0.0016$; $R^2 = 0.996$; $n = 3$). Each data point represents mean $\pm$ standard deviation. (E) Three lactose concentrations of 10, 25, and 50 mg/mL subjected to the Bradford assay; lane 1, water only; lanes 2–4, 10, 25, and 50 mg/mL lactose, respectively. (F) Influence of lactose concentration (10, 25, and 50 mg/mL) at the absorbance of 595 nm. Even high lactose concentration (50 mg/mL) did not affect the Bradford assay. (G) Four BSA concentrations of 2.5–14.9 µg/mL BSA subjected to the Bradford assay; lane 1, water only (blank); lanes 2–5, 2.5, 5, 9.9, and 14.9 µg/mL BSA, respectively. (H) Standard curve of 4 concentrations of BSA with blank (Regression equation, $y = 0.0222x - 0.0094$; $R^2 = 0.993$, $n = 3$). Each data point represents mean $\pm$ standard deviation.

0–14.9 µg/mL showed a concentration-dependent blue color development (Fig. 1G), and the A_{595} values increased in a protein concentration-dependent manner (Fig. 1H). Thus, these results indicate that the Bradford assay can accurately determine protein concentrations in the presence of high lactose concentrations.

Lactose at ≥ 0.1 mg/mL interferes with the Lowry assay: To better understand the effect of lactose on the Lowry assay results, the effects of various lactose concentrations in the presence (filled circles in Fig. 2) or absence (open circles in Fig. 2) of 6.2 µg/mL BSA, mimicking the total protein concentration in JE vaccines, were examined. In both cases, the A_{750} values increased in a lactose concentration-dependent manner, and the A_{750} values increased markedly at lactose concentrations ≥ 0.1 mg/mL in both cases (Fig. 2, Table 1). The A_{750} value for each lactose concentration containing BSA was similar to the sum of that for each lactose concentration plus BSA at 6.2 µg/mL (Table 1), especially, in the range of 0.01–1 mg/mL lactose; the regression curves were almost parallel in this range.

The A_{750} value of each lactose concentration was con-

Fig. 2. Correlation between concentrations of lactose in the presence or absence of 6.2 µg/mL BSA and the A_{750} values in 1 mL sample. Nine lactose concentrations (0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, and 2 mg/mL) were assayed in the presence (filled circles) or absence (open circles) of 6.2 µg/mL BSA. Regression curve of lactose only: $y = 0.0676x + 0.0015$, $R^2 = 0.995$, $n = 3$; regression curve of lactose containing BSA: $y = 0.0622x + 0.0326$, $R^2 = 0.991$, $n = 3$. Each data point represents mean $\pm$ standard deviation.

Table 1. Influence of lactose on the Lowry method for protein concentration determination

Lac. conc. (mg/mL)	Lactose only ¹⁾		Lactose + BSA ²⁾	
	A ₇₅₀	Converted BSA conc. ³⁾ (μ g/mL)	A ₇₅₀	Converted BSA conc. ³⁾ (μ g/mL)
0	0.000 ⁴⁾	~0 ⁵⁾	0.031 $\pm$ 0.001	6.8 $\pm$ 0.1 (100%)
0.01	0.002 $\pm$ 0.002	~0	0.031 $\pm$ 0.001	6.9 $\pm$ 0.1 (101%)
0.02	0.002 $\pm$ 0.002	~0	0.031 $\pm$ 0.003	6.9 $\pm$ 0.3 (101%)
0.05	0.004 $\pm$ 0.001	~0	0.034 $\pm$ 0.001	7.6 $\pm$ 0.3 (112%)
0.1	0.007 $\pm$ 0.001	0.3 $\pm$ 0.6	0.039 $\pm$ 0.001	8.6 $\pm$ 0.3 (126%)
0.2	0.015 $\pm$ 0.001	2.0 $\pm$ 0.6	0.046 $\pm$ 0.002	10 $\pm$ 0.5 (147%)
0.5	0.039 $\pm$ 0.002	7.5 $\pm$ 0.5	0.069 $\pm$ 0.003	16 $\pm$ 0.9 (235%)
1	0.076 $\pm$ 0.002	16 $\pm$ 0.6	0.102 $\pm$ 0.005	23 $\pm$ 1.5 (338%)
2	0.133 $\pm$ 0.005	28 $\pm$ 0.8	0.152 $\pm$ 0.007	35 $\pm$ 2.1 (515%)

¹⁾: Lactose only; open circles in Fig. 2.

²⁾: Lactose + BSA; filled circles in Fig. 2.

³⁾: Value represents concentration extrapolated to BSA protein concentration from each A₇₅₀ value of lactose or lactose + BSA sample, according to the standard curve of BSA.

⁴⁾: Blank.

⁵⁾: ≤ 0 μ g/mL (-1 to 0 μ g/mL), according to the BSA standard curve.

Lac., Lactose; Conc., concentration.

verted into an apparent BSA protein concentration, according to a BSA standard curve. For lactose only, the A₇₅₀ values obtained from 0.1, 0.2, 0.5, 1, and 2 mg/mL lactose samples corresponded to the BSA protein concentrations of 0.3 ± 0.6 , 2.0 ± 0.6 , 7.5 ± 0.5 , 16 ± 0.6 , and 28 ± 0.8 μ g/mL, respectively. Meanwhile, the A₇₅₀ values obtained from lactose samples of 0–0.05 mg/mL corresponded to BSA concentrations of approximately 0 μ g/mL (Table 1).

In lactose samples containing 6.2 μ g/mL BSA, the A₇₅₀ values obtained from 0.1, 0.2, 0.5, 1, and 2 mg/mL lactose samples corresponded to BSA protein concentrations of 8.6 ± 0.3 , 10 ± 0.5 , 16 ± 0.9 , 23 ± 1.5 , and 35 ± 2.1 μ g/mL, respectively. Meanwhile, the A₇₅₀ values obtained from lactose samples of 0, 0.01, 0.02, and 0.05 mg/mL with BSA corresponded to BSA concentrations of 6.8–7.6 μ g/mL (Table 1). The rate of increase is shown in parenthesis in Table 1, and lactose concentrations of ≥ 0.1 mg/mL are indicated as an increase of $\geq 120\%$. Thus, it appeared that lactose concentrations of ≥ 0.1 mg/mL interfered with this assay.

Correlation between the lactose content and residual liquid volume: The Lowry assay according to the MRBP in Japan includes a heated TCA precipitation step designed to remove and/or dilute interfering substances in samples. Here, the correlation between the lactose content and the supernatant volume (residual liquid) after heated TCA precipitation was examined by performing Lowry assays with 50 mg/mL lactose only, or BSA samples of 6.2 μ g/mL in the presence or absence of 50 mg/mL lactose, mimicking the total protein and lactose content in JE vaccines. All the samples were tested at volumes of 2 mL, since the protein determination in JE vaccines will be performed in 2 mL volumes in subsequent studies.

In studies on lactose only, residual volumes of 0.1, 0.2, and 0.4 mL showed A₇₅₀ values of 0.014, 0.063, and 0.16, respectively (Fig. 3A, open bars). The lactose concentrations were estimated to be 0.1, 0.5, and 1.2 mg/mL (Table 2), respectively, according to the regres-

sion curve of lactose only shown in Fig. 2. In contrast, residual volumes of 0 and 0.05 mL had almost zero absorbance at 750 nm. These results indicated that lactose is present in larger residual volumes and consequently interferes with the Lowry assay, whereas lactose interference seems avoidable at residual volumes of ≤ 0.05 mL.

In experiments with BSA only, all the samples with various residual volumes showed almost the same A₇₅₀ values (0.06 in 2 mL sample; Fig. 3B, hatched bars), and the estimated protein content was 6.4–6.6 μ g/mL, which was consistent with the initial protein content of 6.2 μ g/mL (recovery, 103–106%). These results indicated that the residual volume did not affect the Lowry assay in the absence of lactose, allowing for accurate determination of the protein content.

In BSA samples containing 50 mg/mL lactose, increasing residual volumes (0.1, 0.2, and 0.4 mL) led to increased A₇₅₀ values of 0.086, 0.14, and 0.27, respectively (Fig. 3B, filled bars). In contrast, when the residual volumes were 0 or 0.05 mL, A₇₅₀ values were 0.06 for both, and almost the same as those of BSA only, which are described above. These results reinforced the conclusion that lactose interference is avoidable with residual volumes of ≤ 0.05 mL. Residual volumes of 0.1, 0.2, and 0.4 mL indicated BSA concentrations of 9.5 ± 0.6 , 16 ± 1.0 , and 30 ± 1.4 μ g/mL, respectively, with 1.5-, 2.6-, and 4.8-fold increases over the initial protein content. In contrast, when the residual volumes were 0 or 0.05 mL, the protein content was estimated to be 6.0 ± 0.1 or 6.8 ± 0.2 μ g/mL, respectively. These values were almost the same as the initial 6.2 μ g/mL protein content and similar to the protein content measured in the absence of lactose. These results indicated that larger residual volumes led to protein overestimation, while total protein content could be accurately determined without lactose interference when the residual liquid volume was ≤ 0.05 mL after precipitation.

In studies with the same residual liquid volumes, the sums of lactose only and BSA only were similar to the

Fig. 3. Influence of the residual liquid volume after heated TCA precipitation in 2 mL sample. The correlation between the A_{750} values and residual volumes was represented; samples contained 50 mg/mL lactose (open bars in panel A), BSA only (hatched bars in panel B) or both 6.2 μ g/mL BSA and 50 mg/mL lactose (filled bars in panel B); residual volumes after precipitation were set at ~0, 0.05, 0.1, 0.2, and 0.4 mL; the averaged A_{750} value (~0.06 in 2 mL) obtained from 6.2 μ g/mL BSA only is shown in horizontal line. Each data point represents the mean $\pm$ standard deviation.

Table 2. Lactose and BSA concentrations estimated from the A_{750} values in various residual liquid volumes

Residual Liquid (mL)	Lac. only Converted Lac conc. ¹⁾ (mg/mL)	Lac. only Converted BSA conc. ²⁾ (μ g/mL)	BSA only Converted BSA conc. ²⁾ (μ g/mL)	BSA + Lac. Converted BSA conc. ²⁾ (μ g/mL)	Sum of Converted BSA conc. ³⁾ (μ g/mL)	Converted BSA conc. in Table 1 ⁴⁾ (μ g/mL)
~0	~0	~0	6.5 $\pm$ 0.1	6.0 $\pm$ 0.1	6.5	6.8 $\pm$ 0.1
0.05	~0	~0	6.4 $\pm$ 0.1	6.8 $\pm$ 0.2	6.4	6.8 $\pm$ 0.1
0.1	~0.1	1.5 $\pm$ 0.2	6.6 $\pm$ 0.2	9.5 $\pm$ 0.6	8.1	8.6 $\pm$ 0.3
0.2	~0.5	7.2 $\pm$ 0.5	6.6 $\pm$ 0.2	16 $\pm$ 1.0	14	16 $\pm$ 0.9
0.4	~1.2	18 $\pm$ 1.1	6.4 $\pm$ 0.1	30 $\pm$ 1.4	24	$\geq 23 \pm 1.5$ ⁵⁾

¹⁾: Lactose concentrations were estimated from the A_{750} values of various residual liquid volumes in the lactose only sample using the regression curve of lactose only in Fig. 2 (open circles).

²⁾: BSA concentrations were estimated from the A_{750} values of various residual liquid volumes, according to the standard curve of BSA. Lactose only (open bars in Fig. 3A), BSA only (hatched bars in Fig. 3B), and BSA + Lactose (filled bars in Fig. 3B).

³⁾: Sum of converted BSA protein concentrations of Lac. only and BSA only.

⁴⁾: BSA concentrations are the values of lactose (0.1, 0.5, and 1 mg/mL) with BSA of 6.2 μ g/mL in Table 1.

⁵⁾: Description of $\geq 23 \pm 1.5$ is due to lactose concentration (~1.2 mg/mL) in residual liquid of 0.4 mL is greater than that of BSA with lactose of 1 mg/mL.

Abbreviations are in Table 1.

values of BSA with 50 mg/mL lactose. In addition, they were almost the same as the values obtained from 6.2 μ g/mL BSA plus lactose at 0.1, 0.5, and 1 mg/mL, as shown in Table 1. Thus, these results revealed that lactose interferes with the Lowry assay when ≥ 0.1 mg/mL lactose exists in the residual liquid of volume ≥ 0.1 mL. This is consistent with the results of the effects of lactose shown in Fig. 2 and Table 1. These results are summarized in Table 2.

Comparison of heated TCA and DOC-TCA precipitation methods: It has been reported that TCA precipitation does not produce a quantitative precipitation of protein at low levels (1–25 μ g) of protein, while the combined use of DOC and TCA was able to produce quantitative precipitation of protein in the range of 1–50 μ g (3). To obtain further evidence that the method presented here including heated TCA precipitation is useful for accurate protein determination even at low levels of protein, the heated TCA precipitation method was compared with the DOC-TCA precipitation method using 6.2 μ g/mL BSA in the presence and absence of 50

mg/mL lactose. Similar results were obtained in all cases, and the protein content was estimated to be in the range of 6.3–6.9 μ g/mL, almost the same as the initial 6.2 μ g/mL protein content. Thus, these results indicated that the modified method presented here is able to accurately determine protein content by producing quantitative precipitation even at low levels (approximately 5 μ g/mL) of protein. These data are also strongly supported by evidence that the recovery of BSA in 6.2 μ g/mL BSA samples in various residual volumes (Fig. 3B, hatched bars) was 103–106% (6.4–6.6 μ g/mL).

Determination of the total protein content in JE vaccines: To verify whether it is useful to make the residual liquid volume ≤ 0.05 mL after heated TCA precipitation when assessing the JE vaccine, the total protein in JE vaccine was examined when residual volume was approximately 0.05 mL. In 2 vaccine lots, the total protein content was determined to be 4.4 ± 0.3 and 4.6 ± 0.1 μ g/mL, respectively (Table 3). In addition, the total protein content of the same lots determined by the Bradford assay were estimated to be 4.6 ± 0.2 and 4.8 ± 0.2

Table 3. Protein content determined by Lowry and Bradford methods

	Lowry method	Bradford method
Lot 1	4.4 ± 0.3 µg/mL	4.6 ± 0.2 µg/mL
Lot 2	4.6 ± 0.1 µg/mL	4.8 ± 0.2 µg/mL

µg/mL, respectively, almost the same as the levels determined by the present Lowry method. Thus, ≤0.05 mL residual volume after precipitation is important for avoiding the effects of lactose interference during precise determination of the total protein content in JE vaccines.

DISCUSSION

In this study, the effects of lactose on the Lowry assay for protein concentration determination in vaccines was examined in detail. Lactose has been previously reported to interfere with the Lowry assay of a vaccine (14), but the degree to which lactose interfered with the Lowry assay was unclear. In this study, it was demonstrated that ≥0.1 mg/mL lactose caused interference.

The Lowry method is a two-step procedure: the Biuret reaction involving copper reduction in an alkaline solution and a subsequent Folin-Ciocalteu reagent reduction producing a characteristic blue color. Lactose at ≥2 mg/mL has been reported to interfere with the Biuret method for the determination of protein in human milk (16); thus, the Lowry assay is approximately 20 times more sensitive than the Biuret method.

Furthermore, it was important to test whether lactose interference is avoidable in Lowry assays, which are required according to the MRBP in Japan. In this study, the correlation between lactose concentration and residual volume after precipitation revealed that, when the residual liquid volume was ≥0.1 mL and lactose concentration was ≥0.1 mg/mL, the A₇₅₀ values increased in a volume-dependent manner, causing protein overestimation (Figs. 2 and 3, and Table 2). Our results indicated that ≥0.1 mg/mL lactose existing in ≥0.1 mL of supernatant after precipitation would interfere with the assay due to low levels (5 µg/mL) of protein determination and, concurrently, also showed that lactose interference was avoidable with residual volumes of ≤0.05 mL. In fact, accurate estimation of the protein content in JE vaccines with no lactose interference was confirmed by the Bradford method (Table 3).

Other substances in JE vaccines might also potentially interfere with the Lowry method. For example, TCM-199 medium in the JE vaccine (BIKEN) can be expected to be a source of potentially interfering substances. However, in this study, such interference appeared negligible using the present Lowry method, according to MRBP in Japan, by producing residual volumes after precipitation of ≤0.05 mL (Table 3).

In conclusion, the present study indicated that high levels of lactose in JE vaccines interfere with protein determination using the Lowry method and that the interference depends on residual volumes after precipitation. These findings suggest that it is important for the resid-

ual volume to be ≤0.05 mL after precipitation and that the total protein content of JE vaccines is precisely determined by the Lowry method according to the MRBP in Japan. We believe that focus on residual volume, as demonstrated in this study, will also be useful and applicable to other vaccines containing large amounts of potentially interfering substances.

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Conflict of interest None to declare.

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