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Keto acid profiling analysis as ethoxime/*tert*-butyldimethylsilyl derivatives by gas chromatography–mass spectrometry

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ABSTRACT

Organic acids, including keto acids, are key intermediates of central pathways in cellular metabolism. In this study, a comprehensive and reliable method was developed and optimized for the simultaneous measurement of 17 keto acids in various biological samples. The keto acids were converted to solvent extractable forms by ethoximation followed by *tert*-butyldimethylsilylation for direct analysis by gas chromatography–mass spectrometry in selected ion monitoring mode. The proposed method was precise (0.05–8.3, % RSD) and accurate (−10.5 to 5.3, % RE) with low limit of detection (0.01–0.5 ng/mL) and good linearity ($r > 0.995$) in the range of 0.01–5.0 μg/mL. This was suitable for profiling analysis of targeted keto acids in human plasma, urine and rat brain tissue.

1. Introduction

Organic acids are the most diverse and prominent metabolites that exhibit a vital role in the intermediary metabolism. They are involved in major pathways in biological systems such as the tricarboxylic acid (TCA) cycle, fatty acid β-oxidation, and amino acid metabolism. Changes in the organic acids levels are associated with a variety of metabolic disorders such as diabetes mellitus, maple syrup urine disease (MSUD), organic acidopathies, organic acidemias, and ketoacidosis [1–3]. In particular, MSUD is caused by deficiency of the branched-chain α-ketoacid dehydrogenase, which leads to disruption in the conversion of the branched-chain amino acids such as leucine, isoleucine, and valine to α-ketoisocaproic acid, α-keto-β-methylvaleric acid and α-ketoisovaleric acid, respectively. These α-keto acids play an important role in equilibrium with their precursor amino acids [2–5]. Another important keto acid, acetoacetic acid is accumulated during fatty acid metabolism when glucose is not readily available. Acetoacetate serves as a crucial metabolic fuel and plays a key role in sparing glucose utilization, especially, provides an alternative source of energy for the brain during periods of glucose deficiency. Abnormally large quantities of ketone bodies are found in patients with ketoacidosis, therefore ketone bodies have been used as markers of energy metabolism [6]. Pyruvic acid is a precursor of acetyl coenzyme A, which is the primary material for energy production via the TCA cycle. Other keto acids such as α-ketoglutaric acid and oxaloacetic acid are both vital intermediates of amino acid metabolism and the TCA cycle. Abnormal levels of these keto acids and organic acid intermediates are frequently associated with TCA cycle dysfunction and enzyme deficiency, which are causes of various diseases including tumor in human [1,2,7,8].

The most important objective of quantitative analysis of organic acids is to detect negligible changes in metabolite profiles between normal and abnormal, physiological and pathological states [9], which provide valuable information for insight into the metabolic pathways as well as for screening of metabolic disorders. The development of sensitive, rapid and comprehensive techniques is required for simultaneous quantitative measurement of an enormous number of organic acids in physiological fluids. Due to its high sensitivity, high resolution, accuracy and reliability, gas chromatography–mass spectrometry (GC–MS) is a powerful technique and has been widely used for metabolic profiling analysis in

Abbreviations: TCA, tricarboxylic acid; MSUD, maple syrup urine disease; GC–MS, gas chromatography–mass spectrometry; MO/TMS, methoxime/trimethylsilyl; EO, ethoxime; TBDMS, *tert*-butyldimethylsilyl; SIM, selected ion monitoring; IS, internal standard; TDPA, 3,3′-thiodipropionic acid; MTBSTFA, N-methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide; RSD, relative standard deviation; RE, relative error; LOD, limit of detection.

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diagnostic investigation [4,9–14]. Since keto acids are highly polar, they were difficult to extract by organic solvent and are impossible to analyze by GC without derivatization, therefore an appropriate derivatization procedure is necessary to isolate keto acids present in aqueous media, to protect their labile carbonyl groups and to block active protons of carboxylic functions prior to analysis by GC–MS. Derivatization of keto acids has been performed to reduce their polarity and improve their volatility and thermal stability to enable analysis by GC–MS. Keto acids are generally converted to methoxime/trimethylsilyl (MO/TMS) ethers [4,10–14], ethoxime/TMS (EO/TMS) ethers [14] or MO/*tert*-butyldimethylsilyl (MO/TBMDS) derivatives [14–18] prior to separation and detection by GC–MS. Although numerous methods have been developed for analysis of organic acids, only a few keto acids have been analyzed in these studies. Furthermore, no study has reported optimized reaction conditions for EO/TBDMS derivatization of keto acids.

The main purpose of this work was to develop a new protocol based on EO/TBDMS derivatives and GC–MS analysis in selected ion monitoring (SIM) mode for profiling analysis of keto acids. A variety of parameters that can affect the chemical derivatization process were identified and optimized. This derivatization method appeared to be suitable for recovery of keto acids from biological aqueous samples. The superior chromatographic and mass spectral properties of EO/TBDMS derivatives, combined with the sensitivity of GC–MS, provided the method of choice for simultaneous separation and determination of these compounds. Application of the present method allowed quantitative analysis of seventeen keto acids in human urine and plasma and rat brain tissue with acceptable precision and accuracy.

2. Experimental

2.1. Chemicals and reagents

The following 17 keto acid standards were purchased from several vendors including Sigma–Aldrich (St. Louis, MO, USA) and Tokyo Chemical Industry (Kita-ku, Tokyo, Japan): glyoxylic, pyruvic, α-ketobutyric, α-ketoisovaleric, acetoacetic, α-ketovaleric, α-ketoisocaproic, α-keto-β-methylvaleric, α-ketocaproic, α-ketooctanoic, α-keto-γ-methiolbutyric, ketomalonic, oxaloacetic, α-ketoglutaric, α-ketoadipic, β-ketoadipic and γ-ketopimelic acids. Ethoxyamine hydrochloride and 3,3′-thiodipropionic acid (TDPA) were also obtained from Sigma–Aldrich. N-methyl-N-(*tert*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) + 1% *tert*-butyldimethylchlorosilane was provided by Thermo Scientific (Bellefonte, PA, USA). Toluene, diethyl ether, ethyl acetate, and dichloromethane (pesticide grade) were obtained from Kanto Chemical (Chuo-ku, Tokyo, Japan). Sodium hydroxide was supplied by Duksan (Ansan, Gyeonggi-do, South Korea), sulfuric acid was from Samchun Pure Chemical Co. Ltd. (Pyeongtaek, Gyeonggi-do, South Korea). All other chemicals were analytical reagent grade.

2.2. Gas chromatography–mass spectrometry

Derivatized samples were analyzed in both scan and SIM modes by using an Agilent 6890N gas chromatograph interfaced to an Agilent 5975B mass-selective detector (70 eV, electron ionization source). The mass spectra were scanned in range 50–650 μm at a rate of 0.99 scans/s. The temperatures of the injector, interface and ion source were 260, 300 and 230 °C, respectively. An HP Ultra-2 (Agilent Technologies, Santa Clara, CA, USA) cross-linked capillary column coated with 5% phenyl–95% methylpolysiloxane bonded phase (25 m × 0.20 mm I.D., 0.11 μm film thickness) was used for all analyses. Helium was used as the carrier gas at a flow rate of 0.5 mL/min in constant flow mode. Samples (1 μL) were introduced in split-injection mode (10:1), the oven temperature was set initially at 100 °C (2 min), then increased to 250 °C at rate of 5 °C/min and finally programmed to 300 °C at rate of 20 °C/min (5 min). In SIM mode, three characteristic ions for each compound were used for peak confirmation, while one target ion was selected for quantification.

2.3. Preparation of standard solutions

Standard stock solutions of keto acids were individually prepared at 10 mg/mL in distilled water. Keto acids were divided into two groups to ensure that all compounds in the mixture were completely separated from others. Standard working solutions at 0.5, 0.1 and 0.005 mg/mL were made from stock solutions by consecutive dilution with distilled water. A solution of 3,4-dimethoxybenzoic acid in methanol at 0.01 mg/mL was prepared from its stock solution (10 mg/mL) and used as the internal standard (IS). All standard solutions were stored at 4 °C.

2.4. Sequential ethoximation combined tert-butyldimethylsilylation

Mixed standard solutions containing targeted keto acids were spiked to distilled water (1 mL). The carbonyl groups present in keto acids were converted into EO derivatives by reacting with ethoxyamine hydrochloride (5 mg) in neutral conditions and heated at 60 °C for 30 min. The reaction mixture then was acidified to pH 1–2 with 10% sulfuric acid solution, saturated with sodium chloride, and extracted with diethyl ether (3 mL) followed by ethyl acetate (2 mL). After addition of triethylamine (5 μL), the combined extracts were evaporated under a gentle stream of nitrogen (40 °C) to dryness. The residues were added to toluene (20 μL) as solvent, MTBSTFA (20 μL) as silylation reagent and reacted at 60 °C for 30 min to form EO/TBDMS derivatives for direct GC–MS analysis.

2.5. Method optimization

Several variables that may affect the derivatization process, including pH, amount of antioxidant, reaction temperature and time of EO process, were investigated and optimized. For this purpose, IS (0.5 μg) was added to mixed standards (at 1 μg/mL of each) and the EO reaction was subsequently performed followed by TBDMS as described above. Initially, for identification to identify the effect of pH, the EO/TBDMS reactions were conducted in different media with pH ranging from below 2 to above 13. The influence of TDPA as an antioxidant was studied by adding different amounts of TDPA ranging from 0 to 150 μg to the EO reaction. Reaction temperature from 25 °C to 90 °C and reaction time ranging from 10 min to 120 min were also examined to obtain optimal reaction conditions.

2.6. Method validation for assay of keto acids

In order to investigate the applicability of the method to biological samples, all validation experiments were performed in triplicate on the same day under optimized conditions. The calibration curves were constructed in range of 0.01–5.0 μg/mL. Different amounts of standard (0.01, 0.02, 0.05, 0.1, 0.5, 1.0, 2.0 and 5.0 μg) each containing IS (0.2 μg) were converted into EO-TBDMS derivatives as described above. The quantitative analyses were based on the peak area ratios of keto acids to that of IS. The response of each keto acid was calculated as the sum of *syn* and *anti* isomers. The values of slope, intercept and correlation coefficient were obtained by linear regression analysis from the calibration curves constructed based on the peak area ratios versus corresponding concentration ratios of analytes and IS. Intra- and inter-day precision as relative standard