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Measurement of Cortisol in Human Serum by Isotope Dilution Liquid Chromatography/Mass Spectrometry and a Comparison through a Proficiency Testing

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Liquid chromatography/mass spectrometry using isotope dilution method has been established as a primary method for the measurement of cortisol in human serum. Verification of this method was accomplished by the participation in Consultative Committee for Amount of Substance-Metrology in Chemistry (CCQM) pilot study. Two levels of cortisol certified reference materials were prepared and certified by the established method. They were used as sample materials for the proficiency testing. The expanded uncertainty in the measurement of cortisol in human serum was approximately 1.2% at 95% confidence level. The results of the proficiency testing showed a good precision among the participants, but some bias to the certified values. This means that commercial field laboratories should keep traceability chain to SI unit through available reference measurement procedures and/or available reference materials.

Key Words: ID LC/MS, Cortisol, Primary method, Traceability, Proficiency testing

Introduction

Cortisol is the primary glucocorticoid produced by the adrenal cortex gland. Most of the secreted cortisol in circulation (above 90%) is bound to protein, mainly to cortisol-binding globulin (CBG).[1] It is synthesized from cholesterol, and its production is stimulated by pituitary adrenocorticotropic hormone (ACTH), which is regulated by corticotropin releasing factor (CRF). Cortisol has many functions. It helps the body break down food for energy, *i.e*. metabolism, and helps the body manage stress. Cortisol levels can be affected by many conditions, such as physical or emotional stress, strenuous activity, infection, or injury.[2-3]

Normally, cortisol levels rise during the early morning hours and are highest at about 7 a.m. They drop to very low levels in the evening and during the early phase of sleep. The factor controlling this rhythm is not completely defined and can be disrupted by a number of physical and psychological conditions. ACTH and cortisol are secreted independent of the circadian rhythm in response to physical and psychological stress. Elevated cortisol levels and a lack of diurnal variation have been identified with Cushing's disease, phenomena of ACTH hypersecretion. Elevated circulating cortisol levels have also been identified in patients with adrenal tumors. Low cortisol levels are found in primary adrenal insufficiency, identified with Addison's disease, and in ACTH deficiency. Due to the normal circadian variation in cortisol levels, it is difficult to distinguish normal cortisol levels from abnormally-low levels. Therefore, several daily collections are recommended.[1]

Then cortisol is a diagnostic marker for the production of steroid hormones and for diagnosis of adrenal function. Accurate measurement of hormone levels is necessary for proper diagnosis and treatment of many diseases. Therefore a cortisol test is done to find problems related to the pituitary gland or adrenal glands, such as the secretion of excessive or insufficient levels of hormones. Serum concentrations of cortisol range from 30 ng/mL to 230 ng/mL.[1,4]

For the accurate measurement of cortisol in serum, an isotope dilution liquid chromatography/mass spectrometry (ID LC/MS) method was established as a primary method in this study. Primary methods of measurement play an important role in metrology because they provide the essential first link in the chain of traceability from the abstract definition of a unit of the International System of Units (SI) to its practical use in measurement.[5] IDMS is regarded as a primary method in organic analysis due to its precision, accuracy and provision of definable uncertainty values. The advantages of ID LC/MS include a simple sample preparation without derivatization and the use of an isotopic analogue as an internal standard, which enables the matrix effect to be compensated. In IDMS, once equilibration of the analyte and isotopic analogue has been achieved, total recovery of the analyte is not required, as the determined value is based on the measurement of the ratio between the analyte and the isotopic analogue.

Thus far, the GC/MS method has been used as a reference method for the quantification of cortisol. Sieckmann et al had carried out a comparison test among the laboratories for the validation of the ID GC/MS procedures themselves. However, as the GC/MS method requires a more complex sample pretreatment such as derivatization, it has been recently replaced with the LC/MS or LC/MS/MS method.[6-11] Susan *et al*. had published the ID LC/MS and ID LC/MS/MS method as a candidate reference method for the determination of cortisol in serum for the first time. But we used the bracketing calibration method and liquid-liquid extraction for the sample pretreatment in compared to the former method. Our result had more small expanded uncertainty. The LC/MS/MS method is also widely used in the analysis of cortisol in saliva,[3,12] urine.[13-16] The enzyme immunoassay (EIA) method is used in most of the commercial clinical laboratories due to its high selectivity and sensitivity.[17-19]

Verification of the established method was accomplished

through participation in a pilot study arranged by Consultative Committee for Amount of Substance-Metrology in Chemistry (CCQM). The national measurement institutes (NMI) of six countries participated in the CCQM pilot study. The results showed equivalence among the participating laboratories within an acceptable level of uncertainty. Two levels of cortisol CRM were developed, and were certified using the established method. The expanded uncertainty of measurement was 1.2% at a 95% confidence level.

The established ID LC/MS method was compared with commercial methods through proficiency testing.[4]

The two levels of cortisol CRM were distributed to commercial clinical laboratories in the Rep. of Korea for proficiency testing. The measurement method of cortisol of the commercial clinical laboratories was enzyme immunoassay (EIA). The results of the proficiency testing showed a good precision among the participants, but showed some bias to the values certified by ID LC/MS. This bias is believed to result from the absence of traceability. In order to improve the measurement confidence of the commercial field laboratories, the joint committee of traceability in laboratory medicine (JCTLM) recommends that the traceability of values assigned to calibrators and/or control materials must be assured through available reference measurement procedures and/or available reference materials of a higher order.

Experimental

Materials. Cortisol (98.9 ± 0.2%, SRM 921) was supplied by National Institute of Standards and Science (NIST). 90% enriched isotopic cortisol, cortisol-d_3, was bought from Cambridge Isotope Laboratories (Andover, MA, USA). Water (deionized, Millipore Alpha Q) and methanol (HPLC grade, Aldrich, USA) were filtered with a 0.22 μm PVDF filter (Supelco, USA) in a vacuum before use. Ethanol (ACS reagent), dichloromethane (ACS reagent) and ammonium acetate (99.99%) were purchased from Aldrich. As sample materials for the CCQM pilot study, two levels of cortisol serum, male and female, were supplied by NIST. The human serum as a source material for the cortisol CRM was provided by Seoul Clinical Laboratory (Seoul, Rep. of Korea). Serum units, which showed negative in HIV, HBV, HCV, and V.D.S. tests, were collected and pooled as a source material. The pooled serum was frozen at −75 °C. Later the serum was thawed at 4 °C and centrifuged at 9,000 rpm to remove fibrin. It was also filtered through a pre-sterilized 0.22 μm filter (SPGP M10RJ, Millipore) to remove microorganisms. Two levels of cortisol CRM, normal and abnormal level, were prepared. The abnormal level of CRM was prepared by adding of a precise amount of cortisol to the normal level. Finally, 3.0 mL aliquot of the pooled serum was dispensed into 3.5 mL sterilized polypropylene vials. They were capped and stored at −75 °C. The serums were termed as KRISS CRM level I and level II. The serum vials for this study were randomly selected from each set of KRISS CRM.

Preparation of the calibration standard mixture. 20 mM ammonium acetate-methanol (50/50, v/v) mixture was used as a solvent for the preparation of the standard solutions. Four independent cortisol standard solutions were prepared gravimetrically. Approximately 0.6 mg of cortisol was accurately weighed using a microbalance (2 g, Mettler Toledo, Switzerland) and dissolved in 100 mL of solvent that had been weighed using an analytical balance (205 g, Mettler Toledo, Switzerland). Concentrations of the standard solutions were approximately 7 mg/kg. Cortisol-d_3 stock solution was also prepared to a level of 7 mg/kg, and the cortisol-d_3 working solution was diluted to a level of 1 mg/kg from stock solution. In order to apply a bracketing calibration method, calibration standard mixtures with cortisol/cortisol-d_3 weight ratios of 0.9 and 1.1 were prepared by the mixing of each cortisol standard solution and cortisol-d_3 working solution. Therefore eight calibration standard mixtures were prepared from four standard solutions.

Sample preparation. 90% of cortisol in blood is bound to corticosteroid-binding globulin. Therefore it is necessary to release cortisol from the corticosteroid-binding globulin to determine the total cortisol. The processes for the pretreatment of the serum sample are given below.

The serum bottles and all required apparatuses were left in a weighing room for 2 hrs to ensure temperature equilibrium. Five samples were taken for a single measurement. 1 mL of serum sample was weighed and put into a 4 mL amber vial for sample workups. An appropriate amount of cortisol-d_3 solution was added to each aliquot to give a cortisol to cortisol-d_3 ratio of approximately 1:1. Serum and the added cortisol-d_3 solution were determined by weighing the vial before and after adding each substance to the vial. The serum was transferred to a 15 mL falcon tube and equilibrated for 1 hr. Each sample was then acidified to pH 1.5 with 6 mL of 0.5 M phosphoric acid and equilibrated at room temperature for 1 hr. Cortisol was then extracted two times with 5 mL of dichloromethane. The combined dichloromethane extract was dried under a stream of nitrogen at room temperature and the residue was resolved with 200 μL of ethanol. The sample was then filtered with a 0.22 μm centrifugal filter and put into a vial for LC/MS analysis.

LC/MS conditions. The mass spectrometer used in this study was a Finnigan TSQ Quantum Ultra Spectrometer (Thermo, San Jose, USA). It was combined with an Agilent 1200 series HPLC (HP, Waldbronn, Germany) through its electrospray ionization interface. The HPLC column was a Thermo ODS Hypersil C_{18} column (150 × 2.1 mm, 3 μm) guarded with a C_{18} guard cartridge (10 × 2.1 mm, 3 μm). Mobile phase was 20 mM ammonium acetate-methanol (50/50, v/v). The flow rate was 0.4 mL/min and the sample volume was 5 μL. The column was cleansed with methanol for 3 min before the next sample run. The column was kept at 50 °C during the chromatographic run.

Mass tuning was performed in an electrospray positive ionization mode with a mixed solution of cortisol and cortisol-d_3. The cortisol and cortisol-d_3 were monitored at m/z 363.3 and m/z 366.3, corresponding to $[M+H]^+$, respectively. The optimized mass conditions were as follows: the spray voltage was set at 4,900 V; the sheath gas, ion sweep gas and aux gas pressure were set at 60, 8.0 and 55 psi; respectively. The capillary temperature was set at 350 °C.

Measurement protocol. For every measurement, a LC/MS run was conducted in the following order: calibration standard mixture with a weight ratio of 0.9, sample extracts and calibration standard mixture with a weight ratio of 1.1. The LC/MS

run was repeated four or five times for a single measurement.

A bracketing method was applied for the calibration procedure. This procedure involves taking measurements of each sample between measurements of two calibration standards prepared such that their abundances fall just above and below the ion abundances of the sample. The concentration of the cortisol in serum, C, was calculated using the following formula.

$$C = \frac{M_{is-sol,spiked} \cdot C_{s-sol}}{W_s} \cdot [(\frac{AR_{sample} - AR_1}{AR_2 - AR_1}) (MR_{mix,2} - MR_{mix,1}) + MR_{mix,1}]$$

Here, $M_{is\text{-}sol,spiked}$ is the weight of the cortisol-d_3 solution spiked in the sample, $C_{s\text{-}sol}$ is the concentration of the cortisol standard solution (μg/kg), and W_s is the weight of the sample. AR_{sample} is the observed area ratio of cortisol/cortisol-d_3 of the sample from the LC/MS measurement, AR_i is the observed area ratio of cortisol/cortisol-d_3 of the calibration standard mixture i (i = 1,2) from the LC/MS measurement, and $MR_{mix,i}$ is the weight ratio of the cortisol solution/cortisol-d_3 solution in the calibration standard mixture i (i = 1,2) from the LC/MS measurement.

Proficiency testing. Two levels of cortisol CRM, normal (KRISS CRM Level I) and abnormal (KRISS CRM Level II) level, were distributed to commercial clinical laboratories in the Rep. of Korea. All samples were enclosed in dry ice boxes and transferred to clinical laboratories within 2 days. The measurement results of the participants were received within 2 weeks after the transfer, and the results of the proficiency testing were reported to all the participants.

Results and Discussion

The cortisol and cortisol-d_3 were monitored at m/z 363.3 and 366.3, corresponding to $[M+H]^+$ respectively. The LC/MS chromatograms were dominated by the target analytes and were obtained in a good shape for quantification. Figure 1 shows the LC/MS chromatograms of the spiked serum. There were no interfering peaks from the serum matrix, and the peak intensities were also high enough for quantification. The concentration of cortisol and cortisol-d_3 in serum was approximately 0.4 mg/kg, and cortisol and cortisol-d_3 had nearly the same retention time.

In order to test the repeatability of the instrumental measurement within a single measurement, multiple samples that were prepared independently as well as calibration standard mixtures were repeatedly run five times by LC/MS. The relative standard deviations (RSD) of the area ratios of the cortisol/cortisol-d_3 of the serum samples and calibration standard mixtures ranged from 0.2 to 0.6%. This indicates that there was no detectable long-term drift or short-term fluctuation during analysis.

Isotopic interference was checked by comparison of the pure serum and the serum into which cortisol-d_3 was added. If an isotopic analogue is present in a serum sample, IDMS determinations may be subject to significant bias. Figure 2 shows the LC/MS chromatograms of the cortisol and cortisol-d_3 of pure serum. This figure shows that the isotopic analogue was not present in the serum sample.

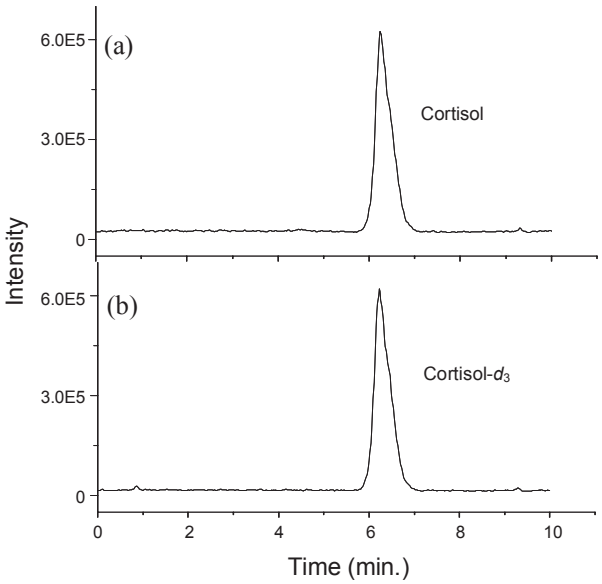

Figure 1. The LC/MS chromatograms of cortisol (a) and cortisol-d_3 (b) in the spiked human serum.

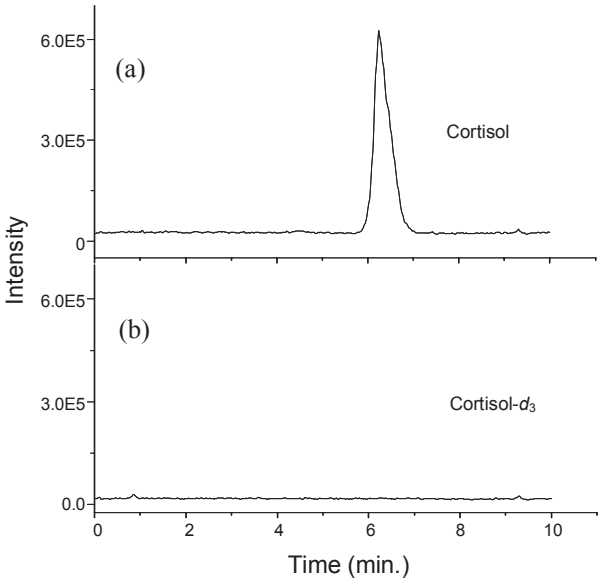

Figure 2. The LC/MS chromatograms of cortisol (a) and cortisol-d_3 (b) in the pure human serum.

The limit of detection of cortisol was estimated to be approximately 1 μg/kg. The measurement concentrations of the analytes were approximately 0.4 mg/kg in this study.

As the calibration standard mixtures are a direct reference for IDMS result, the consistency of the standard solutions and calibration standard mixtures must be verified to ensure traceability to the International System of Units (SI). Therefore, eight calibration standard mixtures, four with a cortisol/cortisol-d_3 weight ratio of 0.9 and four at 1.1, were tested by LC/MS under optimum conditions. The area ratio of cortisol/cortisol-d_3 of each calibration standard mixture was normalized by its weight ratio. This was termed the Response Factor (RF). The RSD of

Table 1. The comparison of relative response factors of the calibration standard mixtures

Calibration mixtures	Weight ratios[a]		Peak area ratios[b]		Response factors[c]	
	0.9:1	1.1:1	0.9:1	1.1:1	0.9:1	1.1:1
1	0.8729	1.0671	0.9966	1.2175	1.1416	1.1410
2	0.8481	1.0771	0.9654	1.2249	1.1383	1.1373
3	0.8675	1.0744	0.9909	1.2272	1.1423	1.1422
4	0.8795	1.0795	1.0059	1.2291	1.1437	1.1386
				Average	1.1406	
				Standard deviation	0.0023	
				Relative standard deviation (%)	0.2	

[a]Weight ratio: weight of cortisol/weight of cortisol-d_3. [b] Peak area ratio: peak area of the cortisol/peak area of cortisol-d_3. [c] Response factor: peak area ratio/weight ratio

Table 2. Measurement results of cortisol in the pilot study (μg/kg)

Samples	Averages by the pilot study	Result by ID LC/MS
Female serum sample	88.21 ± 0.68[a]	87.90 ± 1.27[b]
Male serum sample	105.07 ± 0.78	104.59 ± 1.39

[a]Expanded uncertainty is calculated by Std Dev of lab means divided by square root of n (4). [b]Expanded uncertainty within a 95% confidence level

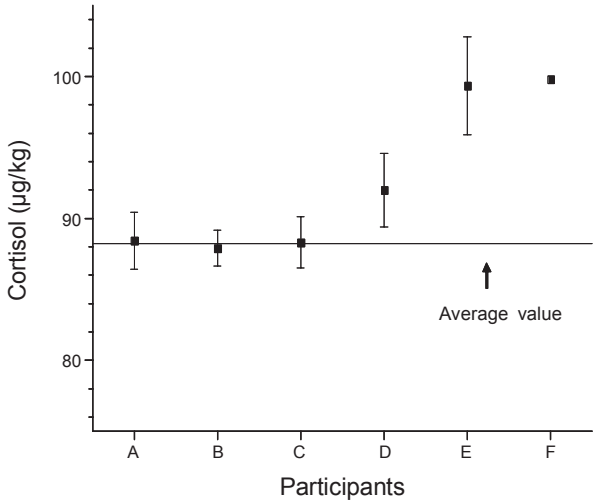

Figure 3. Comparison of cortisol measurement result of female serum in the CCQM Pilot study (Participants represent the NMIs of each country).

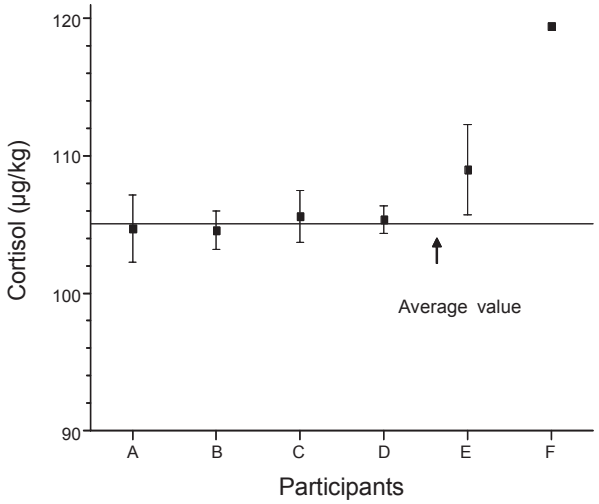

Figure 4. Comparison of cortisol measuremen result of male serum in the CCQM Pilot study(Participants represent the NMIs of each country)

the RF values of eight calibration standard mixtures was 0.2%, as shown in Table 1. This indicates that all of the procedures for the preparation of the standard solutions and the calibration standard mixtures were correct and had a high degree of metrological quality. At both weight ratios of 0.9 and of 1.1, the calibration standard mixtures with the nearest values to average were selected as standards for the quantification of the cortisol in human serum.

Although it has been proven that the IDMS results are traceable to SI units, some degree of validation is always confirmed through the use of a well-characterized standard material or by participation in a key comparison. The validation of this method was performed through the participation in a CCQM pilot study. The NMIs of six countries participated in the CCQM pilot study. The samples for the CCQM pilot study were two types of serum, male and female. The serum samples were analyzed by ID LC/MS, and Table 2 shows the results from the CCQM pilot study. The average value in this comparison is the mean of only five countries, because one country was excluded as an outlier. Figure 3 shows the result of the cortisol measurement of the female serum, and Figure 4 shows the result of the male serum in the CCQM pilot study. In these results, B is our laboratory. In both samples, our ID LC/MS results showed equivalence with the average values among the participants within an acceptable level of uncertainty.

CRM is necessary to maintain and disseminate the traceability to its practical use. Two types of cortisol CRM were developed for this study. The CRMs were certified by ID LC/MS. Five

Table 3. Measurement results of cortisol in KRISS CRM Level I and II by ID LC/MS for three independent periods

Periods	KRISS CRM Level I (μg/kg)	RSD within a single period (%)	KRISS CRM Level II (μg/kg)	RSD within a single period (%)
1	89.30	0.46	393.08	0.61
2	88.17	0.30	392.40	0.16
3	88.78	0.22	393.37	0.14
Average	88.75		392.95	
Combined std. unc.	0.46		1.30	
k	2.45		2.31	
U expanded	1.11		3.00	

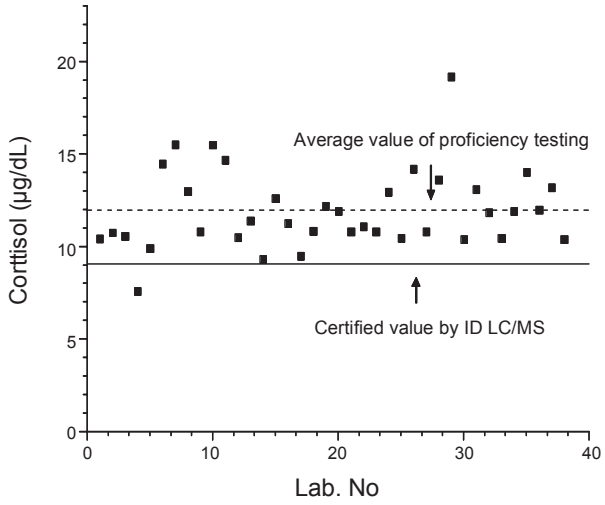

Figure 5. Proficiency testing results of KRISS CRM Level I (Average value: 11.95 ± 2.14 μg/dL (SD), Certified value: 9.07 ± 0.12 μg/dL).

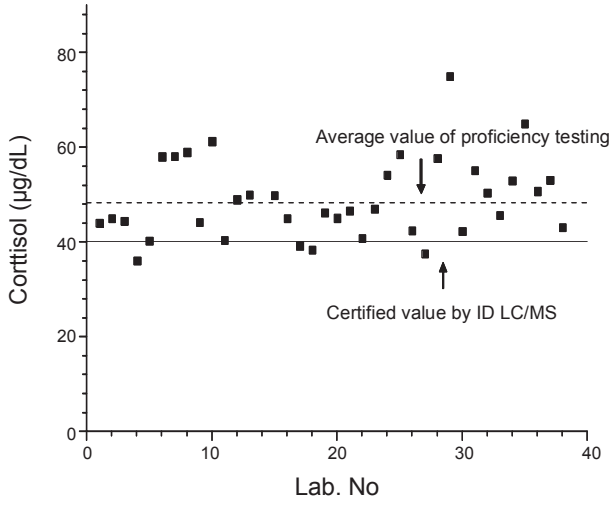

Figure 6. Proficiency testing results of KRISS CRM Level II (Average value: 48.22 ± 7.5 μg/dL (SD), Certified value: 40.16 ± 0.71 μg/dL).

bottles from each KRISS CRM set were selected randomly for a single measurement, and analyses were repeated three times independently. Table 3 shows the results of each independent measurement.

The repeatability of a measurement can be a performance parameter to verify that the method is adequate for use as a primary method. The RSD of the measurement results of multiple samples within a single measurement were less than 0.6% to the mean value. This indicates that this method has good repeatability. The SD of the measurement results of the multiple samples represents the random uncertainty from the weighing of a sample taken for analysis, the weighing of the cortisol-d_3 solution spiked in the sample, and the LC/MS measurements of the calibration standard mixtures and sample extracts.

The SD of several independent measurements represents the reproducibility of measurement. As shown in Table 3, the measurement reproducibility of KRISS CRM Level I was 0.62%, and that of KRISS CRM Level II was 0.13%. This indicates that the ID LC/MS method has a high degree of reproducibility. The certified value of KRISS CRM level I was 88.75 ± 1.11 μg/kg and that of KRISS CRM level II was 392.95 ± 3.00 μg/kg. The expanded uncertainty at a 95% confidence level was 1.2%. In the uncertainty evaluation, the degree of the contribution of each parameter to the uncertainty was evaluated. Among the uncertainty parameters, the area ratios of cortisol/cortisol-d_3 of the calibration standard mixture and the serum sample were checked as the main source of uncertainty. This implies that measurement stability of the instrument is the most important factor for the improvement of the uncertainty level.

KRISS CRM level I and II were distributed to the commercial clinical laboratories in the Rep. of Korea for proficiency testing. Figure 5 shows the proficiency testing result of CRM level I, and Figure 6 shows that of CRM level II. The result of KRISS CRM level I showed some precision among the participants, but showed bias to the certified value. There was approximately 30% difference between the certified value and the average value of the participants. The result of KRISS CRM level II showed better agreement with the certified value such that the difference was about 20%. Since the precisions among the participants were comparatively acceptable, the bias is believed to result from the absence of traceability of the calibrator used for the measurement. Unreliable results from commercial clinical laboratories can result in improper treatment for patients. This implies that the use of well-characterized reference materials or reference methods is very important for the establishment of traceability to its practical use.

Conclusions

The ID LC/MS has been established as a primary method for the measurement of cortisol in human serum. The advantages

of this method include its simple sample preparation without derivatization and its use of an isotopic analogue as an internal standard, which can offset the matrix effect. Validation of this method was accomplished by the participating in a CCQM pilot study. The results of the proposed ID LC/MS showed equivalence with the results of the other participants in the CCQM pilot study. Two types of cortisol CRM were prepared and were certified by ID LC/MS. The expanded uncertainty was approximately 1.2% at a 95% confidence level. The proficiency testing results of the two types of cortisol CRM showed a significant difference between the certified values and the average values from the clinical laboratories. This implies that the results of commercial field laboratories should maintain the traceability chain to SI units through available reference measurement procedures and/or available reference materials.

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