ELSEVIER

Journal of Chromatography B, 751 (2001) 213–220

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Gas chromatographic–mass spectrometric determination of α-ketoisocaproic acid and [$^2H_7$]α-ketoisocaproic acid in plasma after derivatization with *N*-phenyl-1,2-phenylenediamine

Takehisa Matsukawa, Hiroshi Hasegawa*, Yoshihiko Shinohara, Takao Hashimoto

Department of Pathophysiology, School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan

Received 16 May 2000; received in revised form 10 August 2000; accepted 14 August 2000

Abstract

A method for determination of α-ketoisocaproic acid (KIC) and [4,5,5,5,6,6,6-$^2H_7$]α-ketoisocaproic acid ([$^2H_7$]KIC) in rat plasma was developed using gas chromatography–mass spectrometry-selected ion monitoring (GC–MS-SIM). [5,5,5-$^2H_3$]α-Ketoisocaproic acid ([$^2H_3$]KIC) was used as an analytical internal standard to account for losses associated with the extraction, derivatization and chromatography. The keto acids were extracted by cation-exchange chromatography using BondElut SCX cartridge and derivatized with *N*-phenyl-1,2-phenylenediamine to form *N*-phenylquinoxalinone derivatives. Quantitation was performed by SIM of the respective molecular ions at *m/z* 278, 281 and 285 for the derivatives of KIC, [$^2H_3$]KIC and [$^2H_7$]KIC on the electron impact method. The limit of detection was found to be 70 fmol per injection ($S/N=3$) and the limit of quantitation for [$^2H_7$]KIC was around 50 n*M* in rat plasma. Endogenous KIC concentrations in 50 μl of rat plasma were measured with relative intra- and inter-day precision of 4.0% and 3.3%, respectively. The intra- and inter-day precision for [$^2H_7$]KIC spiked to rat plasma in the range of 0.1 to 10 μ*M* gave good reproducibility with relative standard deviation (RSD) of 6.5% and 5.4%, respectively. The intra- and inter-day relative errors (RE) for [$^2H_7$]KIC were less than 6.4% and 3.8%, respectively. The method was applied to determine the plasma concentration of [$^2H_7$]KIC after an intravenous administration of [$^2H_7$]KIC in rat. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Derivatization; GC–MS; α-Ketoisocaproic acid; *N*-Phenyl-1,2-phenylenediamine

1. Introduction

In previous papers [1,2], a gas chromatography–mass spectrometric method employing stable isotopically labeled leucine was uniquely used to examine the pharmacokinetics of D-leucine in rat. After an intravenous administration of D-[$^2H_7$]leucine, about 30% of an administered dose of D-[$^2H_7$]leucine was stereospecifically inverted to the L-enantiomer. D-Leucine is considered to be inverted to L-leucine by two steps as shown in Fig. 1. First, D-leucine undergoes oxidative deamination to α-ketoisocaproic acid (KIC) by D-amino acid oxidase. Subsequently, KIC is asymmetrically reaminated by transaminase to form L-leucine and this process is reversible. KIC may be decarboxylated by branched-chain α-keto acid dehydrogenase resulting in irreversible loss of

*Corresponding author. Tel.: +81-426-76-5699; fax: +81-426-76-5686.

0378-4347/01/$ – see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0378-4347(00)00483-7

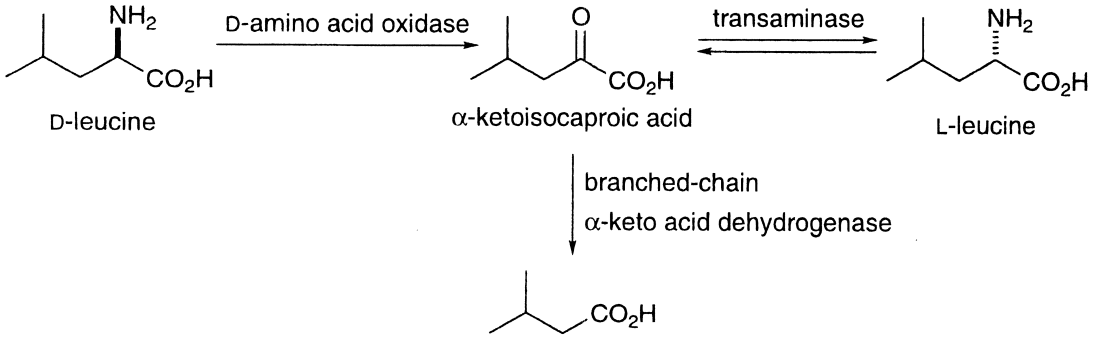

Fig. 1. Proposed metabolic pathway of D-leucine.

leucine. KIC has been shown to be capable of serving as dietary substitutes for L-leucine [3–5], although with less than 100% efficiency. These findings tempted us to examine the pharmacokinetics of KIC to clarify the inversion of D-[$^2H_7$]leucine to the L-enantiomer in detail.

Several methods have been reported for determination of α-keto acids by gas chromatography–mass spectrometry (GC–MS) [6–11]. *O*-(Trimethylsilyl-)quinoxalinol derivatives are the most widely used. The derivatives were obtained by condensation of α-keto acids with 1,2-phenylenediamine to yield quinoxalinol derivatives and the subsequent *O*-trimethylsilylation with trimethylsilylating reagents. The high natural abundance of ^{29}Si and ^{30}Si present in the derivatives results in a high back ground, which may limit the analytical precision and accuracy of selected ion monitoring (SIM) measurements. Fernandes and co-workers [9] have reported the use of *N*-alkylated quinoxalinone derivatives, which avoids the silicon isotopic interferences. However, alkylation of quinoxalinols with *N,N*-dimethylformamide dialkyl acetal has been weakly regioselective causing to produce a mixture of *N*-alkylated quinoxalinones and *O*-alkylated quinoxalinols, which complicated the chromatographic analysis. Cook and Perry [12] reported that the condensation of *N*-phenyl-1,2-phenylenediamine (*N*-Phe-PDA) with pyruvic acid gave 1-phenyl-3-methylquinoxalin-2-one as a single isomer. *N*-Phe-PDA is commercially available, but there has been no reports on the use of this reagent for GC–MS analysis of α-keto acids.

The present paper described a sensitive and specific procedure for simultaneous determination of [$^2H_7$]KIC and endogenous KIC in rat plasma. The method involves derivatization with *N*-Phe-PDA and determination with GC–MS-SIM.

2. Experimental

2.1. Chemicals and reagents

Sodium α-ketoisocaproate (KIC Na) and *N*-phenyl-1,2-phenylenediamine (*N*-Phe-PDA) were purchased from Aldrich (Milwaukee, WI, USA). Sodium [5,5,5-$^2H_3$]α-ketoisocaproate ([$^2H_3$]KIC Na, >98% atom ^2H) was purchased from Isotec (Miamisburg, OH, USA). Sodium [4,5,5,5,6,6,6-$^2H_7$]α-ketoisocaproate ([$^2H_7$]KIC Na) was prepared from commercially available DL-[4,5,5,5,6,6,6-$^2H_7$]leucine (Isotec, >98% atom ^2H) in our laboratory (unpublished result). A strong cation-exchange solid-phase extraction column BondElut SCX (H^+ form, size 1 ml/100 mg) was purchased from Varian (Harbor City, CA, USA). All other chemicals and solvents were of analytical reagent grade and used without further purification.

2.2. Stock solutions

Stock solutions of KIC (15.25 μg/ml, 100 μ*M*), [$^2H_3$]KIC (15.55 μg/ml, 100 μ*M*) and [$^2H_7$]KIC (16.04 μg/ml, 100 μ*M*) were prepared in ethanol. Storage of these solutions at 4°C did not result in any detectable decomposition for more than three months. All analyses were performed by diluting the stock solutions with ethanol.

2.3. Gas chromatography–mass spectrometry-selected-ion monitoring (GC–MS-SIM)

Capillary GC–MS-SIM analysis was carried out on a Shimadzu (Kyoto, Japan) QP1000EX quadrupole gas chromatograph–mass spectrometer equipped with a data processing system. A methylsilicone bonded-phase fused-silica capillary column SPB-1 (15 m×0.25 mm I.D.) with a 0.25 μm thin film (Supelco, Bellefonte, PA, USA) was connected directly into the ion source. Helium was used as the carrier gas at a column head pressure of 0.8 kg/cm^2. A split–splitless injection system Shimadzu SPL-G9 operating in the splitless mode was used at a septum purge flow-rate of 1.0 ml/min and a split vent flow-rate of 30 ml/min. The purge activation time was 2 min after injection. The initial column temperature was set at 120°C. After the sample injection, it was maintained for 2 min, increased at 30°C/min to 250°C and held at 250°C for 2 min. The temperature of the injector was 280°C. The mass spectrometer was operated in electron impact ionization mode at an energy of 70 eV. The ion source temperature was 280°C. Selected-ion monitoring was performed on the molecular ions at *m/z* 278, 281 and 285 for the *N*-phenylquinoxalinone derivatives of KIC, [$^2H_3$]KIC and [$^2H_7$]KIC, respectively.

2.4. Sample preparation for GC–MS-SIM

To 50 μl of rat plasma in a polypropylene microtube (1.5 ml) were added 100 pmol of [$^2H_3$]KIC dissolved in 100 μl of ethanol as an analytical internal standard. The plasma sample was deproteinized and extracted with ethanol (0.5 ml×2) on a vortex mixer for ca. 0.5 min. After centrifugation at 3000 rpm for 10 min, the ethanol solution was transferred into another polypropylene microtube and evaporated at 40°C under a stream of nitrogen. The residue was dissolved in 0.5 ml of 40 m*M* hydrochloric acid and then applied to a BondElut SCX cartridge, which was pre-washed and activated with 3 ml of methanol, 3 ml of a mixture of methanol and 0.1 *M* hydrochloric acid (1:1, v/v) and 3 ml of 0.1 *M* hydrochloric acid. KIC was eluted with 1 ml of water. The waste and effluent were collected in a PTFE-lined screw-cap centrifuge tube (100×16 mm I.D.). After adding 1 ml of 0.2% *N*-Phe-PDA in 6 *M* hydrochloric acid, the resulting solution was heated at 100°C for 1 h. After cooling, the derivative was extracted with 1 ml of a mixture of diethyl ether and hexane (1:1, v/v) twice and the solvent was evaporated to dryness at room temperature under a stream of nitrogen. The residue was reconstituted with 20 μl of a mixture of ether and hexane (1:1, v/v) and a 1–2 μl of the solution was subject to GC–MS.

2.5. Calibration curves and quantitation

To each of a series of standards containing known amounts of [$^2H_7$]KIC (5.0, 10.1, 20.2, 50.4 100.9, 504.3 and 1008.8 pmol) or KIC (0.1, 0.2, 0.5, 1.00 and 2.01 nmol) dissolved in ethanol, [$^2H_3$]KIC (100 pmol) dissolved in ethanol was added as an analytical internal standard. Each sample was prepared in quadruplicate. After evaporation of the solvent at room temperature by a stream of nitrogen, the residue was dissolved in 2 ml of water and the sample was derivatized according to the procedure described above. The derivatized samples were analyzed by GC–MS-SIM in triplicate. The peak area values of each analyte were monitored at *m/z* 278 for KIC, *m/z* 281 for [$^2H_3$]KIC, and *m/z* 285 for [$^2H_7$]KIC. After correcting the peak-area values with the values of mutual contributions as shown in Table 1, the peak area ratios (KIC/[$^2H_3$]KIC and [$^2H_7$]KIC/[$^2H_3$]KIC, respectively) were determined. The curves were obtained by an unweight least-squares linear fitting of the peak-area ratios versus molar ratios on each sample. Plasma concentrations were calculated by comparing the peak-area ratios obtained from the unknown samples with those obtained from the standard mixtures. Quality control samples were prepared by spiking several amounts of [$^2H_7$]KIC to rat plasma to yield three concentrations at 0.1, 1.0 and 10 μ*M*.

Table 1
Mutual contributions to ion intensity of various species in the channels monitored

Compound	*m/z* 278	*m/z* 281	*m/z* 285
KIC	100	0.27	0.02
[$^2H_3$]KIC	1.17	100	0.10
[$^2H_7$]KIC	0.18	0.20	100

2.6. Accuracy and precision

Accuracy and precision were determined by assaying four preparations of 50-μl portions of rat pooled plasma with several concentrations of [$^2H_7$]KIC (0.1, 0.2, 1.0, 2.0 and 10 μ*M*). Following the addition of [$^2H_3$]KIC (100 pmol) dissolved in ethanol as an internal standard, the samples were subjected to clean-up, derivatized and analyzed by GC–MS-SIM according to the procedure described above.

2.7. Drug administration

Sprague–Dawley (S.D.) male rats weighing 250–350 g were used. After fasting for 12 h, [$^2H_7$]KIC (5 mg (36 μmol)/kg weight) in saline (0.5 ml of dosing solution/kg weight) was administered intravenously with a bolus into the femoral vein under anesthesia with ether. Blood sample (150 μl) was collected from the jugular vein using a heparinized syringe at 10 min before dosing and 0.5, 1, 3, 5, 10, 15, 20, 30, 60, 90 and 120 min after dosing. Plasma was separated and stored at −20°C until analysis.

3. Results and discussion

Successful application of stable isotope dilution methodology to the pharmacokinetics and metabolic investigations is dependent upon the availability of compounds labeled at predesigned positions that are chemically and metabolically inert. We have prepared multi-labeled KIC ([$^2H_7$]KIC) containing seven non-exchangeable deuterium atoms at C-4, C-5 and C-6 for use as a biological internal standard (Fig. 2). In addition, a commercially available [5,5,5-$^2H_3$]KIC ([$^2H_3$]KIC) was chosen for an analytical internal standard. In these labeled compounds, the deuterium labels were placed at sufficient distance

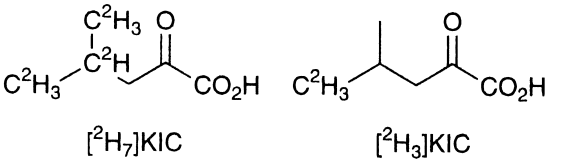

Fig. 2. Structures of [$^2H_7$]KIC and [$^2H_3$]KIC.

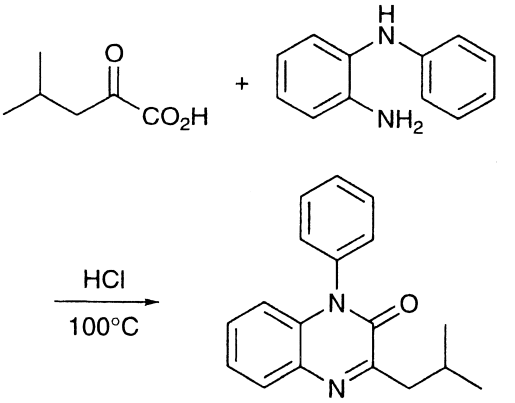

Fig. 3. Preparation of *N*-phenylquinoxalinone derivative of KIC.

from the keto group to avoid loss of the labels under both keto–enol tautomerization [13–15] and transamination process.

KIC was converted to the *N*-phenylquinoxalinone derivative by condensation with *N*-Phe-PDA in a single step as shown in Fig. 3. The derivative showed good chromatographic behavior with a symmetrical peak (Fig. 4). The determination of KIC requires the GC separation with α-keto-β-methylvaleric acid (KMV) which has the identical mass number. Fig. 4 shows the SIM profile at the molecular ion of the *N*-phenylquinoxalinone derivatives of KIC and KMV. The separation of the

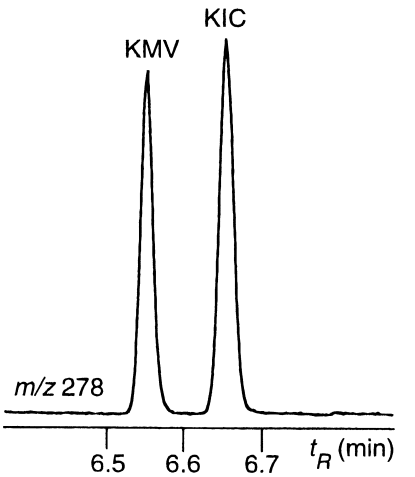

Fig. 4. SIM profile of *N*-phenylquinoxalinone derivatives of KIC and KMV.

derivatives were achieved within 7 min and the R_s value was 3.4.

Fig. 5 shows the electron ionization (EI) mass spectra of *N*-phenylquinoxalinone derivatives of KIC, [$^2H_3$]KIC and [$^2H_7$]KIC, respectively. The molecular ions at *m/z* 278, 281 and 285 for the respective *N*-phenylquinoxalinone derivatives were observed in the relatively high intensities. The *N*-phenylquinoxalinone derivatives gave base peak ions at *m/z* 236 for KIC, at *m/z* 236 and 237 for [$^2H_3$]KIC and at *m/z* 237 for [$^2H_7$]KIC, which were derived by McLafferty rearrangement (Fig. 6) [9,16]. Since the loss of the deuterium labels of both [$^2H_3$]KIC and [$^2H_7$]KIC was observed in the respective base peak ions, the molecular ions on the EI

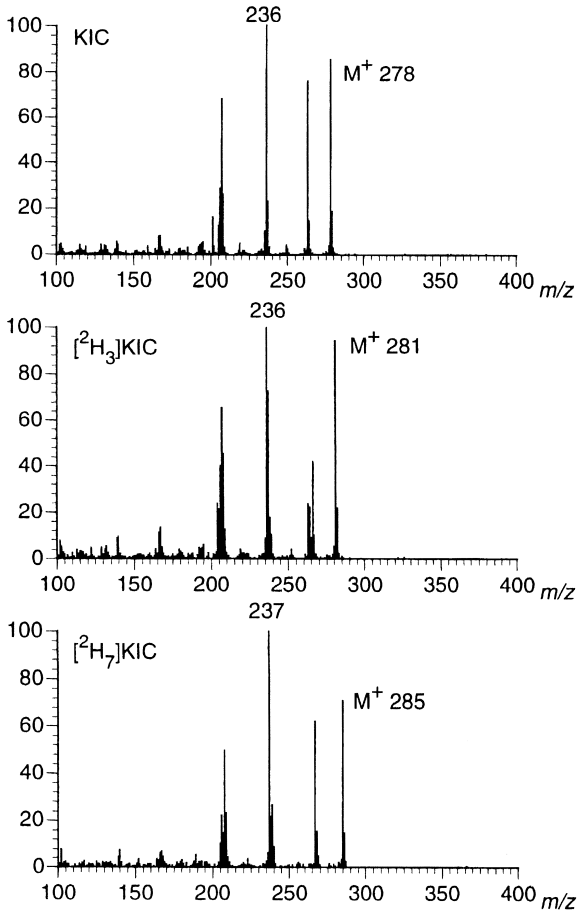

Fig. 5. Electron-impact mass spectra of *N*-phenylquinoxalinone derivatives of KIC, [$^2H_3$]KIC and [$^2H_7$]KIC.

method were chosen for the selected-ion monitoring of *N*-phenylquinoxalinone derivatives. When a signal-to-noise (*S/N*) ratio of at least 3.0 was used as criterion for a significant response, the limit of detection for the present GC–MS-SIM method was found to about 70 fmol per injection for [$^2H_7$]KIC (Fig. 7).

Because of the natural abundance of ^2H, ^{13}C and ^{18}O, small peaks at *m/z* 281 and/or 285 may appear in the mass spectrum of *N*-phenylquinoxalinone derivative of KIC. In addition, there is also the possibility that the derivative of [$^2H_3$]KIC could contribute to the *m/z* 278 and/or 285 peaks and the derivative of [$^2H_7$]KIC to the *m/z* 278 and/or *m/z* 281 peaks. Precise analysis of the GC–MS-SIM data from *N*-phenylquinoxalinone derivatives of KIC, [$^2H_3$]KIC and [$^2H_7$]KIC summarized in Table 1 indicated that no corrections for overlapping ions among the various isotopic compounds in question were necessary. The labeled compounds possessed sufficiently high isotopic purity that the contributions to the other ions were minor. However, there was the possibility that the concentration of [$^2H_7$]KIC was 100–1000 fold smaller than that of endogenous KIC in the dose experiment. In such cases the contributions were significant, and corrections were made based on the values in Table 1.

Calibration curves were prepared from a series of samples containing various amounts of [$^2H_7$]KIC in the range of 5.0–1000 pmol or KIC in the range of 0.1–2.0 nmol. When the peak-area ratios (*y*) were plotted against the mixed molar ratios (*x*), a good correction was found. Unweighted least-regression analysis gave the regression lines, $y=1.18x-0.047$ ($r=0.999$) for [$^2H_7$]KIC and $y=1.17x-0.036$ ($r=0.999$) for KIC.

Ion-exchange chromatography provides a simple method for extracting α-keto acids from biological fluids [17–21]. We have used a cation-exchange cartridge column BondElut SCX to extract leucine from plasma prior to GC–MS analysis [1]. When the same procedure was employed for KIC, KIC was not retained to the cation-exchange column and eluted in the waste and water washing. In this respect, the cation-exchange column would serve as a means of separating KIC and leucine in plasma. Rat plasma spiking [$^2H_7$]KIC (100 pmol) was deproteinized with ethanol and then applied to with BondElut SCX

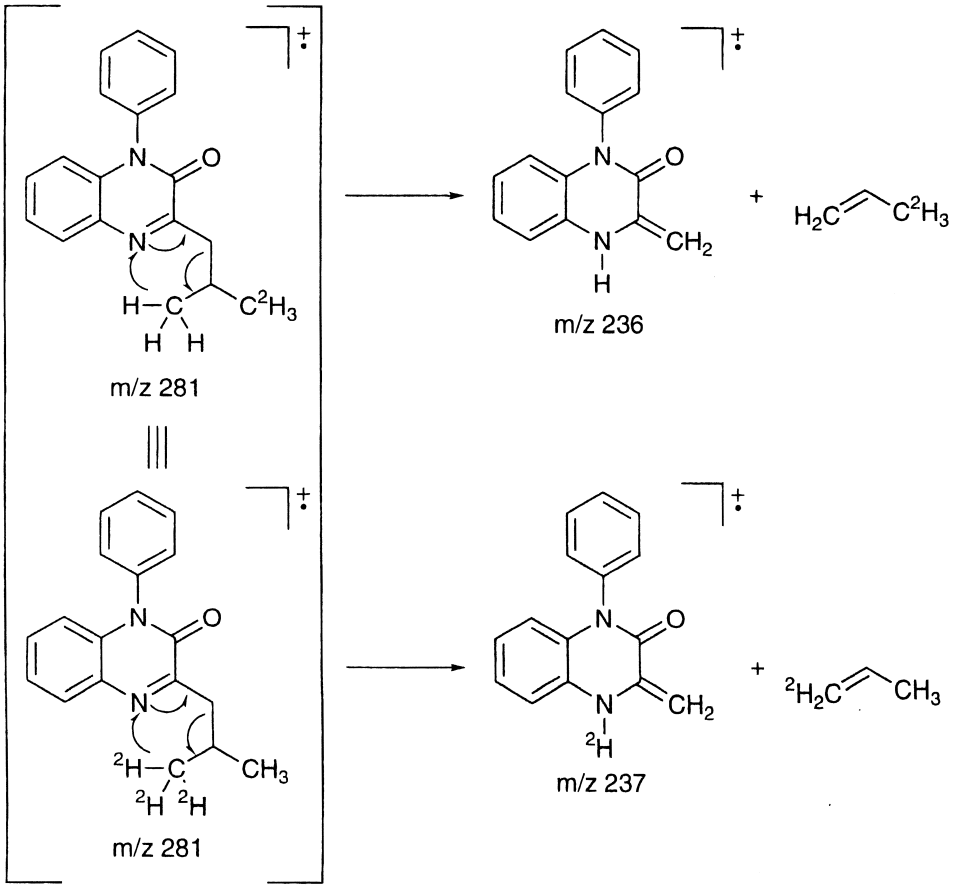

Fig. 6. McLafferty rearrangement of *N*-phenylquinoxalinone derivative of [$^2H_3$]KIC.

column, followed by elution with water to recover the analyte at 62±2% yield (n=3).

Endogenous KIC concentrations in 50 μl of rat plasma were measured with relative intra- and inter-day precision of 4.0% and 3.3%, respectively (Table 2). The intra-day accuracy and precision for [$^2H_7$]KIC spiked to rat plasma in the range of 0.1–10 μ*M* are shown in Table 3. The intra- and inter-day precision for [$^2H_7$]KIC gave good reproducibility with relative standard deviation (RSD) of 6.5% and 5.4%, respectively. The intra- and inter-day relative errors (RE) for [$^2H_7$]KIC were less than 6.4% and 3.8%, respectively. The limit of quantitation (LOQ) for [$^2H_7$]KIC was around 50 n*M* in rat plasma.

The present GC–MS-SIM method was applied for the quantitation of plasma concentrations of [$^2H_7$]KIC and endogenous KIC after a bolus intravenous administration of [$^2H_7$]KIC (36 μmol/kg weight) to S.D. male rats. Representative SIM profiles of plasma samples are shown in Fig. 8. There was no interference from endogenous compounds in the vicinity of the peaks of analytes in the mass fragmentograms. Fig. 9 shows representative plasma concentration-time profiles of [$^2H_7$]KIC, L-[$^2H_7$]leucine formed by amination, and endogenous KIC and L-leucine. Plasma concentration of [$^2H_7$]KIC could be followed for as long as 90 min. Following administration of [$^2H_7$]KIC, L-[$^2H_7$]leucine quickly appeared in the plasma, reached a maximum concentration (14.1±6.4 μ*M*) at approximately 3 min and then gradually decreased. At 120

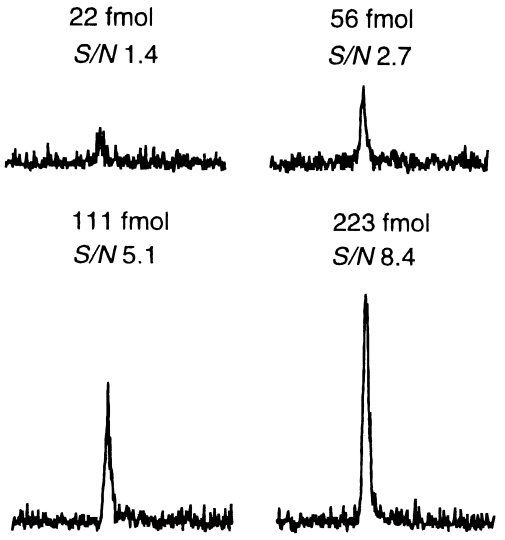

Fig. 7. Sensitivity of *N*-phenylquinoxalinone derivative of [$^2H_7$]KIC by SIM.

Fig. 8. SIM profiles of rat plasma samples at 20 min following an intravenous administration of [$^2H_7$]KIC (36 μmol/kg weight).

Table 2
Intra- and inter-day precision for KIC in rat plasma

	Plasma KIC (μ*M*)	
	Mean±SD	RSD (%)
Intra-day (*n*=3)		
Day 1	22.75±0.64	3.0
Day 2	22.97±0.87	3.8
Day 3	23.29±0.93	4.0
Inter-day		
	22.99±0.76	3.3

min after administration, the concentration of L-[$^2H_7$]leucine was 0.64±0.41 μ*M*. A pharmacokinetic study of KIC including interconversion between KIC and L-leucine is now in progress and will be described in details elsewhere.

The present method provided a sensitive and

Table 3
Intra- and inter-day accuracy and precision for [$^2H_7$]KIC spiked to rat plasma

Spiked (nmol)	Expected (μM)	Intra-day (*n*=4)			Inter-day (*n*=3)		
		Found (μM) (Mean±SD)	RSD (%)	RE (%)	Found (μM) (Mean±SD)	RSD (%)	RE (%)
0.005	0.10	0.095±0.006	6.3	−6.35	0.097±0.002	2.4	−3.80
0.01	0.20	0.204±0.013	6.5	0.99	0.205±0.004	1.8	1.74
0.05	1.01	1.050±0.030	2.8	4.07	1.040±0.029	2.8	3.09
0.10	2.02	1.972±0.063	3.2	−2.26	1.966±0.006	0.3	−2.54
0.50	10.09	10.092±0.186	1.8	0.05	9.788±0.527	5.4	−2.96

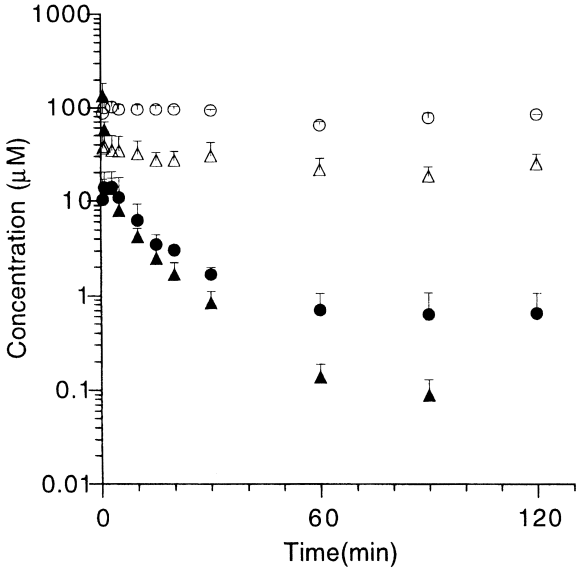

Fig. 9. Plasma concentration versus time profiles for [$^2H_7$]KIC (▲), L-[$^2H_7$]leucine (●) formed, and endogenous KIC (△) and L-leucine (○).

reliable technique for determining the plasma levels of the labeled KIC and endogenous KIC with good accuracy and precision. The method was confirmed to be applicable for assessing the pharmacokinetics of other α-keto acids as well as KIC.

Acknowledgements

This work was supported by Grant for private universities provided by the Promotion and Mutual Aid Corporation for Private Schools of Japan.

References

[1] H. Hasegawa, T. Matsukawa, Y. Shinohara, T. Hashimoto, J. Chromatogr. B 735 (1999) 141.
[2] H. Hasegawa, T. Matsukawa, Y. Shinohara, T. Hashimoto, Drug Metab. Dispos. 28 (2000) 920.
[3] R.K. Chawla, W.J. Stackhouse, A.D. Wadsworth, J. Nutr. 105 (1975) 798.
[4] C.W. Kang, M. Walser, Am. J. Physiol. 249 (1985) E355.
[5] T. Shiota, M. Yagi, M. Walser, Metabolism 38 (1989) 612.
[6] U. Langenbeck, A. Hoinowski, K. Mantel, H.-U. Möhring, J. Chromatogr. 143 (1977) 39.
[7] F. Rocchiccioli, J.P. Leroux, P. Cartier, Biomed. Mass Spectrom. 8 (1981) 160.
[8] K. Tanaka, D.G. Hine, A. West-Dull, T.B. Lynn, Clin. Chem. 26 (1980) 1839.
[9] A.A. Fernandes, S.C. Kalhan, F.G. Njoroge, G.S. Matousek, Biomed. Env. Mass Spectrom. 13 (1986) 569.
[10] D.L. Hachey, B.W. Patterson, P.J. Reeds, L.J. Elsas, Anal. Chem. 63 (1991) 919.
[11] S.H. Lee, S.O. Kim, B.C. Chung, J. Chromatogr. B 719 (1998) 1.
[12] A.H. Cook, C.A. Perry, J. Chem. Soc. (1943) 394.
[13] R.B. Weinberg, M. Walser, Biochem. Med. 17 (1977) 164.
[14] D.E. Matthews, E. Ben-Galim, M.W. Haymond, D.M. Bier, Pediatr. Res. 14 (1980) 854.
[15] P. Schadewaldt, H.-W. Hammen, C. Dalle-Feste, U. Wendel, J. Inher. Metab. Disease 13 (1990) 137.
[16] F.W. McLafferty, Anal. Chem. 28 (1956) 306.
[17] H.P. Schwarz, I.E. Karl, D.M. Bier, Anal. Biochem. 108 (1980) 360.
[18] R.J. Early, J.R. Thompson, T. McAllister, T.W. Fenton, R.J. Christopherson, J. Chromatogr. 310 (1984) 1.
[19] S.L. Nissen, C. Van Huysen, M.W. Haymond, J. Chromatogr. 232 (1982) 170.
[20] F.F. Horber, J. Kahl, L. Lecavalier, B. Krom, M.W. Haymond, J. Chromatogr. 495 (1989) 81.
[21] G.C. Ford, K.N. Cheng, D. Halliday, Biomed. Mass Spectrom. 12 (1985) 432.