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Developing a reference measurement procedure for free glycerol in human serum by two-step gas chromatography–isotope dilution mass spectrometry

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ABSTRACT

Background: Free glycerol in human serum is measured in clinical laboratories using enzymatic methods, which can be affected by interferences from biological samples. These methods are not applicable when stable isotopic tracers are used to determine lipid kinetics. Hence, a reference measurement procedure for free glycerol in human serum is needed.

Methods: A reference measurement procedure based on two-step gas chromatography–isotope dilution mass spectrometry (GC–IDMS) was developed for the measurement of free glycerol in human serum. This procedure involved spiking with $^{13}C_3$-glycerol, protein precipitation and cation exchange SPE, followed by two-step derivatization with 1-butylboronic acid and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide. Tripalmitin certified reference material (CRM) was used as the calibration standard to ensure metrological traceability.

Results: Good precision and accuracy were obtained as demonstrated by relative standard deviation (RSD) of 1.51%–3.33%, with average recoveries over 98%. The relative measurement uncertainty was below 3% with major contributions from the concentration of glycerol calibration solution, choice of ion pair, linear regression, and measurement precision.

Conclusions: With good accuracy and precision, as well as clear metrological traceability, the developed GC–IDMS procedure is useful in producing traceable and accurate measurement of free glycerol in human serum.

Introduction

Glycerol is an important metabolite in fat metabolism. The concentration of glycerol in serum reflects adipose lipolysis as an index of fat mobilization [1]. The level of free glycerol in serum could also be used as a biomarker for increased risk of development of hyperglycemia and type 2 diabetes [2], and for excessive alcohol intake [3]. In addition, glycerol measurement provides "blanking" for enzymatic triglycerides analysis [4]. Glycerol blanking is essential for reference analyses and under certain circumstances where glycerol concentrations are potentially high.

Enzymatic methods are commonly used to determine the level of free glycerol in human serum. However, the results can be affected by various interferences from extraneous sources of reagents and endogenous substances in serum [5]. Furthermore, enzymatic methods are not applicable when stable isotopic tracers are used to determine lipid kinetics [6–9]. Instrumental analytical methods for free glycerol in human serum had been developed. These included GC-FID method [10,11] and GC–MS method [12,13] with trimethylsilyl derivative, and HPLC-UV method with benzoylated derivative [14,15].

Isotope dilution mass spectrometry (IDMS) is recognized to be a primary method [16–18], which has been widely used to establish reference measurement procedures for organic biomarkers such as creatinine [19–21], glucose [22–24], cholesterol [25–27], urea [28,29], uric acid [30,31] and triglycerides [32,33]. A GC–IDMS method for free glycerol measurement was reported previously [13]. However, in this IDMS method, a fixed calibration curve with a wide calibration range of up to 60 mg/L was used. This approach suggested that the isotope ratio in both sample blends and calibration blends could be very different. This was different from IDMS method used as a primary method of measurement where there was a need to match the isotope ratio in both sample blends and calibration blends [34]. In addition, this method made use of commercially available glycerol as the calibration standard without determining its purity. It thus, in the opinion of the authors, did not achieve full metrological traceability.

Abbreviations: IDMS, isotope dilution mass spectrometry; GC–MS, gas chromatography–mass spectrometry; GC–IDMS, gas chromatography–isotope dilution mass spectrometry; GC-FID, gas chromatography with flame ionization detector; HPLC-UV, high performance liquid chromatography with UV detector; CRM, certified reference material; NIST, National Institute of Standards and Technology; RSD, relative standard deviation.

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In this paper, we report a new reference measurement procedure for free glycerol in human serum based on GC–IDMS method where the sample preparation and the derivatization procedure are optimized. In order to achieve metrological traceability, the purity of the glycerol calibration standard would need to be determined. As glycerol is highly hygroscopic, its purity cannot be determined by the commonly used mass balance approach [35]. We have therefore determined the concentration of the glycerol standard solution first, by using certified reference material (CRM) of tripalmitin in modified GC–IDMS experiments, which involves additional hydrolysis and derivatization steps.

Materials and methods

Materials

Tripalmitin CRM (SRM 1595) was purchased from the National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA. It was certified to have a purity of 99.5% with an expanded uncertainty of 0.2%. $^{13}C_3$-Glycerol (^{13}C, 99%, purity ≥98%) was purchased from Cambridge Isotope Laboratories, Inc., Andover, MA, USA. Glycerol (99% GC purity) was purchased from Sigma-Aldrich Pte Ltd., Singapore. Other chemicals and solvents are of HPLC grade or analytical grade (see Supplementary material for details).

Human sera were purchased from Solomon Park Research Laboratories, Kirkland, WA, USA. The materials were kept frozen during shipment, and were stored below −60 °C immediately upon receipt. Samples were thawed and equilibrated to room temperature before use.

Instrumentation

Weighing was carried out on a Mettler Toledo XP205 balance with a readability of 0.01 mg (Mettler-Toledo Inc., Columbus, OH, USA). GC–MS determination was performed on an Agilent 5975C inert XL MSD with a triple axis detector coupled with an Agilent 7890A GC (Agilent Technologies, Santa Clara, CA, USA). See supplementary material for details of other instruments used in this paper.

IDMS procedure

Linear regression IDMS procedure was used throughout the measurements as previously described [24,33]. Two IDMS procedures were performed. One of them was used to determine the concentration of glycerol standard solution (IDMS 1), and the other was used to measure the free glycerol in human serum (IDMS 2). The sample blends and calibration blends in these two IDMS procedures are defined as follows:

G-SB: sample blend in IDMS 1. It was prepared by mixing glycerol and $^{13}C_3$-glycerol standard solutions.

G-CB: calibration blend in IDMS 1. It was prepared by mixing tripalmitin and $^{13}C_3$-glycerol standard solutions.

F-SB: sample blend in IDMS 2. It was prepared by spiking $^{13}C_3$-glycerol standard solution into human serum sample.

F-CB: calibration blend in IDMS 2. It was prepared by mixing glycerol and $^{13}C_3$-glycerol standard solutions.

In this paper, all the solutions were prepared using gravimetric method which includes dissolving solid standard in solvent, dilution of standard solutions, mixing of standard and isotope labeled standard solutions, and spiking isotope labeled standard solution into human serum.

In the determination of free glycerol in serum (IDMS 2), four F-CBs were prepared by mixing glycerol and $^{13}C_3$-glycerol standard solutions with the isotope molar ratios being close to 0.7, 0.85, 1.15 and 1.3, respectively. The isotope molar ratio in F-SB was controlled within the acceptable range of 0.9 to 1.1 with optimum value of 1 by spiking $^{13}C_3$-glycerol standard solution into serum sample. The isotope molar ratio obtained from weighing and the measured isotope ratio by GC–MS should have a linear relationship (Fig. 1). The "isotope molar ratio vs measured isotope ratio" calibration curve with a goodness-of-fit for liner regression, R^2, better than 0.999 was accepted for further calculation.

For the unknown F-SB, the isotope molar ratio can be calculated by the linear regression function:

$$R_M = mR_B + b \tag{1}$$

where

R_M = isotope molar ratio in F-SB
R_B = isotope ratio in F-SB measured by GC–MS
m = slope of the linear regression plot based on the four F-CBs
b = interception on y axis for the linear regression plot.

The isotope molar ratio in F-SB (R_M) is proportional to the concentration of the analyte in serum sample (C_X) as in Eq. (2). The concentration of the analyte in serum can be obtained from Eq. (3) after rearrangement of Eq. (2).

$$R_M = \frac{M_X C_X}{M_Y C_Y} \tag{2}$$

$$C_X = R_M \times \frac{M_Y C_Y}{M_X} \tag{3}$$

where

C_X = molar fraction of analyte (mmol/kg) in serum sample
M_X = mass of serum sample
M_Y = mass of $^{13}C_3$-glycerol internal standard solution added to the serum sample
C_Y = concentration of $^{13}C_3$-glycerol internal standard solution (mmol/kg) added to the serum sample.

C_Y does not affect the value of C_X, as R_M contains a component of C_Z / C_Y (where C_Z is the concentration of glycerol standard solution), which cancel out C_Y but introduce the effect of C_Z on the value of C_X. The complex mathematic conversion was published in our previous paper [24].

The concentration of free glycerol in serum sample in mmol/L can be easily calculated using C_X value in mmol/kg and the density of the serum.

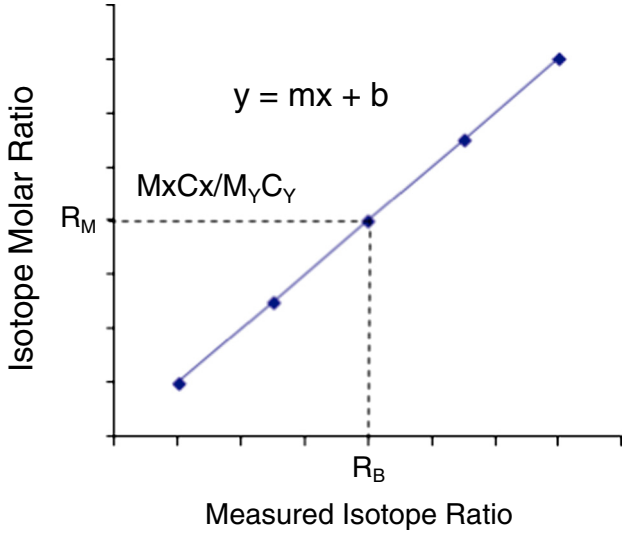

Fig. 1. Isotope molar ratio versus measured isotope ratio in calibration blends and sample blends.

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