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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

deviation (% RSD) were evaluated from measurements performed under the same conditions within-day and between three different days. The accuracy was reported as relative error (% RE), which was determined by comparing the responses of samples spiked with standard at low, medium and high concentrations. Limit of detection (LOD) was estimated from the concentration that gave a response higher than the background signal plus three times its standard deviation.

For evaluation of stability, the derivatized keto acids at 0.1 and 1.0 μg/mL were prepared in triplicate, stored at 4 °C and thawed at room temperature for 1 h prior to injection. The stability of the EO/TBDMS derivatives was evaluated by repeated measurement of peak area ratio of keto acid standards to IS, analyzed for 20 consecutive days at an interval of 1 or 2 days.

2.7. Sample preparation for measurement of keto acids in human urine, human plasma and rat brain tissue

In this study, human urine, human plasma and rat brain tissue were used. Normal urine and plasma specimens were obtained from healthy volunteers and stored at −20 °C without any additives. Before experiments, samples were thawed at room temperature and vortex-mixed (2 min). Four healthy rats were sacrificed and whole brain tissues were rapidly collected and immediately stored in a deep freezer at −80 °C until analyzed. Brain tissues were to added distilled water and finely homogenized in an ice water bath using the T10 basic Ultra-Turrax® disperser (IKA-Werke GmbH & Co.KG, Staufen, Germany), and the homogenates were pooled.

Aliquots of urine (0.2 mL) were processed identically for the sequential EO/TBDMS procedure described above, followed by GC–MS analyses. The plasma sample (0.1 mL) and homogenized brain sample (equivalent to 20 mg of brain tissue) containing IS (0.2 μg) were diluted with distilled water (0.4 mL); acetonitrile (0.5 mL) was then added and vortex-mixed for 2 min. The mixtures were centrifuged (14,000 rpm, 10 min) for protein precipitation and the supernatant layers were subjected to the EO/TBDMS reactions prior to GC–MS analysis.

3. Results and discussion

3.1. Ethoximation with subsequent tert-butyldimethylsilylation

Most keto acids are not stable enough for GC analysis, thus modification of polar functional groups is usually required. Generally, the carbonyl groups of keto acids are derivatized to form either MO or EO derivatives, while the carboxyl substitutes are converted into either TMS esters or TBDMS esters [4,10]. Conversion of keto acids to MO/TBDMS derivatives was developed by Kim et al. [15] and Paik et al. [16–18] in our laboratory and these derivatives appeared to be suitable for analysis by GC–MS. Compared with TMS ethers, TBDMS derivatives are much more stable against hydrolysis than TMS ethers [19], and their mass spectra exhibit intense characteristic ions of $[M-57]^+$ as predictable fragments that are useful for peak interpretation. In addition, MO/TBDMS derivatives show good chromatographic properties [15–18]. Although the MO/TBDMS is more common technique for derivatization of keto acids, we

Table 1
Mass spectral data of keto acids as ethoxime/*tert*-butyldimethylsilyl derivatives.

No.	Compound	Mass spectral data set[a]						
		Mr	[M−15]	[M−57]	[M−85]	*m/z* 103	*m/z* 75	Other characteristic ions
1	Glyoxylic acid[b]	231	216 (2)	174 (100)	146 (44)	103 (2)	75 (24)	73 (16), 175 (11)
2	Pyruvic acid-1[b]	245	230 (0)	188 (34)	160 (2)	103 (100)	75 (42)	73 (31), 59 (15), 58 (11), 104 (10)
2′	Pyruvic acid-2[b]	245	230 (2)	188 (100)	160 (35)	103 (4)	75 (26)	73 (19), 189 (14), 74 (12)
3	α-Ketobutyric acid-1[b]	259	244 (0)	202 (28)	174 (2)	103 (100)	75 (34)	73 (24), 59 (11)
3′	α-Ketobutyric acid-2[b]	259	244 (2)	202 (100)	174 (24)	103 (7)	75 (28)	73 (18), 203 (14)
4	α-Ketoisovaleric acid-1[b]	273	258 (0)	216 (28)	188 (0)	103 (100)	75 (31)	73 (27), 59 (10)
4′	α-Ketoisovaleric acid-2[b]	273	258 (3)	216 (100)	188 (6)	103 (20)	75 (54)	73 (31), 217 (16), 59 (11), 128 (10)
5	Acetoacetic acid-1[b]	259	244 (0)	202 (32)	174 (0)	103 (64)	75 (100)	73 (43), 156 (29), 112 (17)
5′	Acetoacetic acid-2[b]	259	244 (1)	202 (42)	174 (1)	103 (21)	75 (38)	57 (100), 97 (94), 147 (33), 109 (31)
6	α-Ketovaleric acid-1[b]	273	258 (0)	216 (26)	188 (1)	103 (100)	75 (31)	73 (25), 59 (10), 104 (9)
6′	α-Ketovaleric acid-2[b]	273	258 (2)	216 (100)	188 (17)	103 (9)	75 (29)	73 (19), 217 (15), 115 (14)
7	α-Ketoisocaproic acid-1[b]	287	272 (0)	230 (27)	202 (0)	103 (100)	75 (31)	73 (29), 57 (14)
7′	α-Ketoisocaproic acid-2[b]	287	272 (2)	230 (100)	202 (6)	103 (17)	75 (28)	73 (22), 231 (16), 115 (11)
8	α-Keto-β-methylvaleric acid-1[b]	287	272 (0)	230 (33)	202 (1)	103 (100)	75 (32)	57 (36), 73 (31), 59 (10)
8′	α-Keto-β-methylvaleric acid-2[b]	287	272 (4)	230 (100)	202 (8)	103 (21)	75 (28)	73 (26), 115 (17), 177 (16), 231 (14)
9	α-Ketocaproic acid-1[b]	287	272 (0)	230 (25)	202 (1)	103 (100)	75 (32)	73 (25), 104 (9)
9′	α-Ketocaproic acid-2[b]	287	272 (2)	230 (100)	202 (16)	103 (9)	75 (29)	73 (18), 231 (16)
10	α-Ketooctanoic acid-1[b]	315	300 (0)	258 (25)	230 (1)	103 (100)	75 (31)	73 (26), 104 (10)
10′	α-Ketooctanoic acid-2[b]	315	300 (2)	258 (100)	230 (9)	103 (8)	75 (21)	259 (17), 73 (14)
11	α-Keto-γ-methiolbutyric acid-1[b]	305	290 (0)	248 (56)	220 (4)	103 (100)	75 (96)	73 (68), 260 (23)
11′	α-Keto-γ-methiolbutyric acid-2[b]	305	290 (2)	248 (90)	220 (0)	103 (11)	75 (34)	61 (100), 73 (46), 260 (32), 200 (31)
12	Ketomalonic acid[c]	389	374 (2)	332 (81)	304 (0)	103 (6)	75 (12)	73 (100), 147 (26), 84 (21), 333 (20)
13	Oxaloacetic acid-1[c]	403	388 (0)	346 (51)	318 (2)	103 (32)	75 (29)	73 (100), 147 (22), 347 (15), 98 (14)
13′	Oxaloacetic acid-2[c]	403	388 (2)	346 (82)	318 (0)	103 (7)	75 (24)	73 (100), 147 (39), 347 (21), 98 (20)
14	α-Ketoglutaric acid-1[c]	417	402 (3)	360 (84)	332 (2)	103 (31)	75 (58)	73 (100), 147 (23), 361 (22), 156 (20)
14′	α-Ketoglutaric acid-2[c]	417	402 (3)	360 (100)	332 (0)	103 (8)	75 (41)	73 (96), 147 (34), 361 (26), 156 (24)
15	α-Ketoadipic acid-1[c]	431	416 (2)	374 (50)	346 (3)	103 (46)	75 (73)	73 (100), 147 (17), 375 (14), 175 (12)
15′	α-Ketoadipic acid-2[c]	431	416 (2)	374 (64)	346 (0)	103 (13)	75 (68)	73 (100), 147 (36), 156 (24), 170 (19)
16	β-Ketoadipic acid-1[c]	431	416 (3)	374 (100)	346 (0)	103 (14)	75 (53)	73 (97), 375 (26), 147 (22), 376 (11)
16′	β-Ketoadipic acid-2[c]	431	416 (4)	374 (100)	346 (0)	103 (10)	75 (46)	73 (83), 375 (27), 147 (21), 376 (10)
17	γ-Ketopimelic acid[c]	445	430 (5)	388 (100)	360 (0)	103 (12)	75 (45)	73 (72), 389 (28), 129 (16), 147 (14)

Ultra-2 capillary column (25 m × 0.20 mm I.D., 0.11 μm d_f), was initially set 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).

[a] *m/z* values with relative abundances of ions (%) in parentheses.

[b] As mono-ethoxime/mono-*tert*-butyldimethylsilyl derivatives.

[c] As mono-ethoxime/di-*tert*-butyldimethylsilyl derivatives.

Keto acids as *syn* and *anti* isomers were denoted 1 and 2.

Table 2
Selected ions for keto acids as ethoxime/*tert*-butyldimethylsilyl derivatives in SIM mode by GC–MS.

No.	Compound	Selected ions (*m*/*z*)
1	Glyoxylic acid[a]	146, 174, 216
2	Pyruvic acid-1[a]	103, 188, 230
2′	Pyruvic acid-2[a]	160, 188, 230
3	α-Ketobutyric acid-1[a]	103, 202, 244
3′	α-Ketobutyric acid-2[a]	174, 202, 244
4	α-Ketoisovaleric acid-1[a]	103, 216, 258
4′	α-Ketoisovaleric acid-2[a]	188, 216, 258
5	Acetoacetic acid-1[a]	156, 202, 233
5′	Acetoacetic acid-2[a]	202, 233, 244
6	α-Ketovaleric acid-1[a]	103, 216, 258
6′	α-Ketovaleric acid-2[a]	188, 216, 258
7	α-Ketoisocaproic acid-1[a]	103, 230, 272
8	α-Keto-β-methylvaleric acid-1[a]	103, 230, 272
8′	α-Keto-β-methylvaleric acid-2[a]	220, 230, 272
7′	α-Ketoisocaproic acid-2[a]	202, 230, 272
9	α-Ketocaproic acid-1[a]	103, 230, 272
9′	α-Ketocaproic acid-2[a]	202, 230, 272
10	α-Ketooctanoic acid-1[a]	245, 258, 300
10′	α-Ketooctanoic acid-2[a]	230, 258, 300
11	α-Keto-γ-methiolbutyric acid-1[a]	248, 260, 290
11′	α-Keto-γ-methiolbutyric acid-2[a]	248, 260, 290
12	Ketomalonic acid[b]	260, 332, 374
13	Oxaloacetic acid-1[b]	331, 346, 388
13′	Oxaloacetic acid-2[b]	147, 346, 388
14	α-Ketoglutaric acid-1[b]	147, 360, 402
14′	α-Ketoglutaric acid-2[b]	147, 360, 402
15	α-Ketoadipic acid-1[b]	374, 386, 416
15′	β-Ketoadipic acid-2[b]	328, 374, 416
16	α-Ketoadipic acid-1[b]	374, 386, 416
16′	β-Ketoadipic acid-2[b]	328, 374, 416
17	γ-Ketopimelic acid[b]	388, 430, 445
	3,4-Dimethoxybenzoic acid (IS)	165, 195, 239

The underlined ions were used as quantification ions.
[a] As mono-ethoxime/mono-*tert*-butyldimethylsilyl derivatives.
[b] As mono-ethoxime/di-*tert*-butyldimethylsilyl derivatives.
Keto acids as *syn* and *anti* isomers were denoted 1 and 2.

attempted to develop and optimize a new derivatization method, and the EO/TBDMS derivatives of keto acids showed superior properties. The difference between the EO/TBDMS and the MO/TBDMS method is that, in the first derivatization, keto acids are converted into EO and MO derivatives, respectively. They differ on one CH_2 group, and this difference provided some advantages. Firstly, polarity of EO derivative of a keto acid is less than that of corresponding MO derivative. Therefore, EO derivatives are easier extracted with organic solvents, which enhance extraction efficiency. Secondly, the introduction of EO moiety resulted in higher molecular mass derivatives compare to MO/TBDMS. The monitoring of high mass ions is preferred in quantitative analysis because the influence of background interference from complex matrixes may be avoidable. Moreover, EO/TBDMS derivatives exhibited a good stability and permitted development of a sensitive GC–MS method.

In this study, the carbonyl groups in keto acids were initially inactivated by conversion into their EO derivatives and then extracted with organic solvents. The carboxyl groups were subsequently derivatized with MTBSTFA as the silylation reagent to form EO/TBDMS derivatives. The electron impact mass spectral data of seventeen keto acids as EO/TBDMS derivatives are summarized in Table 1. For fifteen asymmetrical keto acids except for glyoxylic acid, two peaks were observed for each compound, corresponding to *syn* and *anti* isomers. The mass spectral patterns between the two forms were different due to the difference in geometric structure; in contrast, symmetrical keto acids including ketomalonic and γ-ketopimelic acids had one isomer and thus gave a single peak in the chromatogram. Keto acids with a single carboxyl group were elucidated from mono-EO/mono-TBDMS derivatives, while dicarboxylic acids including ketomalonic, oxaloacetic, α-ketoglutaric, α-ketoadipic, β-ketoadipic and γ-ketopimelic acids were mono-EO/di-TBDMS derivatives. The molecular ions of most keto acids were absent, except for γ-ketopimelic, α- and β-ketoadipic acids. All mass spectra exhibited characteristic fragments ions of $[M-15]^+$ and $[M-57]^+$, which were formed by loss of CH_3 and $C(CH_3)_3$ from molecular ions, respectively. These specific fragments ions were predictable and allowed rapid structural interpretation. The peaks at $[M-85]^+$ were formed by elimination of C_2H_4 from the $[M-57]^+$ ions. For most keto acids, $[M-57]^+$ ions were either base peaks or prominent ions and were selected for quantification by SIM mode (Table 2), whereas $[M-15]^+$ ions were observed with low intensity (less than 5%). The EO/TBDMS derivatives of keto acids were clearly identified on the basis of the ratio of their $[M-15]^+$ and $[M-57]^+$ ions, and permitted development of a sensitive and selective GC–MS–SIM method. Prominent ions of *m*/*z* 103 and *m*/*z* 75 were assumed to be $[(CH_3)_2SiOCHO]^+$ and $[(CH_3)_2SiOH]^+$, respectively.

3.2. Method optimization

Oxime formation has been widely used for derivatization of keto acids under alkaline conditions [15–18] or in the presence of pyridine [11–14,20]. However, an excess amount of pyridine

Table 3
Effect of pH on the derivatization of keto acids as ethoxime/*tert*-butyldimethylsilyl derivatives.

No.	Compound	Normalized values[a] (mean ± SD)				
		pH < 2	pH = 4	pH = 7	pH = 10	pH > 13
1	Glyoxylic acid	0.99 ± 0.01	1.03 ± <0.01	1.0 ± 0.01	1.51 ± 0.04	0.05 ± 0.01
2	Pyruvic acid	0.94 ± 0.04	1.25 ± 0.04	1.0 ± 0.03	1.15 ± 0.08	1.44 ± 0.05
3	α-Ketobutyric acid	0.96 ± 0.04	1.26 ± 0.04	1.0 ± 0.03	1.13 ± 0.08	0.91 ± 0.02
4	α-Ketoisovaleric acid	0.62 ± 0.02	1.19 ± 0.01	1.0 ± 0.01	0.98 ± 0.05	0.72 ± 0.03
5	Acetoacetic acid	0.30 ± 0.02	0.61 ± 0.03	1.0 ± 0.02	0.50 ± 0.04	0.38 ± 0.02
6	α-Ketovaleric acid	0.97 ± 0.03	1.16 ± 0.02	1.0 ± 0.02	1.07 ± 0.06	0.88 ± 0.02
7	α-Ketoisocaproic acid	0.98 ± 0.03	1.12 ± 0.02	1.0 ± 0.02	0.89 ± 0.05	0.72 ± 0.02
8	α-Keto-β-methylvaleric acid	0.98 ± 0.03	1.12 ± 0.02	1.0 ± 0.02	0.89 ± 0.05	0.72 ± 0.02
9	α-Ketocaproic acid	0.94 ± 0.02	1.07 ± 0.02	1.0 ± 0.02	0.99 ± 0.04	0.84 ± 0.01
10	α-Ketooctanoic acid	0.95 ± 0.01	0.96 ± <0.01	1.0 ± 0.01	1.04 ± 0.04	0.95 ± 0.01
11	α-Keto-γ-methiolbutyric acid	1.04 ± 0.01	0.87 ± 0.02	1.0 ± <0.01	0.64 ± 0.01	0.04 ± <0.01
12	Ketomalonic acid	1.16 ± <0.01	1.00 ± 0.04	1.0 ± 0.06	0.75 ± 0.05	0.77 ± 0.01
13	Oxaloacetic acid	1.26 ± 0.01	1.05 ± 0.03	1.0 ± 0.02	0.64 ± 0.06	0.76 ± 0.06
14	α-Ketoglutaric acid	0.86 ± <0.01	0.94 ± 0.01	1.0 ± <0.01	0.76 ± 0.03	0.67 ± 0.03
15	α-Ketoadipic acid	0.87 ± <0.01	0.96 ± <0.01	1.0 ± <0.01	0.91 ± 0.01	0.75 ± 0.02
16	β-Ketoadipic acid	0.87 ± <0.01	0.97 ± <0.01	1.0 ± <0.01	0.92 ± 0.01	0.75 ± 0.02
17	γ-Ketopimelic acid	0.02 ± <0.01	1.00 ± 0.01	1.0 ± 0.01	0.06 ± <0.01	0.02 ± <0.01

[a] All measurements were expressed as normalized values of peak area ratios to the corresponding mean values at pH = 7 (mean ± SD).

Table 4
Effect of reaction temperature on the derivatization of keto acids as ethoxime/*tert*-butyldimethylsilyl derivatives.

No.	Compound	Normalized values[a] (mean ± SD)				
		Room temp.	50 °C	60 °C	80 °C	90 °C
1	Glyoxylic acid	0.92 ± 0.03	0.93 ± 0.02	1.0 ± 0.01	0.99 ± 0.03	0.99 ± <0.01
2	Pyruvic acid	0.93 ± 0.06	0.77 ± 0.11	1.0 ± 0.04	0.71 ± 0.03	0.64 ± 0.06
3	α-Ketobutyric acid	0.94 ± 0.07	0.80 ± 0.11	1.0 ± 0.04	0.40 ± 0.02	0.20 ± 0.02
4	α-Ketoisovaleric acid	1.12 ± 0.04	0.96 ± 0.05	1.0 ± 0.01	0.39 ± 0.03	0.18 ± 0.01
5	Acetoacetic acid	1.01 ± 0.35	0.75 ± 0.10	1.0 ± 0.07	0.80 ± 0.07	0.84 ± 0.12
6	α-Ketovaleric acid	0.95 ± 0.05	0.86 ± 0.07	1.0 ± 0.02	0.43 ± 0.02	0.22 ± 0.02
7	α-Ketoisocaproic acid	1.01 ± 0.04	0.90 ± 0.04	1.0 ± 0.01	0.56 ± 0.02	0.35 ± 0.01
8	α-Keto-β-methylvaleric acid	1.01 ± 0.04	0.90 ± 0.04	1.0 ± 0.01	0.55 ± 0.02	0.35 ± 0.01
9	α-Ketocaproic acid	0.96 ± 0.03	0.92 ± 0.03	1.0 ± 0.01	0.45 ± 0.02	0.24 ± 0.01
10	α-Ketooctanoic acid	0.96 ± 0.04	1.00 ± 0.02	1.0 ± 0.02	0.50 ± 0.02	0.29 ± 0.02
11	α-Keto-γ-methiolbutyric acid	0.55 ± 0.08	1.02 ± 0.14	1.0 ± 0.05	1.32 ± 0.16	0.84 ± 0.07
12	Ketomalonic acid	1.42 ± 0.14	1.00 ± 0.09	1.0 ± 0.04	0.09 ± 0.01	0.02 ± 0.01
13	Oxaloacetic acid	0.31 ± 0.03	0.59 ± 0.05	1.0 ± 0.01	1.16 ± 0.12	1.32 ± 0.08
14	α-Ketoglutaric acid	1.02 ± 0.06	0.90 ± 0.03	1.0 ± 0.02	0.85 ± 0.07	0.67 ± 0.01
15	α-Ketoadipic acid	1.02 ± 0.06	0.94 ± 0.02	1.0 ± 0.02	0.73 ± 0.06	0.46 ± 0.01
16	β-Ketoadipic acid	1.02 ± 0.06	0.93 ± 0.02	1.0 ± 0.02	0.72 ± 0.06	0.45 ± 0.01
17	γ-Ketopimelic acid	0.60 ± 0.07	0.88 ± 0.06	1.0 ± 0.03	1.12 ± 0.09	0.90 ± 0.01

[a] All measurements were expressed as normalized values of peak area ratios to the corresponding mean values at 60 °C (mean ± SD).

may impair the GC column stationary phase as well as produce by-products. In addition, it is not easy to extract or clean-up these highly polar compounds in biological fluids into an organic solvent phase prior to this derivatization step. In this study, the conversion of keto acids into solvent extractable form as their EO derivatives efficiently enhanced the recovery of these components from aqueous matrix. Derivatization is one of the most critical steps in sample preparation for analysis by GC–MS. A variety of variables may affect yield of EO reaction and should be investigated in order to enhance the reaction yield and determine optimal conditions.

3.2.1. Effect of pH

The effect of pH on the EO/TBDMS derivatization of keto acids is shown in Table 3. For most keto acids, the yields of the EO reaction were highest when the reaction medium was either neutral or mild acidic pH. The reaction productivities for most keto acids were decreased in both basic and strong acidic conditions, probably due to decomposition of labile keto acids. The abundance of glyoxylic acid and α-keto-γ-methiolbutyric acid (a sulfur-containing oxocarboxylic acid) above pH 13 and of γ-ketopimelic acid at strong acid (pH < 2) and basic pH was drastically reduced. These results indicated that the EO reaction can be performed under mild conditions in aqueous solutions; neither basic nor acidic conditions are required. Therefore, we suggest that EO/TBDMS reactions for keto acids should be performed in neutral pH medium.

3.2.2. Effect on amount of TDPA

α-Keto acids are easily decomposed, dimerized and decarboxylated [4,10]. In order to stabilize these compounds during ethoximation reaction, TDPA was used as an antioxidant at concentrations varying from 0 to 150 μg/mL. The reaction yields increased when the concentration of TDPA increased. The addition of TDPA yielded higher responses for most keto acids that contained a single carboxyl group, whereas responses for dicarboxylic acids were similar. The response of α-keto-γ-methiolbutyric acid was drastically increased in the presence of TDPA. The addition of TDPA as an antioxidant played a significant role in the derivatization process and enhanced the reaction yield.

3.2.3. Effect of reaction temperature and time

The influence of reaction temperature was studied by performing experiments at room temperature, 50, 60, 80 and 90 °C for 30 min, and the peak area ratios of keto acids to that of IS were compared. The reaction temperature at 60 °C appeared to be the most suitable for reaction of ethoxiamine with keto acids (Table 4). Except for α-keto-γ-methiolbutyric and γ-ketopimelic

Table 5
Effect of reaction time on the derivatization of keto acids as ethoxime/*tert*-butyldimethylsilyl derivatives.

No.	Compound	Normalized values[a] (mean ± SD)				
		10 min	30 min	60 min	90 min	120 min
1	Glyoxylic acid	0.52 ± 0.04	1.0 ± 0.07	1.06 ± 0.02	1.09 ± 0.08	1.01 ± 0.02
2	Pyruvic acid	0.21 ± 0.05	1.0 ± 0.20	1.32 ± 0.02	1.21 ± 0.11	1.14 ± 0.04
3	α-Ketobutyric acid	0.25 ± 0.06	1.0 ± 0.17	1.18 ± 0.02	1.04 ± 0.09	1.10 ± 0.04
4	α-Ketoisovaleric acid	0.61 ± 0.03	1.0 ± 0.07	0.86 ± 0.01	0.80 ± 0.05	0.83 ± 0.03
5	Acetoacetic acid	1.03 ± 0.05	1.0 ± 0.01	0.78 ± 0.02	0.80 ± 0.02	1.06 ± 0.02
6	α-Ketovaleric acid	0.33 ± 0.05	1.0 ± 0.14	1.09 ± 0.02	1.01 ± 0.07	1.10 ± 0.03
7	α-Ketoisocaproic acid	0.50 ± 0.03	1.0 ± 0.10	1.02 ± 0.02	0.95 ± 0.06	1.03 ± 0.02
8	α-Keto-β-methylvaleric acid	0.50 ± 0.03	1.0 ± 0.10	1.02 ± 0.02	0.95 ± 0.06	1.03 ± 0.02
9	α-Ketocaproic acid	0.43 ± 0.03	1.0 ± 0.11	1.01 ± 0.01	0.98 ± 0.06	1.06 ± 0.02
10	α-Ketooctanoic acid	0.70 ± 0.04	1.0 ± 0.04	0.89 ± 0.01	0.86 ± 0.06	0.77 ± 0.01
11	α-Keto-γ-methiolbutyric acid	0.71 ± 0.01	1.0 ± 0.02	0.95 ± 0.09	1.08 ± 0.09	0.90 ± 0.03
12	Ketomalonic acid	1.14 ± 0.02	1.0 ± 0.04	0.65 ± 0.01	0.56 ± 0.01	0.52 ± <0.01
13	Oxaloacetic acid	0.63 ± 0.02	1.0 ± 0.03	1.30 ± 0.08	1.35 ± 0.03	1.50 ± 0.06
14	α-Ketoglutaric acid	0.94 ± 0.02	1.0 ± 0.04	0.95 ± 0.01	1.00 ± 0.01	0.95 ± 0.01
15	α-Ketoadipic acid	0.97 ± 0.01	1.0 ± 0.04	0.92 ± 0.02	0.96 ± 0.01	0.90 ± 0.01
16	β-Ketoadipic acid	0.98 ± 0.02	1.0 ± 0.04	0.92 ± 0.02	0.95 ± 0.01	0.90 ± 0.01
17	γ-Ketopimelic acid	0.73 ± 0.02	1.0 ± 0.04	0.97 ± 0.04	1.04 ± 0.01	1.03 ± 0.01

[a] All measurements were expressed as normalized values of peak area ratios to the corresponding mean values at 30 min (mean ± SD).

acids, which reached maximum reaction yield at 80 °C, and oxaloacetic acid at 90 °C, the highest reaction yields of most compounds were obtained at 60 °C. The yield from most keto acids was significantly decreased when the reaction mixture was heated to 80 °C and 90 °C, probably due to decomposition of unstable keto acids or loss of volatile EO derivatives at high temperature. The ketomalonic acid peak nearly disappeared when the reaction was performed at 80 °C and 90 °C. Therefore, the optimum temperature was around 60 °C, and another experimental set was carried out at 60 °C for 10, 30, 60, 90 and 120 min to assess the effect of reaction time. As summarized in Table 5, the maximum responses of keto acids were obtained at either 30 min or 60 min. Improvements in yields were not achieved by increasing or decreasing the heating time. A longer reaction time (90 min) reduced the yield, while a shorter heating (10 min) resulted in lower yield with the exception of ketomalonic acid. There were no considerable differences in response between reaction times of 30 min and 60 min, therefore 30 min were selected for subsequent experiments.

The obtained results suggested that reaction temperature was an important factor that could affect the EO derivatization process in aqueous phase; 60 °C was the most suitable temperature. The reaction time also had some effect; however it did not strongly affect the reaction yield of keto acids. In summary, the optimal conditions for EO reaction from this work were achieved in aqueous media at neutral pH in the presence of ethoxyamine (5 mg) and TDPA (100 μg) at 60 °C for 30 min.

3.2.4. Stability of the EO/TBDMS derivatives

The stability of the EO/TBDMS derivatives was determined by repeated evaluation of the peak area ratio of keto acid standards to IS that were stored in the refrigerator at 4 °C and thawed at room temperature for 1 h prior to injection. All the EO/TBDMS derivatives of keto acids exhibited good stability for at least 20 days when stored at 0 °C in sealed vials.

3.3. Method validation for measurement of keto acids

The SIM chromatogram of keto acids from the standard mixture is shown in Fig. 1. The calibration curves of 17 keto acids ranging from 0.01 to 5.0 μg/mL under optimal conditions were linear with regression coefficients better than 0.995 and good LODs (0.01–0.5 ng/mL), precision and accuracy. The intra-day and inter-day precision (% RSD) of this profiling method was 0.3–4.3 and 0.05–8.3 at different levels of 0.5 and 5.0 μg/mL, respectively. The accuracy (% RE) measured at two different concentrations were −10.3 to 4.8 for intra-day and −10.5 to 5.3 for inter-day. These were acceptable for quantitative measurement of keto acids from biological samples (Table 6).

3.4. Profiling analysis of keto acids

Good chromatographic profiles were obtained when the present method was applied to analyze urine, plasma and tissue samples (Fig. 1). A total of 17 keto acids were positively identified and quantified from these samples by GC–MS in SIM mode.

In conclusion, a selective and sensitive GC–MS method for measurement of keto acids as EO/TBDMS derivatives was developed. The present method exhibited several advantages: it allowed simultaneous determination of keto acids from various biological samples, and its overall linearity, precision, and accuracy indicated suitability and applicability of the method for recovery and quantitative analysis of these compounds from aqueous matrixes. The method also appeared to be sensitive with LODs at nanogram levels. We confirmed that the method can be applied for profiling analysis of keto acids present in urine, plasma and tissue for detection of biological markers as well as rapid monitoring of

Table 6
Validation data of keto acids as ethoxime/*tert*-butyldimethylsilyl derivatives.

No.	Compound	Linearity (r)[a]	LOD[b] (ng/mL)	Added (μg/mL)	Intra day		Inter day		Added (μg/mL)	Intra day		Inter day	
					Precision (% RSD)[c]	Accuracy (% RE)[d]	Precision (% RSD)	Accuracy (% RE)		Precision (% RSD)	Accuracy (% RE)	Precision (% RSD)	Accuracy (% RE)
1	Glyoxylic acid	0.9990	0.5	0.5	0.3	−1.0	1.2	0.2	2.0	2.8	−4.9	1.1	−5.9
2	Pyruvic acid	0.9981	0.2	0.5	3.2	0.8	0.6	−1.0	2.0	3.8	−7.6	1.8	−9.5
3	α-Ketobutyric acid	0.9974	0.01	0.5	1.6	−4.3	2.2	−6.1	2.0	1.8	2.5	3.2	−1.2
4	α-Ketoisovaleric acid	0.9993	0.04	0.5	2.1	−2.6	4.5	0.4	2.0	1.0	−3.9	0.7	−4.2
5	Acetoacetic acid	0.9997	0.07	0.5	3.8	4.8	8.3	2.9	2.0	3.3	0.4	2.0	2.2
6	α-Ketovaleric acid	0.9970	0.02	0.5	2.4	−8.6	1.9	−6.7	2.0	2.6	2.6	1.6	3.8
7	α-Ketoisocaproic acid	0.9973	0.1	0.5	0.5	−2.5	2.1	−0.3	2.0	1.3	−9.8	2.0	−9.5
8	α-Keto-β-methylvaleric acid	0.9973	0.2	0.5	0.5	−2.6	2.1	−0.3	2.0	1.4	−9.8	2.0	−9.5
9	α-Ketocaproic acid	0.9967	0.2	0.5	0.6	−8.7	4.0	−4.3	2.0	2.8	2.8	0.05	2.7
10	α-Ketooctanoic acid	0.9972	0.04	0.5	1.0	−5.6	2.5	−3.2	2.0	4.3	−8.8	1.5	−9.6
11	α-Keto-γ-methiolbutyric acid	0.9994	0.2	0.5	1.2	−9.3	2.3	−7.2	2.0	1.4	0.5	0.2	0.4
12	Ketomalonic acid	0.9957	0.02	0.5	0.9	−9.6	7.7	−0.8	2.0	1.1	2.4	2.5	5.3
13	Oxaloacetic acid	0.9999	0.4	0.5	3.7	4.3	5.1	−0.6	2.0	0.7	2.1	3.1	4.9
14	α-Ketoglutaric acid	0.9975	0.03	0.5	0.2	−8.3	4.1	−4.2	2.0	1.7	−8.1	0.5	−7.6
15	α-Ketoadipic acid	0.9951	0.01	0.5	1.4	−9.8	1.3	−10.5	2.0	1.4	3.3	0.9	4.4
16	β-Ketoadipic acid	0.9951	0.01	0.5	1.3	−10.3	1.8	−10.2	2.0	1.3	3.5	1.0	4.2
17	γ-Ketopimelic acid	0.9974	0.01	0.5	0.8	−8.6	2.2	−6.7	2.0	1.0	2.4	1.0	1.5

[a] Correlation coefficients in the calibration range of 0.01–5.0 μg/mL.
[b] Limit of detection.
[c] Relative standard deviation.
[d] Relative error.

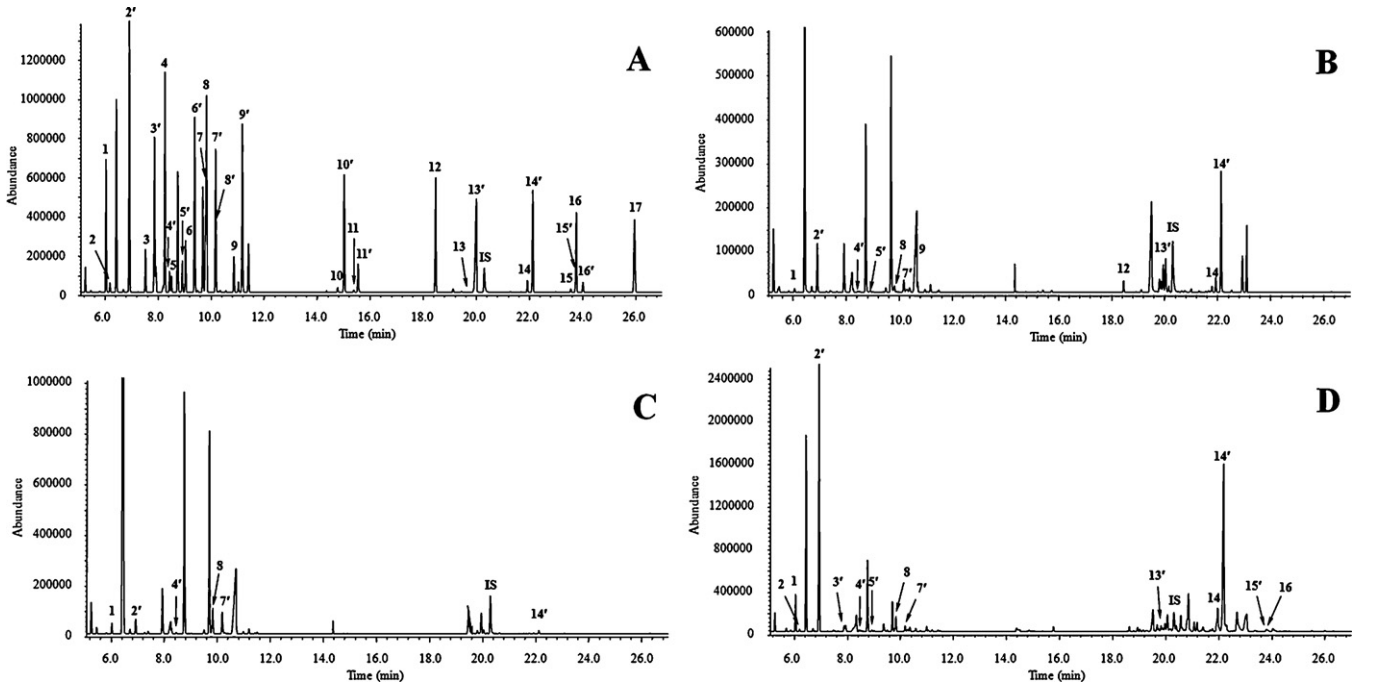

Fig. 1. SIM chromatogram of keto acids as ethoxime/*tert*-butyldimethylsilyl derivatives of standard mixture (A), rat brain tissue (B), normal human urine (C) and normal human plasma (D): IS, 3,4-dimethoxybenzoic acid; 1, glyoxylic acid; 2, pyruvic acid; 3, α-ketobutyric acid; 4, α-ketoisovaleric acid; 5, acetoacetic acid; 6, α-ketovaleric acid; 7, α-ketoisocaproic acid; 8, α-keto-β-methylvaleric acid; 9, α-ketocaproic acid; 10, α-ketooctanoic acid; 11, α-keto-γ-methiolbutyric acid; 12, ketomalonic acid; 13, oxaloacetic acid; 14, α-ketoglutaric acid; 15, α-ketoadipic acid; 16, β-ketoadipic acid; 17, γ-ketopimelic acid.

levels associated with diseases such as the metabolic disorders. The method will be useful for investigating the changes in the metabolic profile in abnormal physiological states.

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