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Quantification of plasma myo-inositol using gas chromatography–mass spectrometry

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ABSTRACT

Background: Myo-inositol (MI) deficiency is associated with an increased risk for neural tube defects (NTDs), mental disorders and metabolic diseases. We developed a gas chromatography–mass spectrometry (GC–MS) method to detect MI in human plasma, which was accurate, relatively efficient and convenient for clinical application.
Methods: An external standard method was used for determination of plasma MI. Samples were analyzed by GC–MS after derivatization. The stable-isotope labeled internal standard approach was used to validate the method's accuracy. Alpha fetal protein (AFP) was detected by chemiluminescence immunoassay.
Results: The method was validated by determining the linearity, sensitivity and recovery rate. There was a good agreement between the internal standard approach and the present method. The NTD-affected pregnancies showed lower plasma MI ($P = 0.024$) and higher AFP levels ($P = 0.001$) than control. Maternal MI level showed a better discrimination in spina bifida subgroup, while AFP level showed a better discrimination in anencephaly subgroup after stratification analysis.
Conclusions: We developed a sensitive and reliable method for the detection of clinical plasma MI, which might be a marker for NTDs screening, and established fundamental knowledge for clinical diagnosis and prevention for the diseases related to disturbed MI metabolism.

1. Introduction

Inositol or cyclohexane-1, 2, 3, 4, 5, 6-hexol is a compound with the formula of $C_6H_{12}O_6$. Myo-inositol (MI) is the most important form among the nine stereo-isomers of a C_6 alditol and is a polyalcohol derived from glucose. It is synthesized in only a few organs such as the testes, kidney and brain [1,2]. MI is a primary component of living cells, serving as precursor for inositol phosphates, phosphatidylinositol (PI) and phosphatidylinositol phosphate (PIP) lipids [3]. It plays an important role in cell morphogenesis and cytogenesis, lipid synthesis, formation of cell membrane structures and cell growth [4,5]. It also plays a key role in some important intracellular signaling pathways and altered MI metabolism was associated with many clinical diseases, such as neural tube defects (NTDs) [6,7], Down syndrome [8], Alzheimer's disease [9] and diabetic glomerulopathy [10]. Furthermore, the result of four randomized controlled trials (RCTs) on MI supplementation to preterm infants showed that MI supplementary to preterm infants could significantly reduce adverse neonatal outcomes including neonatal/infant death, retinopathy of prematurity and intraventricular haemorrhage retinopathy of prematurity [11]. Recent clinical studies showed that MI combined with alpha lipoic acid supplementation could reduce insulin resistance and increase HDL-C in postmenopausal women affected by metabolic syndrome [12]. Therefore, a sensitive and fast method for the clinical detection of MI could be useful.

MI can be detected by 3 different methods, including enzymatic method, high-performance liquid chromatography-mass spectrometry and gas chromatography–mass spectrometry. The high sensitive enzymatic assay is based on the principle of NADH cycling [13,14]. It consists of several steps such as destruction of endogenous NADH, MI oxidation, destruction of excess NAD^+ and NADH cycling. Another simple and sensitive enzymatic method uses thio-NAD^+, NADH and thermostable MI

Abbreviations: MI, myo-inositol; TMCS, trimethylchlorosilane; HMDS, hexamethyldisilazane; *N*,*N*-DMF, *N*,*N*-dimethylformamide; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate.

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dehydrogenase to quantitate MI in biological samples [15]. However, bacterial cultures are needed in the method, which makes the clinical detection more complicated. Moreover, the enzymatic methods are often complicated for the interferences from detergents, salts and other compounds from biological samples [16]. HPLC-MS is another accurate and sensitive method for quantitative detection. Due to the complexity of the biological matrix, sample should undergo complex and time-consuming pretreatment process [17], which makes the method unsuitable for the large-scale clinical application. The methods of GC or GC/MS have been reported for biological MI detection, and showed good accuracy and sensitivity [18–20]. However, none of these methods could be operated using the low volume samples (<100 μl) available from patients, especially infants.

2. Materials and methods

2.1. Chemicals and reagents

MI (Sigma-Aldrich), isotope labeled MI ([$^2H_6$] myo-inositol (J&K Scientific Ltd.); trimethylchlorosilane (TMCS), hexamethyldisilazane (HMDS) and *N*,*N*-dimethyl formamide (*N*,*N*-DMF) (Alfa-Aesar) were purchased. All the other chemicals were from Fisher Scientific.

2.2. GC–MS

Analysis of MI was performed on an Agilent 7890A gas chromatography equipment combined with 5975C mass spectrometer (Agilent Technologies). A fused silica HP-5MS capillary column (length, 30 m; i.d., 0.25 mm; film thickness, 0.25 μm; Agilent) was used. The helium as carrier gas was set at a constant flow rate of 1 ml/min. 1 μl aliquot of sample was injected in the splitless mode. The initial oven temperature was 140 °C, increased by 20 °C/min to 220 °C, and then 5 °C/min to 260 °C for 5 min. The total analysis time was 17 min. The most abundant fragment ion (*m*/*z* 73.1) was used for quantification under the electron impact ionization at 70 eV in the selected ion monitor (SIM) mode. The mass spectrum of MI derivative was shown in Fig. 1.

2.3. Preparation of calibration standards and quality control samples

Plasma MI determination was performed by external standard method. Stock solution of MI was prepared by dissolving the appropriate amount of substance in deionized water at 500 mg/l, and derivatized as described in Section 2.4 to yield the MI derivative solution to 2 mg/l. Consideration the effect of plasma matrix, calibration solutions were prepared by diluting the MI derivative solution with 1 ml derivative human plasma pool to the following concentrations: 20, 50, 100, 200 and 500 μg/l. For validation study, quality control (QC) samples were fortified by adding different concentrations of standards into 50 μl human plasma pool to obtain fortified plasma samples. All stock solution, working solutions and QC samples were divided and stored at −80 °C.

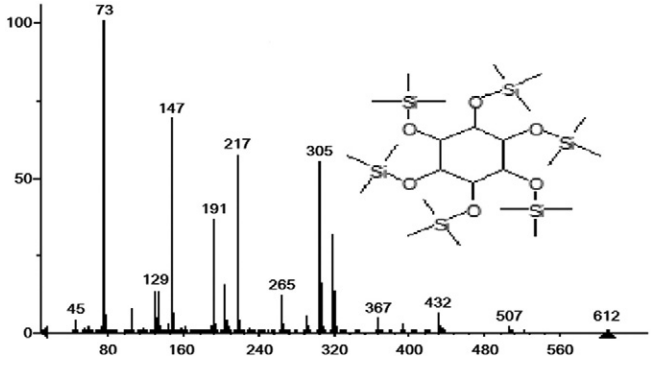

Fig. 1. Mass spectrum of MI (200 μg/l) derivatives under the selected ion monitoring mode.

2.4. Sample selection and pretreatment

Pregnant women from Lvliang mountain area of Shanxi Province in North China were recruited at local hospital [21]. Maternal plasma from 20 NTD-affected and control pregnancies were randomly selected (Supplementary Table 1).

Ten milliliters of anhydrous ethanol was added to the 30 μl plasma sample, and evaporated dry at 70 °C by the rotary evaporator. Five milliliters of TMCS/HMDS/*N*,*N*-DMF (1: 2: 8, v/v/v) was added and derived at 70 °C for 1 h, then added 5 ml hexanes and 10 ml saturated NaCl solution after cool down to room temperature. The mixtures were vortexed for 1 min and centrifuged at 6,000 rpm at room temperature for 5 min. The supernatant was evaporated to be dry under a stream of nitrogen at 40 °C. The residues were reconstituted with 1 ml hexanes and injected into the GC–MS system. The concentration of MI in human plasma was within the linear range and has been diluted 20 times before GC–MS detection.

2.5. Method verification with stable-isotope labeled method

A stable-isotope labeled MI ([$^2H_6$] myo-inositol) was added to the plasma samples with a range of (unlabeled) MI enrichments to confirm that the daily calibration curve and external standard approach was yielding accurate results. The derivatized internal standard solutions (0.1 mg/l) were mixed with the range of (unlabeled) MI to obtain the working standard solution: 20, 50, 100, 200 and 500 μg/l. QC samples at 3 different fortified MI levels with the isotope labeled MI were detected in triplicates. Three plasma samples were selected randomly to evaluate the MI level by the internal standard approach and external standard approach, respectively.

2.6. Alpha fetal protein (AFP) detection

Twenty-five microliters of plasma was used for the detection by the protocol of Diagnostic Kit of AFP with chemiluminescence quantitative immunoassay (Beijing Yuande Bio-Medical Engineering Co., Ltd.). Briefly, standard sample and plasma was mixed with enzyme conjugate respectively and incubated at 37 °C for 2 h. After washing 5 times, chemiluminescent solution was added and relative luminosity was detected to quantify the plasma AFP levels by Chemiluminescent Immunoenzyme Assay Access Immunoassay system II (Beckman Coulter).

2.7. Statistical analysis

All statistical analyses were performed with SPSS 16.0. The paired *t*-test was used to analyze the agreement between internal standard approach and external standard method. An independent *t*-test and CV were used to compare the biochemical parameters between the NTD subgroups and control. A $P < 0.05$ was considered as significantly different.

3. Results

3.1. The optimized method

The optimized derivatization conditions were as follows: 5 ml mixture of TMCS/HMDS/*N*,*N*-DMF at 70 °C for 60 min, shaking at 10 min intervals. The MI concentration was proportional to the injection volume and the minimal plasma volume for detection was 30 μl.