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Gas chromatographic–mass spectrometric determination of urinary oxoacids using *O*-(2,3,4,5,6-pentafluorobenzyl)oxime-trimethylsilyl ester derivatization and cation-exchange chromatography

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Abstract

We introduced a new combined method to isolate, purify and quantify oxoacids in human urine. Preparation of *O*-(2,3,4,5,6-pentafluorobenzyl) oximes of oxoacids at pH 2 to 3 was followed by cation-exchange column chromatography for removing the biological interferences. The effluent with water was extracted with ethyl acetate and the oxoacids were quantitatively converted into their trimethylsilyl derivatives for detection by gas chromatography–mass spectrometry. Good quality control data were obtained through precision and accuracy tests. Analytical recoveries (53.5~99.8%) were quantitative for a wide variety of oxoacids. This method was used for the measurement of 18 oxoacids in the urine of healthy volunteers.

Keywords: Derivatization, GC–MS; Oxoacids; *O*-PFBOximes; TMS-ester

1. Introduction

Oxoacids have been known to play an important part in the metabolism of valine, leucine, isoleucine, etc. [1]. An abnormal pattern of oxoacids profile is a valuable biochemical marker of hereditary metabolic disease such as diabetes mellitus [2], uremia [3], maple syrup urine disease [4], phenylketonuria [5] and unspecified mental retardation [6]. However, the oxo group makes an extraction from biological fluid and identification by gas chromatography–mass spectrometry (GC–MS) difficult. In this case, it is necessary to convert the oxo function to a stable form prior to esterification of the carboxylic acid group. Methyloximes of trimethylsilyl (TMS) esters [7], trimethylsilyloximes of trimethylsilyl esters [8], *O*-trimethylsilylquinoxalinol derivatives [9] and 2,4-dinitrophenylhydrazone methyl esters [10] have been used. Recently, *O*-(2,3,4,5,6-pentafluorobenzyl) oximes (*O*-PFBOximes) were shown to be more satisfactory derivatives for the quantitative determination of oxoacids as compared to the commonly used *O*-ethoximes-TMS-esters [11].

As for an extraction method for the isolation of these acids, solvent extraction and anion-exchange methods have been widely used. However, because of their lack of specificity and coextraction with large amounts of amino acid, Sweetmann et al. [12]

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introduced the silicic acid column chromatographic method, which gave specific isolation but was somewhat laborious and time consuming for routine analysis in most clinical laboratories. Therefore, we introduced the combined method, which includes cation-exchange column and partition chromatography, and suggested that this efficient method was suitable for the quantitative analysis of organic acids [13].

In this study, we extend our work on organic acids by describing a simple and improved method for the separation and quantification of oxoacids in urine by GC–MS.

2. Experimental

2.1. Chemicals

O-(2,3,4,5,6-Pentafluorobenzyl) hydroxylamine (PFBHA) hydrochloride and all oxoacid standards were purchased from Sigma (St. Louis, MO, USA). All chemicals and solvents were of guaranteed grade and used without further purification. Deionized water was distilled before use. Dowex 50W x 8 (strongly acidic cation-exchange resin, H^+) was purchased from Sigma. Silylating reagents, MSTFA (*N*-methyl-*N*-trimethylsilyltrifluoroacetamide) and TMCS (trimethylchlorosilane) were purchased from Sigma. Ethyl acetate was high purity ''HPLC solvent'' grade.

2.2. Sample collection

Urine specimens were obtained from healthy volunteers, 16 women (22~53 years) and 20 men (23~51 years), each with an unremarkable medical history. No dietary restrictions were applied except for total abstinence from alcoholic beverages for 24 h. All samples were stored at −20°C without additives: before analysis they were thawed and creatinine content was determined by the Jaffé reaction.

2.3. Gas chromatography–mass spectrometry

A Hewlett-Packard GC–MS system consisting of a Model 5890A gas chromatograph, a Model 5970B mass-selective detector and a HP G1701AA MSD ChemStation was used. A fused-silica capillary column coated with cross-linked 5% phenyl methyl silicone (Ultra-2, 25 m×0.2 mm I.D., 0.33 μm film thickness) was also used. The GC temperature program was as follows: initial temperature was 80°C, increased to 160°C at a rate of 4°C/min, held for 2 min, then to 174°C at a rate of 2°C/min, then to 260°C at a rate of 5.4°C/min and finally to 300°C at a rate of 10°C/min and held for 6 min. The split ratio was 1:12, injection port temperature was 300°C, transfer line temperature was 300°C and ion source temperature was 200°C. The mass spectrometer was operated at 70 eV in the electron impact (EI) mode using SCAN or SIM (selected ion monitoring). The selected ion groups for the identification of 18 oxoacids in SIM mode are listed in Table 1. The dwell time for each ion was set at 50 ms.

2.4. Sample extraction

2.4.1. Preparation of O-PFBOxime derivatives

Urine samples and aqueous standards were processed identically. *O*-PFBOximes were prepared by adding 5 mg of PFBHA hydrochloride to 1 ml of urine containing 1.0 μg of α-ketocaproic acid and 2.0 μg of tricarballylic acid as internal standards. The pH of the samples was adjusted to between 2 and 3 with 0.5 *M* sulfuric acid according to a pH stick indicator, and the samples were allowed to stand at room temperature for 2 h.

2.4.2. Cation-exchange column chromatographic procedures

The cation-exchange column chromatography (CEC) procedure was modified slightly from the method previously described [2]. Preconditioned Dowex 50W x 8 resin was poured into a pasteur pipet (I.D. 0.5 cm) up to 3 cm of height. As soon as urine samples were introduced, the effluent with 2 ml of water was collected without waste. Then, *O*-PFBOxime-derivatized oxoacids were extracted twice with 5 ml of ethyl acetate and the solvent was evaporated in vacuo. The residue was dried in a vacuum desiccator over diphosphorus pentaoxide–potassium hydroxide (P_2O_5–KOH).

Table 1
Characteristic ions and retention times of *O*-PFBOxime-TMS-ester derivatized oxoacids for detection by GC–MS

	Characteristic ions (m/z)	Retention time (min)
Glyoxlyic acid	341, 326, 181	18.26
Pyruvic acid	355, 340, 181	19.15
α-Ketoisovaleric acid	383, 255, 366	20.22, 20.95 (4.4:1)
α-Ketobutyric acid	369, 352, 181	20.31
α-Ketovaleric acid	383, 255, 366	21.52
Acetoacetic acid	369, 354, 255	21.48, 2.43 (1:1.5)
α-Keto-3-methylvaleric acid	397, 200, 382	22.54, 22.97 (6.4:1)
α-Ketoisocaproic acid	397, 200, 382	22.45, 23.56 (1:3.3)
Succinic semialdehyde	369, 279, 354	25.36
Dihydroxy acetone	429, 339, 181	25.48
γ-Ketovaleric acid	383, 266, 181	26.69
α-Ketooctanoic acid	425, 308, 181	31.93
α-Keto-4-methiolbutyric acid	415, 400, 218	33.04
Oxalacetic acid	471, 456, 181	33.68
α-Ketoglutaric acid	485, 470, 181	35.63
β-Phenylpyruvic acid	431, 416, 181	35.22, 36.17 (1:18)
α-Ketoadipic acid	484, 302, 181	37.66
p-Hydroxyphenylpyruvic acid	519, 277, 181	41.13, 42.13 (1:33)

Quantitative ions are underlined.
Values in parentheses show the ratio of the areas of double peaks due to *syn* and *anti* forms of the oxo-benzoxime group.

2.4.3. Derivatization into O-PFBOxime-TMS-ester of oxoacids

The residue was dissolved in 50 μl of TMS reagent mixture (MSTFA–TMCS, 100:1, v/v) and heated at 60°C for 30 min. After heating, 2-μl aliquots were injected onto the GC column using an autosampler.

2.5. Quality control

For quality control, each series was analyzed using 1 ml of a pooled urine (blank sample) and with the same pooled urine fortified with 1.0, 2.0 and 5.0 μg/ml of glyoxylic acid, pyruvic acid, α-ketovaleric acid, oxalacetic acid, α-ketoglutaric acid and *p*-hydroxyphenylpyruvic acid, and with 0.5, 1.0 and 2.0 μg/ml of α-ketobutyric acid, α-ketoisovaleric acid, acetoacetic acid, α-keto-3-methylvaleric acid, succinic semialdehyde, α-ketoisocaproic acid, β-phenylpyruvic acid and α-ketoadipic acid, and with 0.1, 0.2 and 0.5 μg/ml of dihydroxy acetone, α-ketooctanoic acid and α-keto-4-methiolbutyric acid. Three aliquots at each concentration were analyzed on three occasions over a three-week period.

3. Results and discussion

3.1. Extraction and analysis

The proposed method has the advantage of simple sample preparation, which consists of *O*-PFBOxime formation, column and partition chromatography with only a single pH adjustment (pH 2~3).

O-PFBOximes were prepared by adding PFBHA hydrochloride in weakly acidic media (pH 2~3) at room temperature. As can be seen in Table 1, α-ketoisovaleric acid, acetoacetic acid, α-keto-3-methylvaleric acid, α-ketoisocaproic acid, β-phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid formed a double peak, corresponding to *syn* and *anti* isomers [14]. This stereoisomerism is known to make quantitative work with oxoacids difficult. In the case of *O*-PFBOximes-TMS-esters, however, one or the other isomer greatly predominated because of a great steric hindrance of the *O*-(2,3,4,5,6-pentafluorobenzyl) group [11] and quantification was not unduly affected. In addition, the molecular mass of the *O*-PFBOximes-TMS-esters exceeds the non-derivatized oxoacids by more than 267 mass units. On

the GC chromatogram, all oxoacids were well separated from biological interferences.

The isolation of *O*-PFBOxime derivatized oxoacids is based on cation-exchange column chromatography and liquid partition chromatography at the same pH for *O*-PFBOxime formation, which is a modification of our previous method [13]. In this step, unreacted PFBHA as well as the interferences derived from biological matrix, e.g., amino acids, urea and creatinine [13] are retained on the cation-exchange column. However, *O*-PFBOxime derivatized oxoacids of interest are eluted as their neutralized forms. Then, to enhance the specificity on the GC chromatogram, TMS-ester derivatives are prepared by adding a mixture of MSTFA–TMCS (100:1, v/v).

By using the above method, oxoacids are simultaneously determined by GC–MS with selected ion monitoring (SIM) mode. Characteristic ions and retention times of the *O*-PFBOxime derivatives of 18 oxoacid TMS-esters are given in Table 1.

The molecular ions of the derivatives of oxoacids are readily observed. However, these molecular ions decrease with increasing chain length. A useful ion to confirm molecular mass is at M–15, which arises by loss of a methyl radical from a TMS function. The base peak is mostly at *m/z* 181 or *m/z* 73; the former corresponds to the pentafluorobenzyl group and the latter is caused by the TMS group and is accompanied by an intense ion, *m/z* 75. For the most part, the rearrangement ion of $(CH_3)_2Si=^+O$-PFB also occurs at *m/z* 255. When the alkyl chain is sufficiently long to contain a hydrogen gamma to the nitrogen atom to the oxime, the following rearrangement takes place and the ion at *m/z* 355 is formed, see Scheme 1.

In the cases of long-chain mono and di-acids, the ions at M–117 (M–CO_2TMS) and M–197 (M–OPFB) are produced. The characteristic ions at *m/z* M–90 (M–TMSOH) and *m/z* 147 [TMS-$^+$O=$Si(CH_3)_2$], a TMS rearrangement ion, are presented in the spectrum of oxo di-acids.

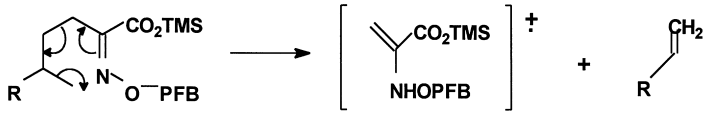
3.2. Precision and recovery

The intra- and inter-day reproducibility of the urine profiling is given in Table 2. Precision and accuracy data from supplemented artificial urine, as judged from relative standard deviations (R.S.D.s) (<10%), were satisfactory. Recovery studies were performed three times at 0.05, 2.00 and 10.00 μg/ml concentration for each oxoacid. Table 2 represents the overall analytical recoveries of oxoacids using this method. As shown in Table 2, analytical recoveries exceeding 60% were mostly found. Although the extraction recoveries of α-ketooctanoic acid and β-phenylpyruvic acid were only 53.5% and 61.9%, respectively, they could be sufficiently detected in biological samples.

3.3. GC–MS profiles in urine

Fig. 1 shows the effects of a proposed method on the resulting GC–MS analysis. The total ion chromatogram of *O*-PFBOxime-TMS-ester derivatized standard oxoacids is shown in Fig. 1a, demonstrating the good chromatographic separation of 18 oxoacids in a single run. Although some oxoacids are co-eluted, they could be analyzed because of the specificity of the SIM mode with two or three ions on the basis of characteristic ions and retention times in Table 1. Using the solvent extraction method, the peak of PFBHA, which reacts with silylating reagents, is at 17.22 in Fig. 1b. Fig. 1c shows that the oxoacids were well separated from each other and the other constituents of the urine.

The concentration range of oxoacids in control urine (16 females and 20 males) was simultaneously determined by GC–MS with the above extraction method and is described in Table 3.



m/z 355

Scheme 1.

Table 2
Recovery, precision and accuracy data for determination of oxoacids

Oxoacids [Recovery (%)±S.D.]	Added amounts (μg/ml)	Within-day		Day-to-day		Oxoacids [Recovery (%)±S.D.]	Within-day		Day-to-day	
		Found (mean±S.D.) (μg/ml)	R.S.D. (%)	Found (mean±S.D.) (μg/ml)	R.S.D. (%)		Found (mean±S.D.) (μg/ml)	R.S.D. (%)	Found (mean±S.D.) (μg/ml)	R.S.D. (%)
Glyoxylic acid	Blank	0.50±0.03	6.0	0.54±0.05	9.3	Pyruvic acid	1.89±0.08	4.2	2.09±0.10	4.8
(91.6±1.03)	1.0	1.86±0.09	4.8	1.96±0.15	7.7	(86.5±5.87)	2.92±0.07	2.4	2.92±0.13	4.5
	2.0	2.28±0.08	3.5	2.53±0.06	6.3		3.80±0.12	3.2	3.84±0.15	3.9
	5.0	4.55±0.11	2.4	4.34±0.13	3.0		6.97±0.14	2.0	7.32±0.14	1.9
α-Ketoisovaleric acid	Blank	0.14±0.01	7.1	0.14±0.01	7.1	α-Ketobutyric acid	0.38±0.02	5.5	0.38±0.03	6.8
(89.0±5.02)	0.5	0.72±0.05	6.9	0.76±0.06	7.9	(87.4±1.04)	0.61±0.02	3.3	0.68±0.03	4.4
	1.0	1.28±0.08	6.3	1.30±0.09	6.9		0.96±0.02	2.1	1.04±0.04	3.8
	2.0	2.46±0.12	4.9	2.59±0.15	5.8		2.11±0.03	1.4	2.29±0.05	2.2
α-Ketovaleric acid	Blank	0.79±0.04	4.9	0.86±0.05	5.5	γ-Ketovaleric acid	0.58±0.02	4.1	0.62±0.03	4.8
(92.1±3.43)	1.0	1.69±0.07	4.0	1.79±0.08	4.2	(96.9±9.25)	1.47±0.03	2.3	1.49±0.04	2.4
	2.0	2.67±0.12	4.4	2.76±0.13	4.7		2.29±0.05	2.2	2.35±0.06	2.5
	5.0	5.41±0.13	2.4	5.84±0.15	2.6		5.68±0.06	1.1	5.95±0.07	1.2
Acetoacetic acid	Blank	0.31±0.00	0.6	0.32±0.00	0.8	α-Keto-3-methylvaleric	0.29±0.01	2.4	0.29±0.01	3.4
(94.1±6.77)	0.5	0.69±0.00	0.4	0.69±0.00	0.4	acid (70.3±8.81)	0.65±0.01	1.5	0.66±0.01	1.9
	1.0	1.13±0.00	0.6	1.16±0.01	0.8		1.05±0.01	1.1	1.06±0.02	0.9
	2.0	2.65±0.00	0.2	2.65±0.01	0.4		1.79±0.01	0.4	1.81±0.01	0.5
α-Ketoisocaproic acid	Blank	0.13±0.00	2.6	0.13±0.00	2.8					
(96.6±2.80)	0.5	0.48±0.01	2.1	0.50±0.01	2.4					
	1.0	0.85±0.02	2.5	0.91±0.03	2.7					
	2.0	1.81±0.04	2.2	1.92±0.05	2.7					
Dihydroxy acetone	Blank	0.28±0.01	2.7	0.29±0.01	3.4	α-Ketooctanoic acid	0.26±0.04	15.4	0.27±0.05	18.9
(96.6±8.01)	0.1	0.35±0.00	1.2	0.35±0.01	2.9	(53.5±5.08)	0.37±0.03	7.3	0.38±0.04	11.3
	0.2	0.42±0.01	1.3	0.42±0.01	2.8		0.32±0.02	7.2	0.40±0.05	11.5
	0.5	0.83±0.01	0.6	0.90±0.01	0.7		0.83±0.02	2.8	0.94±0.04	4.5
α-Keto-4-	Blank	0.10±0.01	10.0	0.11±0.01	11.8	β-Phenylpyruvic acid	0.35±0.01	4.0	0.35±0.02	4.9
methiolbutyric acid	0.1	0.16±0.01	6.3	0.17±0.02	9.4	(61.9±13.0)	0.44±0.02	3.4	0.44±0.02	4.1
(75.8±12.5)	0.2	0.20±0.01	5.0	0.22±0.02	6.8		0.51±0.01	2.7	0.54±0.02	3.9
	0.5	0.58±0.02	4.1	0.60±0.03	4.7		0.80±0.01	1.4	0.81±0.01	1.7
Oxalacetic acid	Blank	5.11±0.21	4.2	5.42±0.24	4.4	α-Ketoglutaric acid	13.2±0.96	7.3	14.2±1.03	7.3
(96.9±14.4)	1.0	6.71±0.16	2.4	8.97±0.22	2.5	(91.7±13.4)	14.1±0.92	6.5	16.3±1.07	6.6
	2.0	7.49±0.21	2.3	9.55±0.30	3.1		17.1±0.85	5.0	19.7±0.97	4.9
	5.0	9.24±0.13	1.4	11.92±0.16	1.3		21.3±0.58	2.7	22.6±0.78	3.5
α-Ketoadipic acid	Blank	0.94±0.04	4.4	0.97±0.05	4.8	Succinic semialdehyde	0.61±0.03	4.4	0.70±0.04	5.1
(99.8±11.8)	0.5	1.36±0.05	3.7	1.40±0.06	4.3	(95.4±1.79)	1.01±0.03	3.0	1.02±0.03	2.9
	1.0	1.67±0.06	3.6	1.79±0.08	4.5		1.31±0.04	3.1	1.33±0.04	3.0
	2.0	2.40±0.03	1.3	2.53±0.04	1.5		2.27±0.05	2.2	2.56±0.07	2.7
p-Hydroxyphenyl-	Blank	0.69±0.04	6.2	0.69±0.05	7.0					
pyruvic acid	1.0	1.41±0.05	3.8	1.50±0.06	4.3					
(71.7±8.11)	2.0	2.49±0.06	2.4	2.70±0.09	3.4					
	5.0	5.15±0.10	1.9	5.61±0.17	3.0					

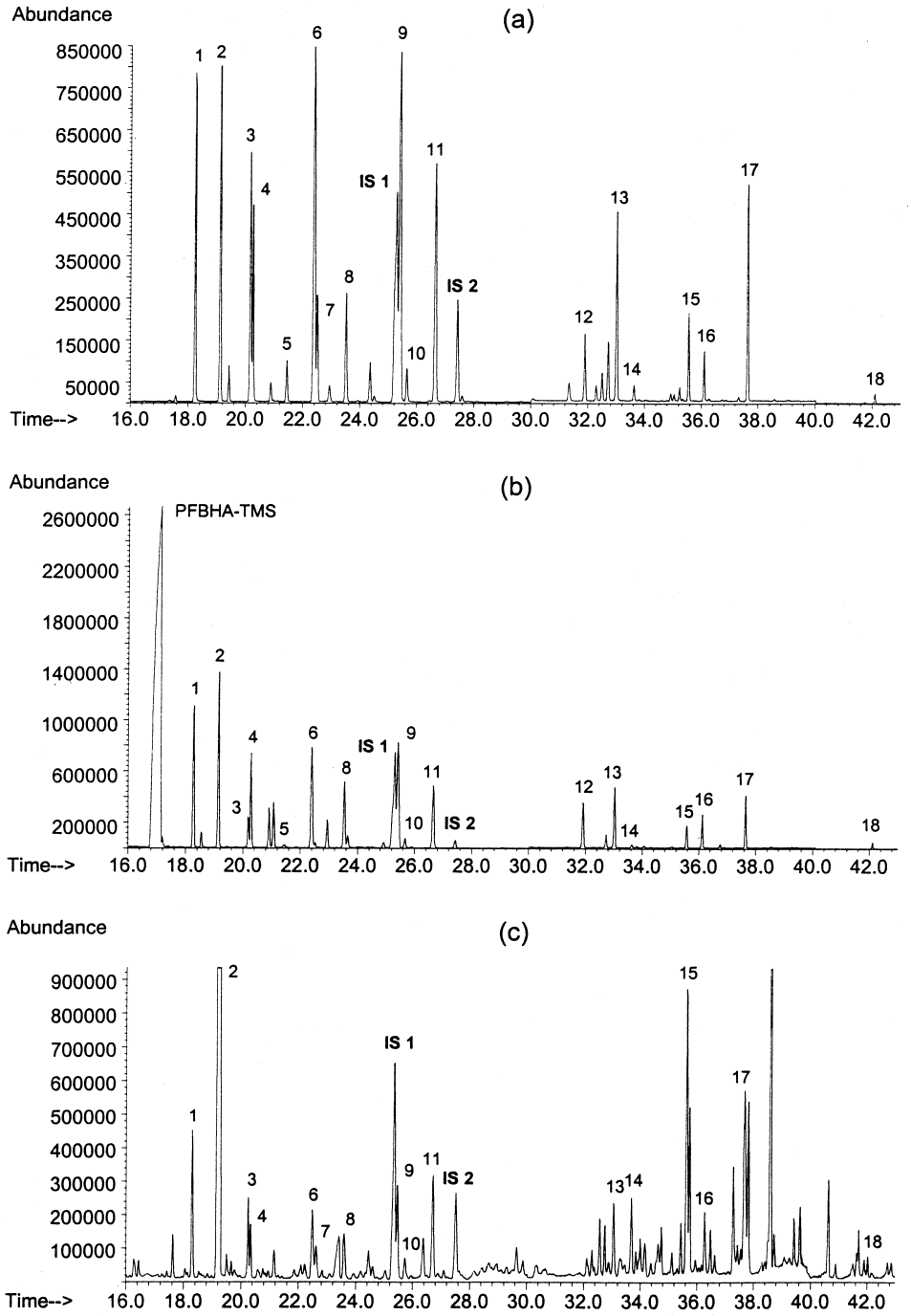


Fig. 1. GC–MS chromatograms of (a) standard oxoacids (2.0 μg/ml) from cation-exchange method, (b) standard oxoacids (2.0 μg/ml) from the solvent extraction method, and (c) oxoacids in the urine of a normal person from the cation-exchange method. (For peak identities, see Table 1).

Table 3
Concentration range of oxoacids in normal urine

Oxoacids	Concentration range (mmol/mol of creatinine)			
	Male (n=20)		Female (n=16)	
	Range	Mean	Range	Mean
Glyoxlyic acid	0.46~3.26	1.27	0.63~3.22	1.88
Pyruvic acid	0.54~8.67	2.13	0.62~3.30	1.68
α-Ketoisovaleric acid	0.00~0.54	0.13	0.01~0.45	0.07
α-Ketobutyric acid	0.05~2.20	0.39	0.04~0.51	0.13
α-Ketovaleric acid	0.03~3.31	0.79	0.04~5.63	1.13
Acetoacetic acid	0.01~0.58	0.15	0.02~0.82	0.20
α-Keto-3-methylvaleric acid	0.11~0.55	0.24	0.12~1.42	0.54
α-Ketoisocaproic acid	0.02~0.45	0.13	0.03~0.34	0.13
Succinic semialdehyde	0.14~0.54	0.35	0.28~0.51	0.39
Dihydroxy acetone	0.02~0.18	0.07	0.05~0.16	0.10
γ-Ketovaleric acid	0.31~1.92	0.87	0.33~2.75	1.28
α-Ketooctanoic acid	0.06~0.35	0.13	0.09 ~0.71	0.25
α-Keto-4-methiolbutyric acid	0.02~0.23	0.06	0.03~0.17	0.07
Oxalacetic acid	0.51~4.88	2.27	1.16~5.94	2.20
α-Ketoglutaric acid	0.18~14.3	2.87	0.42~18.3	2.98
β-Phenylpyruvic acid	0.05~0.67	0.17	0.10~0.76	0.24
α-Ketoadipic acid	0.02~0.57	0.17	0.04~0.81	0.21
p-Hydroxyphenylpyruvic acid	0.15~8.74	1.65	0.23~2.50	0.66

In conclusion, with the cation-exchange chromatography and *O*-PFBOximes-TMS-ester derivatization, quantitative profiles of oxoacids were obtained by GC–MS, and the competing endogenous substances and any excess derivatizing agents on GC–MS chromatograms were effectively excluded. The within-day and day-to-day reproducibility and the analytical recoveries from spiked urine samples demonstrate the usefulness of this method. A concentration range of 18 oxoacids in normal urine are established on the basis of this method.

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