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High accuracy analysis of glucose in human serum by isotope dilution liquid chromatography-tandem mass spectrometry

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ABSTRACT

Background: An isotope dilution mass spectrometry (IDMS) technique has been developed for high accuracy analysis of glucose in human serum. Currently, all the IDMS methods for glucose analysis are based on gas chromatography–mass spectrometry (GC–MS). In this study, isotope dilution liquid chromatography-tandem mass spectrometry (ID LC-MS/MS) was investigated.

Methods: NIST SRM 965b glucose in frozen human serum was analyzed by linear regression IDMS based on both LC-MS/MS and GC–MS. Serum samples were spiked with isotope labeled glucose and deproteinized by acetonitrile. For LC-MS/MS measurement, the supernate was injected directly after filtration and dilution. For GC–MS measurement, the supernate was evaporated to dryness and went through a two-step derivatization before injection.

Results: All measurements had good precision with CVs of <1%. Results from GC–MS agreed very well with results from LC-MS/MS, with a difference of <0.7%. The final reporting values in this study, based solely on LC-MS/MS, were within the certified ranges. The relative expanded uncertainties were within the range of 1.37% to 1.69% for the 4 levels of glucose, which were comparable with uncertainties from the certificate.

Conclusions: The IDMS method based on LC-MS/MS is precise and accurate. It does not require lengthy derivatization steps and thus, greatly simplifies the sample preparation procedure.

1. Introduction

Blood glucose measurement is used for the diagnosis and treatment of hyperglycemia (high blood sugar level) and hypoglycemia (low blood glucose level). In human body, blood sugar levels are regulated by the liver and maintained within precise limits. A fall in blood glucose to a critical level (approximately 2.5 mmol/l) leads to dysfunction of the central nervous system. Although glucose level well above normal for short periods may not produce any permanent effects or symptoms, chronic levels slightly above normal can produce a wide variety of serious complications over a period of years including kidney damage, neurological damage, cardiovascular damage, and damage to the retina [1–3]. Accurate measurement of blood glucose level is therefore very important as it ensures proper diagnosis and treatment.

Routine biochemical measurement methods are usually performed on autoanalyzers based on enzymatic reactions using hexokinase, glucose oxidase, or other enzymes that act on glucose. The reaction product is then measured by spectrophotometry. In general, enzymatic methods can be affected by interferences that exist in the complex biological fluids. Such methods may not be specific enough and differences among the various methods can be large. Results obtained between laboratories or at different times often show significant variation due to factors such as different method principles and test reagents [4]. To accomplish harmonization and to establish accuracy in routine clinical chemistry laboratory tests, reference method based on isotope dilution mass spectrometry (IDMS) needs to be developed.

Measurement of glucose in human serum by IDMS had been reported since the 1970s [5,6]. In 1982, the National Bureau of Standards, (currently known as National Institute of Standards and Technology, NIST) described two IDMS methods for measurement of glucose in serum [7]. Both methods involved complicated and lengthy sample preparation procedures. In one method, glucose reacted with acetone to form diacetone glucose (DAG). DAG was then separated by a series of thin-layer chromatography steps from interferences and injected into GC–MS. In the other method, serum sample was first deproteinized by ethanol and the supernate was evaporated, deionized using resins, and freeze-dried. Subsequently, glucose underwent two-step derivatization where it first reacted with 1-butylboronic acid and then with acetic anhydride to form 1,2:5,6-bis(butylboronate)-6-acetate (glucose BBA).

Abbreviations: IDMS, isotope dilution mass spectrometry; ID LC-MS/MS, isotope dilution liquid chromatography-tandem mass spectrometry; ID GC–MS, isotope dilution gas chromatography–mass spectrometry; CRM, Certified reference material; SRM, Standard reference material; MRM, multiple reaction monitoring; SIM, selected ion monitoring.

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This method was modified in 2010, eliminating the deionization step and thus reducing the sample preparation time [8]. Pelletier et al. reported a different derivatization procedure first with methoxyamine hydrochloride and then with N,O-bis(trimethylsilyl)trifluoroacetamide [9]. In 1992, Magni et al. deproteinized serum samples with acetonitrile and evaporated the supernate. Glucose was then derivatized first with hydroxylamine hydrochloride and then with acetic anhydride [10].

So far, all IDMS methods reported on the analysis of glucose in human serum are carried out by gas chromatography–mass spectrometry, which requires two-step derivatization of glucose. It is well known that liquid chromatography-mass spectrometry (LC-MS) has the advantage that analytes can be injected directly without derivatization and hence, greatly simplifies the sample preparation procedure. Since 2002, LC-MS methods have been developed for the analysis of glucose using electrospray with two modes of ionization [11–14]. In positive mode, $[M+Na]^+$ was monitored, corresponding to $m/z=203$. In negative mode, either $[M-H]^-$ or $[M+HCOO]^-$ was monitored. In the present study, IDMS based on liquid chromatography-tandem mass spectrometry was developed for the analysis of glucose in human serum. NIST SRM 965b was analyzed and the results were compared with those obtained by gas chromatography–mass spectrometry and with the certified values.

2. Materials and methods

2.1. Materials

Ammonium formate as well as formic acid (eluent additives for LC-MS), sodium azide (ReagentPlus, 99.5%), hydroxylamine hydrochloride (ACS reagent, 99.0%), pyridine (anhydrous, 99.8%), acetic anhydride (ACS reagent), D-(−)-fructose (99%), D-(+)-galactose (99%), and D(+)-mannose (99%) were from Sigma-Aldrich Pte Ltd, Singapore. HPLC grade methylene chloride and acetonitrile were from J.T. Baker, Phillipsburg, NJ. Purified water was from Milli-Q Integral system (Resistivity = 18.2 MΩ cm). D-glucose certified reference material (SRM 917c) purchased from NIST, Gaithersburg, MD, US is certified to have a purity of 99.7% with uncertainty of 0.3% at 95% confidence level. Isotope labeled D-glucose, $^{13}C_6$-D-glucose (U-13C6, 99%), was purchased from Cambridge Isotope Laboratories, Inc., Andover, MA.

2.2. Instrumentation

Sample weighing was performed on Mettler Toledo XP205 balance with a readability of 0.01 mg and maximum capacity of 220 g (Mettler-Toledo Inc., Columbus, OH). LC-MS/MS analysis was performed on AB Sciex Qtrap® 5500 MS/MS instrument (AB Sciex Pte. Ltd, Foster City, CA) coupled with Shimadzu Prominence UFLCXR LC system comprising a CBM-20A system controller, a CTO-20AC column oven, 2 LC-20ADXR pumps and a SIL-20AC autosampler (Shimadzu Scientific Instruments, Columbia, MD). GC–MS analysis was performed on Agilent 5975C inert XL MSD with triple axis detector coupled with Agilent 7890A GC (Agilent Technologies, Santa Clara, CA). Solutions were evaporated under nitrogen using Stuart sample concentrator (Bibby Scientific Limited, Staffordshire, UK). Centrifugation was carried out using Sartorius Centrifuge, Sigma 3-16P (Sartorius Stedim Biotech, Aubagne, France).

2.3. Preparation of standard solutions

D-glucose (about 90 mg) was weighed into a 40 ml amber glass vial. Sodium azide solution (30 ml, 0.1%) was weighed into the same container. The solution was sonicated for 5 min and vortexed. $^{13}C_6$-D-glucose was weighed and dissolved according to the same procedure. Stock solutions were diluted gravimetrically to approximately 1000 μg/g in 0.1% sodium azide solution. The working standard solutions were stored at −30 °C and warmed to room temperature before use.

2.4. Preparation of sample blends

Sample blends were prepared by adding $^{13}C_6$-D-glucose to serum samples so that the ratio of unlabeled and isotope labeled glucose was close to 1. $^{13}C_6$-D-glucose solution (around 100 μl) was weighed into 2 ml plastic centrifuge tube, followed by 100–300 μl of serum sample. The mixture was vortexed and left to stand for 2 h for equilibration. Enough acetonitrile (approximately 3 fold of aqueous volume) was added for protein precipitation. The mixture was vortexed and centrifuged. The supernate was filtered through 0.22 μm syringe filter. For LC-MS/MS analysis, samples were diluted to approximately 800 ng/g with 90:10 acetonitrile:water. For GC–MS analysis, the aforementioned samples were evaporated to dryness under nitrogen at 50 °C. Hydroxylamine hydrochloride in pyridine (0.2 mol/l, 400 μl) was added and the mixture was vortexed and heated at 90 °C for 40 min. After the sample was cooled down, 450 μl of acetic anhydride was added. The mixture was vortexed and heated at 90 °C for 60 min. The derivatized sample was evaporated to dryness under nitrogen at 50 °C. The residue was reconstituted in 2 ml of methylene chloride. The solution was filtered through 0.22 μm syringe filter and diluted to approximately 18 μg/g with methylene chloride.

2.5. Preparation of calibration blends

Four calibration blends were prepared by combining different aliquots of $^{13}C_6$-D-glucose working standard solution (2100 μl ~ 3300 μl, approximately 1000 μg/g) and D-glucose working standard solution (2650 μl, approximately 1000 μg/g) gravimetrically to give a ratio of unlabeled and labeled glucose of 0.8, 0.9, 1.1 and 1.2, respectively. For LC-MS/MS analysis, samples were diluted to approximately 800 ng/g with 90:10 acetonitrile:water. For GC–MS analysis, samples were derivatized following the same procedure as sample blends.

2.6. LC-MS/MS measurements

Liquid chromatography was performed using Unison UK-Amino column (2.0 × 100 mm, 3-μm particle diameter, Imtakt Corporation, Kyoto, Japan). Mobile phase A is 5 mmol/l ammonium formate with 0.05% formic acid, and mobile phase B is acetonitrile. Samples (10 μl) were injected and analyzed under isocratic condition consisting 60% mobile phase B at a flow rate of 0.3 ml/min. To separate the hexoses, liquid chromatography was also performed using a longer Unison UK-Amino column (2.0 × 250 mm) with 90% of mobile phase B at a flow rate of 0.2 ml/min. The autosampler tray temperature was set at 4 °C. Ions were generated with turbo spray in the negative ion mode and detected by multiple reaction monitoring (MRM). Selected ion monitoring (SIM) was also used in method evaluation to scan for interferences with the same parameters as for MRM except it does not have Q3 mass, Collision Energy and Collision Exit Potential. Nitrogen was the only gas used. Curtain gas was set at 20 psi. The ion source gas 1 and ion source gas 2 were both at settings of 45 psi. The turbo gas temperature was set at 450 °C. The ion spray voltage was set at 4500 V. Collision gas was set at Medium. The entrance potential was set at 7 V and the declustering potential was set at 50 V. Dwell time was set at 120 ms for all ion pairs. For D-glucose, ion pairs 225/89 and 225/59 were monitored. The collision energy was set at 21 V and 30 V, respectively. The collision exit potential was set at 7 V and 11 V, respectively. For $^{13}C_6$-D-glucose, ion pairs 231/92 and 231/61 were monitored. The collision energy was set at 16 V and 26 V, respectively. The collision exit potential was set at 25 V and 28 V, respectively.

To minimize the impact of the instrument drift on the analysis results, the injection sequence was set as follows. The calibration blends were analyzed first, followed by sample blends, and then by sample and calibration blends in the reverse order. The whole injection sequence was then repeated three times. Hence, each solution