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Development and Validation of Primary Method for the Determination of Glucose in Human Serum by Isotope Dilution Liquid Chromatography Tandem Mass Spectrometry and Comparison with Field Methods

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Glucose is a common medical analyte measuring in human serum or blood samples. The development of a primary method is necessary for the establishment of traceability in measurements. We have developed an isotope dilution liquid chromatography tandem mass spectrometry as a primary method for the measurement of glucose in human serum. Glucose and glucose-$^{13}C_6$ in sample were ionized in ESI negative mode and monitored at mass transfers of *m/z* 179/89 and 185/92 in MRM, respectively. Glucose was separated on NH_2P-50 2D column, and the mobile phase was 20 mM NH_4OAc in 30% acetonitrile/70% water. Verification of this method was performed by the comparison with NIST SRMs. Our results agreed well with the SRM values. We have developed two levels of glucose serum certified reference material using this method and distributed them to the clinical laboratories in Korea as samples for proficiency testings. The expended uncertainty was about 1.2% on 95% confidence level. In proficiency testings, the results obtained from the clinical laboratories showed about 3.6% and 3.9% RSD to the certified values. Primary method can provide the traceability to the field laboratories through proficiency testings or certified reference materials.

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

Introduction

Glucose is a sugar monomer and an important carbohydrate in biology. Glucose is also a common medical component measuring in serum or blood samples. The glucose level in the blood is maintained within a fairly narrow range under diverse conditions by regulatory hormones such as insulin, glucagon, or epinephrine. Eating or fasting has an effect on the glucose level in a blood sample. Relatively high glucose levels may be a sign of prediabetes or diabetes mellitus. Hyperglycemia shows ketoacidosis or hyperosmolar coma, retinopathy, renal failure, neuropathy, and atherosclerosis. Atherosclerosis may result in a stroke, gangrene, or coronary artery disease. Therefore, accurate measurement of blood glucose level is very important for proper diagnosis and treatments.[1,2]

Glucose can be measured by several different methods in clinics. Routine methods for the measurement of glucose in body fluid are performed on the autoanalyzer based on an enzymatic reaction using hexokinase and glucose oxidase, or other enzymes that act on glucose. The reaction product is then measured by spectrophotometry. Enzymatic methods cannot be reference methods, because they can be affected by interferences that exist in the complex biological matrixes. Therefore, the development of a primary method is essential to improve the reliability of routine clinical measurement.

The primary methods of measurement play an important role in metrology because they provide the essential first link in the chain of traceability from the definition of a unit of the International System of Units (SI) to its practical use in measurement. In order to be considered as primary method, two conditions should be provided. First, the method must be specific to a defined material. Second, the values of all parameters, or corrections that depend on other species or the matrix, must be known or calculable within appropriate uncertainty levels. One method with the potential to be primary is isotope dilution mass spectrometry (IDMS).[3,4]

The most important advantage of IDMS is its use of an isotopic analogue as an internal standard. Because isotopic analogues have physical and chemical properties that are identical to those of the analyte, excluding the mass, they can compensate for analyte transformation which can occur during sample pretreatment and instrumental sensitivity changes during periodical measurements. In IDMS, once the equilibration of the analyte and isotopic analogue has been achieved, the 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, IDMS can be a primary method because of its precision, accuracy and its provision of definable uncertainty values. The isotope dilution gas chromatography/mass spectrometry (ID GC/MS) method has been used as a primary method for the quantification of glucose in serum.[5-11] However, because the GC/MS method requires complex sample pretreatment such as derivatization, the isotope dilution liquid chromatography tandem mass spectrometry (ID LC/MS/MS) method

was established recently as an alternate primary method. Yizhao Chen *et al.* used ID LC/MS/MS for the analysis of glucose in serum and compared ID LC/MS/MS with ID GC/MS. For LC/MS/MS analysis, they purified serum samples by protein precipitation with acetonitrile, and selected $[M+HCOO^-]^-$ as a Q1 ion for MRM transition.[12] The main dvantage of ID LC/MS/MS is its simple sample preparation without derivatization.[12-14]

We also have developed the ID LC/MS/MS method as a primary method to determine the level of glucose in serum. Glucose has several stereoisomers such as galactose, mannose and fluctose which can be separated by LC analysis. We found out only very small amount of mannose in serum by ion chromatography which can be neglected to the content of glucose as a marker for the diagnostic of diabetes mellitus.

In order to verify the proposed method, the glucose levels of NIST SRM 652a were analyzed. The measured results were found to be in good agreement with the certified values within the measurement uncertainty. Two levels of glucose serum were prepared and certified using this developed method. Uncertainty evaluation was performed according to ISO guide.[15,16] These were named CRM 01 and 02. This CRM samples were distributed to the commercial clinical laboratories in Korea for proficiency testings. Field measurement results can be compared with reference values through proficiency testings.[17] The field clinical laboratories used spectrophotometry as a determination method. The proficiency testing results showed some variance in both samples. In order to improve the reliability of the measurement results of field laboratories, JCTLM (Joint Committee for Traceability in Laboratory Medicine) recommends that the clinical laboratories have the measured results traced to a higher order reference.

Experimental

Materials. Two levels of glucose serum were prepared from pool of healthy human serum free from HIV, HCV, and HBV through filtering with 0.2 μm filter. Two levels of glucose serum were named as certified reference material (CRM) 01 and 02. CRM 01 of reference level was prepared directly from pooled serum, and the high level, CRM 02, was prepared through spiking glucose into the pooled serum. Serum samples were randomly selected from the CRM batches. D-glucose, which was used as a standard material throughout this study, was from NIST SRM 917C with a certified purity of (99.7 ± 0.3)%. The isotopic labeled glucose, glucose-$^{13}C_6$, was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). Acetonitrile (HPLC grade) was obtained from Burdick and Jackson (Muskegon, MI, USA). Pure water was prepared by the distillation of deionized water obtained from a Millipore Corp Milli-Q RG purification system. Ammonium acetate was from Showa Chemical Co. (Tokyo, Japan). NIST SRM 965a, glucose in frozen human serum, was used for the verification of the developed method.

Standard Solutions. We gravimetrically prepared four independent glucose standard solutions of about 1,000 mg/kg in water. A glucose-$^{13}C_6$ standard solution of about 300 mg/kg was also prepared gravimetrically. Calibration standard mixtures were prepared through the addition of glucose-$^{13}C_6$ solution to each of the four glucose standard solutions, to have a 1:1 weight ratio of glucose/glucose-$^{13}C_6$. We tested the self-consistency of the standard solutions and the calibration standard mixtures through intercomparison by LC/MS/MS. A calibration standard mixture showing a proper reliance among the calibration standard mixtures was selected and used as a standard. The preparation and verification of the standard solutions and the calibration standard mixtures were independently carried out whenever sample measurements were performed at repeatable intervals.

Sample Preparation. Frozen serum samples were thawed and equilibrated at room temperature for 2 h. For each analysis, 0.2 mL of serum sample was taken into a 4 mL amber vial. Then, an appropriate amount of the glucose-$^{13}C_6$ standard solution was spiked into the sample vial to make a 1:1 weight ratio of glucose/glucose-$^{13}C_6$ within the sample. The amounts of serum and the glucose-$^{13}C_6$ solution taken into the vial were precisely weighted. The vial was thoroughly shaken using a vortex machine and then equilibrated for two hours. The samples were filtered with a 3,000 MWCO (molecular weight cut off: Millipore, Bedford, MA) filter for 30 min at 13,000 rpm in order to remove large molecules such as proteins in the serum. The filtered samples were diluted with mobile phase and taken into sample vials for analysis by LC/MS/MS.

LC/MS/MS Conditions. The LC/MS/MS system used for this study was an API 4000 mass spectrometer (Applied Biosystems, USA) combined with a HPLC (Agilent Technologies 1200 series, Germany) through its Turbo V^{TM} Ion source interface. Ionization was performed in negative MRM (multiple reaction monitoring) mode. Glucose and glucose-$^{13}C_6$ were monitored at mass transfers of *m/z* 179/89 and 185/92 respectively. Collision gas was 4 psi; curtain gas was 30 psi; GS1 was 40 psi; and GS2 was 40 psi. The ESI probe was operated at −3.5 kV; and the DP was −45.5 V; EP was −4.5 V; collision energy was −12 V; collision cell exit potential was −22 V; and the desolvation temperature was 400 °C. The column was Shodex Asahipak NH_2P-50 2D (Shiseido, 2.1 × 150 mm, 5 μm). The mobile phase was 20 mM ammonium acetate in acetonitrile/water (v/v, 30/70), and the flow rate of the mobile phase was 0.8 mL/min. The LC column was kept at room temperature during the chromatographic run.

Measurement Protocol. Four calibration standard mixtures were first tested by LC/MS/MS. Then, one of them, having good repeatability, was selected and used as a standard for the sample measurement. In the sample measurement, LC/MS/MS was run with the calibration standard solution first and then with the serum samples. The alternating measurements of the isotope amount ratio on the calibration standard solution and serum samples were repeated five times each. The mean value of the five measurements will minimize the effects of any instrumental drift. In this experiment, the concentration of glucose in serum, C, was calculated by

following formula:

$$C = \frac{M_{is\text{-}glu,spiked} \cdot AR_{sample} \cdot M_{glu,std} \cdot C_{glu,std}}{W_s \cdot AR_{std} \cdot M_{is\text{-}glu,std}}$$

Where $M_{is\text{-}glu,spiked}$ is the weight of the glucose-$^{13}C_6$ solution spiked in the sample; $M_{glu,std}$ is the weight of glucose solution in the calibration standard mixture; $M_{is\text{-}glu,std}$ is the weight of the glucose-$^{13}C_6$ solution in the calibration standard mixture; $C_{glu,std}$ is the concentration of the glucose standard solution (mg/kg); W_s is the weight of the sample; AR_{sample} is the observed area ratio of the glucose/glucose-$^{13}C_6$ in the sample from the LC/MS/MS measurement; AR_{std} is the observed area ratio of the glucose/glucose-$^{13}C_6$ in the calibration standard mixture from the LC/MS/MS measurement.

Calibration Procedures. Several different approaches to calibration are available for IDMS measurements. Graphical method, bracketing method, single point calibration and exact signal matching method are available for IDMS measurements. The graphical method using a calibration curve is most useful for analyzing a number of samples varying in concentration. However, the graphical method is not likely to prove as accurate as the bracketing or exact signal matching method for individual samples. In order to obtain highly accurate results, we used the exact signal matching method for calibration. The exact signal matching method involves a number of preparations of the spiked sample until a match is produced, and is follow as: Initially, an estimate of the concentration of the analyte in the sample is made. We estimated the initial concentration of sample by spectrometry. Isotope amount ratios of the spiked sample and the calibration standard mixture were determined by LC/MS/MS. An improved estimate of the glucose concentration in sample can then be calculated from the data. This step was repeated using the latest value for the concentration of the glucose until the determined concentration value converges towards a limiting value. In practice this procedure involves a number of preparations of the spiked sample until a match is produced.

Results and Discussion

LC/MS/MS Performance. For glucose is a very polar compound having 5 hydroxyl groups, the molecular ions were formed in negative mode easily. $[M\text{-}H]^-$ molecular ions were formed predominantly in 20 mM ammonium acetate (acetonitrile/water, v/v, 30/70) mobile phase as shown in Figure 1. Deprotonation site of the molecular ions is the hydrogen of hydroxyl group bonded to sixth carbon, because hydroxyl groups bonded to ring structure are relatively more stable. Molecular ion of glucose, Q1, was fragmented in collision cell, and the several intensive product ions, Q3 daughter ions, were obtained in MRM mode. The main fragmented product ions were *m/z* 59, 71 and 89. Because *m/z* 89 showed the highest intensity among them, glucose was monitored at the mass transfer of *m/z* 179/89. Glucose-$^{13}C_6$ was monitored at the mass transfer of *m/z* 185/92.

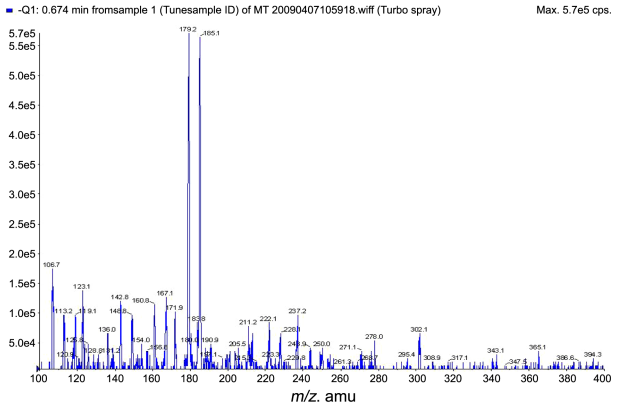

Figure 1. Q1 scan spectrum of glucose and glucose-$^{13}C_6$ in 20 mM ammonium acetate (acetonitrile 30/water 70).

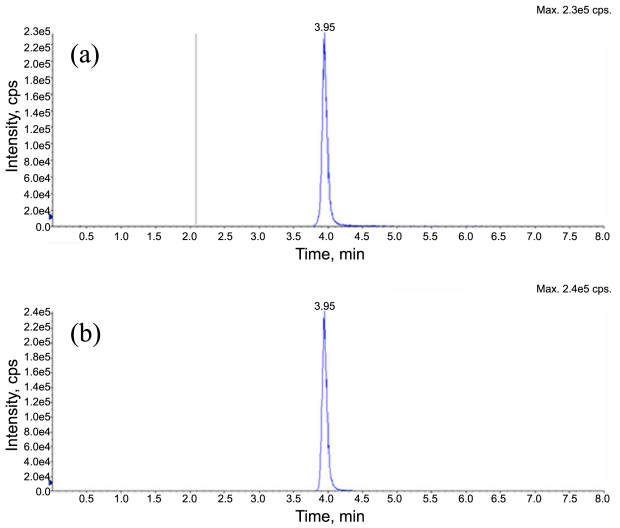

Figure 2. MRM mass chromatograms of glucose (a) and glucose-$^{13}C_6$ (b) in isotope spiked-serum.

Figure 2 shows the MRM mass chromatograms of glucose and glucose-$^{13}C_6$ in isotope spiked-serum. The peak shapes of the two target materials were sharp and showed no interfering peaks from the serum matrix. The area ratios of glucose/glucose-$^{13}C_6$ from repeated sample runs overnight showed no detectable long-term drift. The relative standard deviation of five repeated runs was less than 0.5%.

Verification of Calibration Standard Mixture. The standard solutions and the calibration standard mixtures should be verified to trace the IDMS results to the SI units because gravimetry used in preparing standard solutions and calibration standard mixtures is acknowledged as a primary method traceable to SI units and the calibration standard mixture is a direct reference to the IDMS results. For sample measurement in single period, four independent standard solutions were prepared as described in the experimental section. From each standard solution, each four calibration standard mixtures, having ratios of 1:1 of glucose/glucose-$^{13}C_6$, were prepared. The four calibration standard mixtures were tested by LC/MS/MS under the optimum analytical conditions used for sample analysis. The area ratio of glucose/glucose-$^{13}C_6$ on their corresponding MRM chromato-

grams of each calibration standard mixture was normalized by its weight ratio, as prepared gravimetrically. As shown in Table 1, the normalized area ratios of four calibration standard mixtures, named as response factor, showed agreement within 0.24% RSD. This indicates that there were no mistakes in the procedures for the preparation of the standard solutions and the calibration standard mixtures. In some cases, the test results could identify any significant mistake that occurred in the preparation of a specific standard solution and a calibration standard mixture within a batch.

Method Validation and Analytical Quality Check. The method should be validated to verify if its performance parameters are adequate for use as a primary method. The followings are the experimental results we carried out to evaluate if the method has an adequate quality as a primary method.

Repeatability. To test the within-a-short-period repeatability of the sample preparation processes of the primary method, multiple aliquots from glucose serum CRM were went through with the sample preparation processes independently, and analyzed together by LC/MS/MS with the calibration standard mixture. The relative standard deviation of the measurement results of the multiple aliquots was less than 0.5% of the mean value. This indicates that this method has good repeatability. When serum samples were measured by LC/MS/MS on different days, the results also agreed within 0.5% RSD.

Reproducibility. Serum samples were measured several times with reasonable intervals. For the measurements in each period, all of the measurement processes were carried out independently, including the preparation and verification of a new set of standard solutions and calibration standard mixtures, sample preparation, and LC/MS/MS analysis. The relative standard deviation of the measurement results was about 0.55%, indicating that this method has a high degree of reproducibility.

Validation by Well-Characterized Reference Materials. Though it was proved that the primary method can trace the measurement results to the SI unit, validation by using well-characterized standard material or published methods is necessary. We analyzed NIST SRM 965a, which is a glucose serum SRM having four levels of glucose certified by ID GC/MS. The certified values and the measured values by our method are shown in Table 2. The measured results showed good agreement with the certified values.

Table 1. Relative response factors of the calibration standard mixtures

No.	Conc. ratios (glu/glu-$^{13}C_6$)	Area ratios (glu/glu-$^{13}C_6$)	Relative Response Factors[a]
1	1.003	1.005	1.002
2	1.002	1.004	1.002
3	1.009	1.006	0.997
4	0.999	1.000	1.001
Average			1.001
Standard Deviation			0.002
Relative Standard Deviation (%)			0.24

[a]Relative Response Factor: Area ratio/Concentration ratio

Table 2. Certified values and measurement results of in NIST SRM 965a

NIST SRMs	Certified values (mg/dL)	Measured values (mg/dL)
Level I	34.56 ± 0.36	34.61 ± 0.38
Level II	78.50 ± 0.86	78.59 ± 0.84
Level III	122.1 ± 1.3	120.0 ± 1.3
Level IV	292.6 ± 3.5	291.5 ± 3.2

Uncertainty. Two levels of glucose serum CRM were prepared and certified by this method. The measured values and uncertainties are shown in Table 3. For a primary method to be fit for its purpose, the uncertainty of the measurement result, at a given level of confidence, should be evaluated and must be confirmed to be at least manifold smaller than that of the method generally used in field laboratories. We used the commercial program, PUMA (program for uncertainty-calculation in measurement & analysis), for the uncertainty calculation. Sources of uncertainty in IDMS are the parameters of calculation formula. Because the uncertainties by $M_{is\text{-}glu,spiked}$, $M_{glu,std}$, $M_{is\text{-}glu,std}$, $C_{glu,std}$, and W_s are derived from weighting, B-type uncertainty was used. The main uncertainty sources were from AR_{sample} and AR_{std}. Because the uncertainty contributions by the area ratios of glucose/glucose-$^{13}C_6$ are mainly derived from instrumental fluctuation, it is difficult to be reduced by the improvement of technical skills. In order to reduce the uncertainties, instrumental maintenance and performance should be carried out regularly. In this case, the random uncertainty from repeatability in a single period and the uncertainty from the purity of the glucose reference material are not included in the measurement uncertainty budget. Then, the standard uncertainty of the measurement result was estimated by combining the uncertainty of the PUMA calculation, the uncertainty of the repeatability, and the uncertainty in the purity of the glucose standard material. The measurement results and uncertainty evaluations of CRM 01 and 02 are shown in

Table 3. Measurement results of glucose in human serum CRM by ID LC/MS/MS

Sample No	CRM 01 (mg/kg)	CRM 02 (mg/kg)
1	807.0	1284
2	806.9	1288
3	804.7	1277
4	805.9	1285
5	803.7	1287
Average (mg/kg)	805.7	1284
Std. unc. (%)	0.07	0.15
PUMA unc. (%)	0.5	0.3
Purity unc. (%)	0.3	0.3
Std. sol. unc. (%)	0.24	0.24
Comb. std. unc. (%)	0.63	0.51
ν	19	19
k	2	2
Exp. unc. (%)	1.3	1.0

Table 3. The expanded uncertainties of CRM 01 and 02 were about 1.2%, indicating that the method has a high metrological quality as a primary method.

Comparison with Field Method. Proficiency testings are necessary to improve the accuracy of the measurement results of the field laboratories. The two levels of glucose serum CRM were distributed to about 180 clinical laboratories in Korea for proficiency testings.

The results from the field laboratories were obtained and compared with the certified values. The results are shown in Figures 3 and 4. The measurement ability of each laboratory can be approved by participation in regular proficiency testings. Figure 3 and Figure 4 are very meaningful because they show the state-of-the-art of glucose measurement for the diagnostic of diabetes mellitus by commercial clinical laboratories in Korea. CRM 02 showed more busted variance than CRM 01. The relative standard deviations were about 3.6% and 3.9% to each mean value, respectively. This high RSD suggests that the results from the diagnostic laboratories should be calibrated with well-characterized reference materials or reference methods to improve the variance. But the certified values and the mean values from the results of proficiency testings were agreed well with each other. This means that it is acceptable to use the average value as a reference value when reference materials having reference value are not available for proficiency testings. In order to provide more accurate diagnostic results to clients, the traceability chain should be kept from the final results to SI units through an unbroken line. The certified reference materials developed from this primary method can be used as a primary reference material providing the traceability to improve the measurement accuracy of field laboratories.

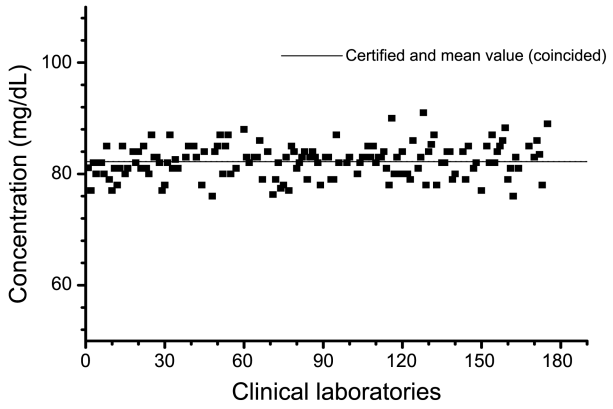

Figure 3. Schematic result of proficiency testing of CRM 01.

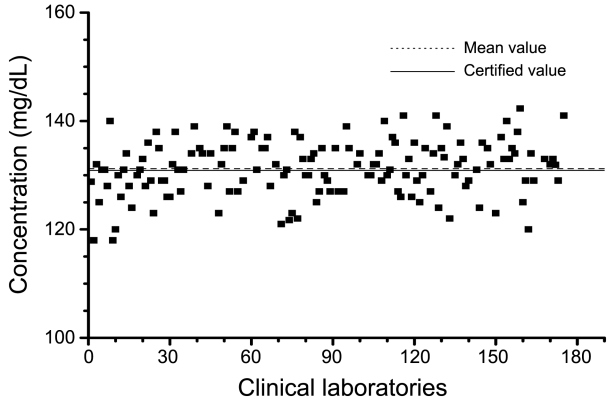

Figure 4. Schematic result of proficiency testing of CRM 02.

Conclusion

An ID LC/MS/MS method was proposed as a primary method for the determination of glucose in serum. This ID LC/MS/MS method needs only filtration as sample pretreatment procedure. ID LC/MS/MS has many advantages, such as simple sample preparation, easy application to diverse compound and selective detection without matrix interferences in comparison with the previous IDMS method based on GC/MS. This method was verified through comparison with the NIST SRM. We developed the two levels of glucose serum CRM on the basis of this method and used them as samples for proficiency testings. Through the proficiency testings, it was possible to view the state-of-the-art of glucose measurement by commercial clinical laboratories in Korea. The certified reference materials developed from this primary method can be used as a primary reference material providing the traceability to improve the measurement accuracy of clinical laboratories.

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References

1. David, B. S.; Sacks, M. B.; Ch, B.; F. A. C. P. *Carbohydrates*; In: Burtis, C. A.; Ashwood, E. R.; Bruns, D. E., Eds.; *Tietz Textbook of Clinical Chemistry*, 3rd ed.; Philadelphia, WB Saunders, 1999; 750-808.
2. Sacks, D. B. *Clin. Chem.* **1997**, *43*, 2230.
3. De Bivère, P. *Anal. Proceedings* **1993**, *30*, 328.
4. Heumann, K. G. *J. Mass Spectrom. Rev.* **1992**, *11*, 41.
5. Schaffer, R. *Pure Appl. Chem.* **1976**, *45*, 75.
6. White, E.; Welch, V. M.; Sun, T. *Biomed Mass Spectrom.* **1982**, *9*, 395.
7. Pelletier, O.; Arratoon, C. *Clin. Chem.* **1987**, *33*, 1397.
8. Magni, F.; Paroni, R.; Bonini, P. A.; Kienle, M. G. *Clin. Chem.* **1992**, *38*, 381.
9. De Leenheer, A. P.; Thienpont, L. M. *Int. J. Mass Spectrom. Rev.* **1992**, *11*, 249.
10. Phinney, C. S.; Murphy, K. E.; Welch, M. J. *Fresenius' J. Anal. Chem.* **1998**, *361*, 71.
11. Prendergast, J. L.; Sniegoski, L. T.; Welch, M. J.; Phinney, K. W. *Analytical and Bioanalytical Chemistry* **2010**, *397*(5), 1779.
12. Chen, Y.; Liu, Q.; Yong, S.; Lee, T. K. *Clin. Chim. Acta* **2012**, *413*(7-8), 808.
13. McIntosh, T. S.; Davis, H. M.; Matthews, D. E. *Anal. Biochem.* **2002**, *300*, 163.
14. Taormina, R.; Baca, J. T.; Finegold, D. N.; Asher, S. A.; Grabowski, J. J. *J. Am. Soc. Mass Spectrom.* **2007**, *18*, 332.
15. ISO/IEC Guide 98-3: ISO, Geneva, 2008.
16. Jongoh, C.; Euijin, H.; Hunyoung, S.; Byungjoo, K. *Accredit. Qua. Ass.* **2003**, *8*, 13.
17. Bjorkhem, I.; Bergman, A.; Falk, O. *Clin. Chem.* **1981**, *27*, 733.