Analytical Methods

Analysis of intracellular α-keto acids by HPLC with fluorescence detection

Journal:	*Analytical Methods*
Manuscript ID	AY-ART-03-2020-000556.R1
Article Type:	Paper
Date Submitted by the Author:	08-Apr-2020
Complete List of Authors:	Fujiwara, Takuya; University of Tokyo Hattori, Ayuna; National Cancer Center Research Institute Ito, Takahiro; Kyoto University Funatsu, Takashi; The University of Tokyo Tsunoda, Makoto; University of Tokyo,

ARTICLE

Analysis of intracellular α-keto acids by HPLC with fluorescence detection

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

Takuya Fujiwara,[a] Ayuna Hattori,[b] Takahiro Ito,[c] Takashi Funatsu[a] and Makoto Tsunoda*[a]

Branched-chain keto acids and branched-chain amino acids are metabolites of branched-chain amino acid aminotransferase (BCAT), which catalyzes reversible transamination between them. We found that BCAT plays an important role in the progression of myeloid leukaemia, and a method for the analysis of intracellular α-keto acids including branched-chain keto acids was necessary to further investigate their role. In this study, we developed a method to analyze six α-keto acids (α-ketoglutaric acid (KG), pyruvic acid, α-ketobutyric acid, α-ketoisovaleric acid, α-ketoisocaproic acid, and α-keto-β-methylvaleric acid) in K562 cells by HPLC with fluorescence detection, using 1,2-diamino-4,5-methylenedioxybenzene (DMB) as a derivatization reagent. Because split peaks of DMB-KG were observed when injection samples were too acidic, the derivatization solution was diluted with NaOH solution to obtain a single peak. Limits of detection and limits of quantification were 1.3-5.4 nM and 4.2-18 nM, respectively. Intracellular concentrations of α-keto acids were 1.55-316 pmol/1 × 10^6 K562 cells. The developed method realized reproducible and sensitive analysis of intracellular α-keto acids. Thus, the method could be used to elucidate the role of BCAT in myeloid leukaemia.

1. Introduction

α-Keto acids are known as intermediates involved in many metabolic pathways, such as amino acid metabolism, glycolysis, and citric acid cycle. Branched-chain keto acids (BCKAs) are synthesized from branched-chain amino acids (BCAAs) by branched-chain amino acid aminotransferase (BCAT), which transfers the amino group of glutamic acid.[1] While glutamic acid is converted to α-ketoglutaric acid (KG) by removing the amino group, BCKAs, e.g., α-ketoisovaleric acid (KIV), α-ketoisocaproic acid (KIC), and α-keto-β-methylvaleric acid (KMV), are converted to BCAA valine, leucine, and isoleucine, respectively. In addition, BCAT catalyzes the reverse reaction that synthesizes glutamic acid and BCKAs from KG and BCAAs, respectively.

We recently found that BCAT plays a significant role in the development of chronic myeloid leukaemia. BCAT is indispensable during the progression of chronic leukaemia cells by BCAA production.[2] To track BCAT activity in cells, the quantification of intracellular metabolites, such as glutamic acid, KG, BCKAs, and BCAAs, is needed. Although many analytical methods for observing amino acids have been developed,[3-6] there are not so many for studying α-keto acids (HPLC with fluorescence detection,[7-12] LC-MS,[13-15] and GC-MS[16]). While intracellular α-keto acids have been quantified by HPLC with fluorescence detection using o-phenylenediamine (OPD) derivatization,[11,12] the precise quantification of four α-keto acids produced by BCAT (KG, KIV, KIC, and KMV) has not yet been realized.

In this study, 1,2-diamino-4,5-methylenedioxybenzene (DMB) was used as the derivatization reagent to improve sensitivity. This is possible because α-keto acids derivatized with DMB produce stronger fluorescence than those derivatized with OPD. A typical derivatization reaction of α-keto acids with DMB is shown in Fig. 1a, and structures of the analytes are shown in Fig. 1b. Pyruvic acid (PV) and α-ketobutyric acid (KB)

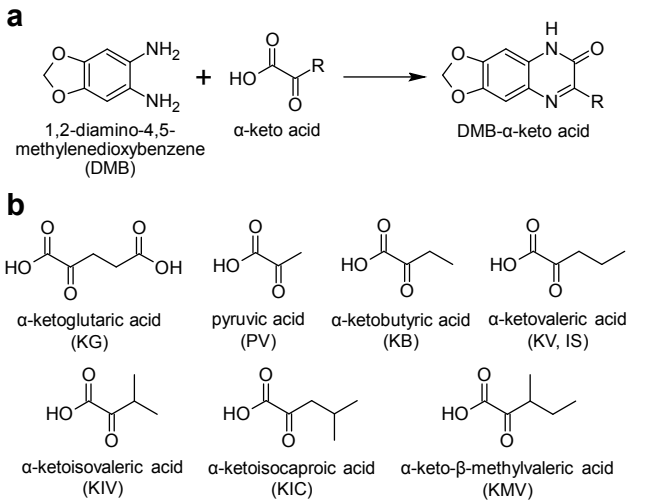


Fig. 1 (a) Derivatization reaction of DMB with α-keto acid and (b) chemical structures of α-keto acids.

[a] *Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo 113-0033, Japan. E-mail: makotot@mol.f.u-tokyo.ac.jp*
[b] *Division of Hematological Malignancy, National Cancer Center Research Institute, Tokyo 104-0045, Japan*
[c] *Institute for Frontier Life and Medical Sciences, Kyoto University, Kyoto 606-8507, Japan, and Department of Biochemistry & Molecular Biology, University of Georgia, Athens 30602, USA.*

were also measured because they are metabolites of amino acids. PV is synthesized from alanine by transamination, and KB is a degradation product of threonine. Using DMB as the derivatization reagent, six α-keto acids in K562 cells were successfully quantified.

2. Experimental

2.1. Chemicals

α-Ketoglutaric acid (KG), pyruvic acid (PV), α-ketobutyric acid (KB), 2-mercaptethanol, sodium sulfite, and hydrochloric acid were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). α-Ketovaleric acid (KV) was from Tokyo Chemical Industry (Tokyo, Japan). α-Ketoisovaleric acid (KIV), α-ketoisocaproic acid (KIC), and α-keto-β-methylvaleric acid (KMV) were from Sigma-Aldrich (St. Louis, MO, USA). 1,2-Diamino-4,5-methylenedioxybenzene dihydrochloride (DMB·2HCl) was obtained from Dojinkagaku Laboratories (Kumamoto, Japan). MeOH (HPLC grade) was from Merck KGaA (Darmstadt, Germany). A Milli-Q system (Merck) was used for water purification.

2.2. Cell samples

K562 human blast crisis CML cells were obtained from ATCC, and cell line authentication testing was performed by ATCC standardized sort tandem repeat analysis to verify its identity. K562 cells maintained in Roswell Park Memorial Institute 1640 medium (RPMI-1640) with 10% FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin. K562 cells were treated with 80% methanol containing KV as internal standard. After removing insoluble particles by centrifugation, supernatants were collected and dried at 30-45°C. The cell solution was prepared by dissolving dried cell sample (1 x 10^6 cells) in 100 μL of water.

2.3. Derivatization conditions

DMB solution was prepared by adding 1.6 mg of DMB·2HCl to 1.0 mL of solution, which contained 4.9 mg of sodium sulfite, 70 μL of 2-mercaptethanol, and 58 μL of concentrated HCl in 0.87 mL of H_2O. DMB solution (40 μL) was added to 40 μL of α-keto acids aqueous solution in the sealed tube. The solution was heated at 85 °C for 45 min. After cooled on ice for 5 min, the solution was diluted fivefold with 65 mM NaOH aqueous solution, and 25 μL was injected into HPLC. In initial condition, the reaction solution was not diluted after cooled on ice and injection volume was 5 μL.

2.4. HPLC conditions

The HPLC system (Jasco) was composed of a PU-980 pump, an LG-1580-02 ternary gradient unit, a DG-980-50 3-line degasser, AS-2057 PLUS autosampler, CO-1560 column oven, and FP-1520S fluorescence detector. Separation was conducted on Inertsil ODS-4V column (250 × 3.0 mm, 5.0 μm) (GL Sciences, Tokyo, Japan). Fluorescence detection was performed at excitation and emission wavelengths of 367 nm and 446 nm, respectively. Mobile phases were (A) MeOH/H_2O (30/70, v/v) and (B) MeOH. Gradient elution was performed as follows: 0 min 0%B, 10 min 0%B, 20 min 50%B, 50 min 50%B (initial condition: 0 min 0%B, 10 min 0%B, 15 min 50%B, 50 min 50%B). A flow rate was 0.3 mL/min and the column temperature was maintained at 40°C.

2.5. Method validation

Calibration curves, limits of detection (LOD), limits of quantification (LOQ), accuracy, and precision were calculated. The calibration curves were obtained using standard samples of respective concentrations (KG, KIV and KMV: 50 nM-5 μM, KB and KIC: 10 nM-1 μM, PV: 100 nM-10 μM), and α-ketovaleric acid (KV) was used as an internal standard. The ratios of peak areas of DMB-keto acids against internal standard were plotted against their concentrations (μM), then the slope, intercept and correlation coefficient of the calibration curves were calculated by least-square regression. LOD and LOQ were calculated at a signal to noise ratio (S/N) = 3 and 10, respectively. The intra-day and inter-day precisions were calculated by five repetitive measurements on the same day and on successive days, respectively.

3. Results and discussion

3.1. Separation of six DMB-α-keto acids

Based on previous studies,[8] derivatization of α-keto acids with DMB and separation of DMB-α-keto acids were initially performed. The derivatization and separation conditions are described in the experimental section. As shown in Fig. 2, six kinds of α-keto acids (KG, PV, KB, KIV, KIC, and KMV)

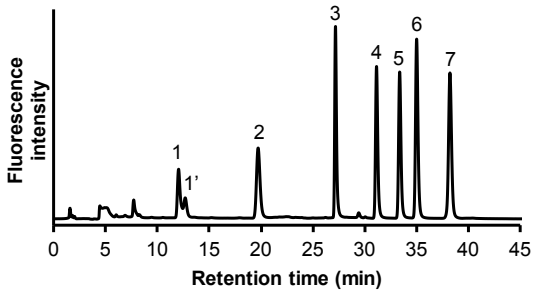


Fig. 2 Chromatogram of DMB-α-keto acids under the initial condition. Mobile phase: (A) MeOH/H_2O (30/70, v/v) and (B) MeOH, gradient elution: 0 min 0%B, 10 min 0%B, 15 min 50%B, 50 min 50%B. Peaks: 1 and 1', DMB-KG (0.5 μM) split into two peaks; 2, DMB-PV (1.0 μM); 3, DMB-KB (1.0 μM), 4, DMB-KV (1.0 μM); 5, DMB-KIV (2.0 μM); 6, DMB-KIC (1.0 μM); 7, DMB-KMV (2.0 μM).

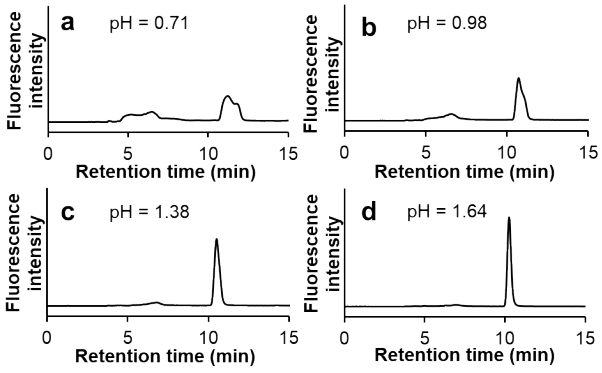


Fig. 3 Chromatograms of DMB-KG when changing the dilution rate. Dilution rate: (a) 1, (b) 2, (c) 5, and (d) 10 with water. pHs of the sample solutions were described in the figure. Mobile phase: (A) MeOH/H_2O (30/70, v/v) and (B) MeOH, gradient elution: 0 min 0%B, 10 min 0%B, 20 min 50%B. Sample: (a) 0.5 μM (b) 1.0 μM, (c) 2.5 μM, and (d) 5.0 μM KG aqueous solution.

derivatized with DMB were successfully separated. However, the peak of DMB-KG was split into two peaks, and the peak shapes and heights were not reproducible. Although other mobile phase conditions were examined ($MeCN/H_2O$, $MeOH/H_2O$/TFA, MeOH/phosphate buffer solution), the splitting of the DMB-KG peak persisted.

3.2. Investigation of DMB-KG peak splitting

To obtain a single peak of DMB-KG, derivatization conditions were re-investigated. Considering that DMB-KG was the only α-keto acid possessing a carboxyl group among the six analytes, we hypothesized that the peak splitting is likely associated with the acidity of the sample solution. Hence, the relationship between the peak shape and acidity of the sample was explored. After the derivatization, the reaction solution pH was 0.71. The solution was then diluted with water 2, 5, and 10 times to change the acidity of the injection sample. These samples were then compared to the original, undiluted solution. As shown in Fig. 3, gradient transition from two peaks to a single peak was observed as the dilution rates increased.

Upon discovering that a single peak for DMB-KG was obtained by diluting with water, and the peak shape improved with more dilutions, the use of a more basic solution to adjust acidity was tested. Aqueous NaOH was selected as the basic solution because a basic buffer was not suitable to buffer 0.4 M HCl. When NaOH concentrations were varied between 35 and 95 mM, with five-fold dilution, a concentration of 65 mM gave the highest peak. Under the initial separation conditions, the DMB-KMV peak overlapped with a blank peak. To separate these peaks, the gradient span was increased from 10 min (5-15 min) to 15 min (5-20 min). Fig. 4a shows the chromatogram resulting from the optimized conditions.

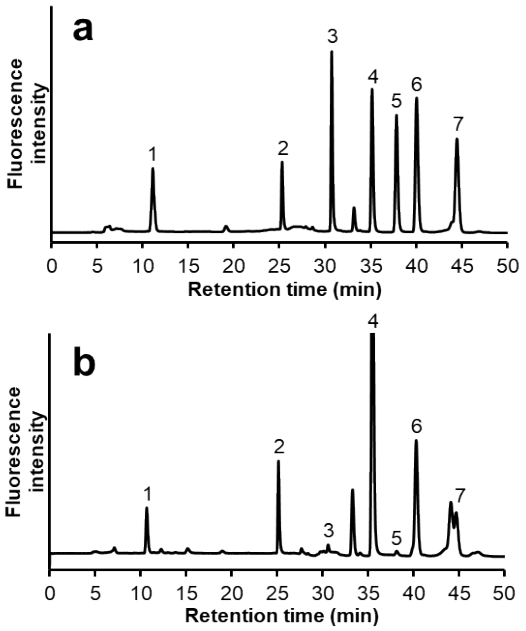
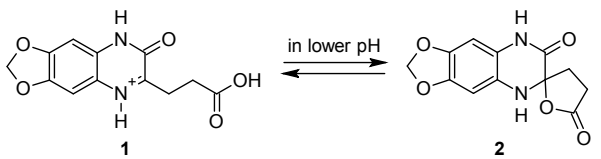


Fig. 4 Chromatogram of DMB-α-keto acids in (a) standard sample and (b) K562 cell sample under the optimized conditions, wherein sample was diluted five-fold with 65 mM NaOH aqueous solution. Peaks: 1, DMB-KG; 2, DMB-PV; 3, DMB-KB, 4, DMB-KV; 5, DMB-KIV; 6, DMB-KIC; 7, DMB-KMV.



Scheme 1 Proposed structural change of DMB-KG. Compound **1** is protonated DMB-KG. As acidity increases, structural change with resolving cation might occur.

As mentioned above, sample acidity was associated with the peak shape of DMB-KG. We hypothesized that structural change caused this peak splitting; namely, a possible intramolecular cyclization reaction due to the iminium cation of DMB-KG present under acidic conditions (Scheme 1). Therefore, we attempted to gather structural information of

Table 1 LOD, LOQ, and linearity for developed method.

α-keto acids	LOD (nM)	LOQ (nM)	linearity (μM, r^2>0.999)
KG	1.3	4.2	0.05-5
PV	1.9	6.2	0.1-10
KB	1.8	5.9	0.01-1
KIV	4.6	15	0.05-5
KIC	2.0	6.6	0.01-1
KMV	5.4	18	0.05-5

Table 2 Intraday precision and accuracy in K562 cell samples (n = 5).

α-keto acids	added (nM)	measured (mean±SD, nM)	RSD (%)	accuracy (%)
KG	0	1559±12	0.8	-
	500	2118±28	1.3	112
	1000	2465±14	0.6	91
	2000	3635±118	3.2	104
	4000	6108±107	1.8	114
PV	0	3602±123	3.4	-
	1250	4911±132	2.7	105
	2500	6193±178	2.9	104
	5000	8730±97	1.1	103
	10000	14270±102	0.7	107
KB	0	16±1	4.9	-
	12.5	30±2	5.1	118
	25	43±3	6.3	109
	50	65±3	4.7	99
	100	122±5	3.9	106
KIV	0	64±3	4.3	-
	12.5	79±1	1.0	118
	25	85±5	5.7	86
	50	111±3	2.7	94
	100	170±4	2.3	106
KIC	0	493±7	1.3	-
	125	632±5	0.8	111
	250	731±12	1.7	95
	500	994±12	1.2	100
	1000	1557±15	1.0	106
KMV	0	453±16	3.5	-
	125	593±8	1.3	112
	250	689±16	2.4	94
	500	977±18	1.9	105
	1000	1579±25	1.6	113

Table 3 Interday precision and accuracy in K562 cell samples (n = 5).

α-keto acids	added (nM)	measured (mean±SD, nM)	RSD (%)	accuracy (%)
KG	0	950±148	15.6	-
	500	1595±173	10.9	129
	1000	2150±143	6.7	120
	2000	2944±111	3.8	100
	4000	4752±76	1.6	95
PV	0	2979±378	12.7	-
	1250	4443±343	7.7	117
	2500	5479±252	4.6	100
	5000	7582±192	2.5	92
	10000	11986±239	2.0	90
KB	0	10±1	12.1	-
	12.5	25±2	9.5	119
	25	35±1	2.8	100
	50	55±3	5.9	89
	100	102±5	5.4	92
KIV	0	48±7	14.1	-
	12.5	64±6	9.3	127
	25	81±4	4.5	134
	50	106±8	7.9	116
	100	150±11	7.5	102
KIC	0	370±6	1.6	-
	125	465±7	1.4	76
	250	585±13	2.2	86
	500	774±17	2.2	81
	1000	1217±24	2.0	85
KMV	0	301±23	7.8	-
	125	469±13	2.9	134
	250	617±15	2.4	126
	500	818±20	2.4	103
	1000	1268±32	2.5	97

DMB-KG in varying acidities. However, NMR spectra could not be obtained due to low solubility of DMB-KG in D_2O.

3.3. Method validation

Table 1 shows the validation data of the developed method. For DMB-α-keto acids, good linearity was obtained. LOD and LOQ values were 1.3-5.4 and 4.2-18 nM, respectively. Sensitivity was at least 6 times higher than that of OPD derivatization.[7,10-12] Intraday precision and interday precision were 0.6-6.3% and 1.4-15.6%, respectively (Tables 2 and 3). Intraday and interday accuracies were 86-118% and 76-134%, respectively. This validation data shows that the developed method is sufficient for routine analysis of cell samples.

3.4. Application to cell samples

The developed method was applied to K562 cell samples. A typical chromatogram of cell sample is shown in Fig. 4b. Quantified intracellular content of KG, PV, KB, KIV, KIC, and KMV were 125±24, 316±48, 1.55±0.27, 4.08±1.36, 45.1±4.3, and 41.4±4.8 pmol/1 x 10^6 K562 cells, respectively (n = 5). These concentrations were in the same order as that in previous reports of K562 cells and neutrophils.[11,12]

4. Conclusion

In this study, an accurate and precise quantification method for analyzing six α-keto acids (KG, PV, KB, KIV, KIC, and KMV) was developed using DMB derivatization. Reproducible separation of DMB-α-keto acids was realized by diluting the reaction mixture with a basic solution. As an application of this method, α-keto acid content in K562 cell samples was quantified. The proposed method could show further value in quantifying samples with more interference peaks or lower α-keto acid content. Furthermore, this developed method has the potential to elucidate the role of BCAT in myeloid leukaemia.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research (C) (17K08234) from the Japan Society for the Promotion of Science (JSPS), the Center of Innovation Program from the Japan Science and Technology Agency (JST) (JPMJCE1305) to MT; by the grant from Japan Agency for Medical Research and Development (AMED) (JP19cm010614) to AH and MT; by the grants from the American Cancer Society (RSG-1703201-DDC), the Yasuda Medical Foundation and the Daiichi Sankyo Foundation of Life Science to TI.

References

1 J. T. Brosnan and M. E. Brosnan, *J. Nutr.* 2006, **136**, 207-211.
2 A. Hattori, M. Tsunoda, T. Konuma, M. Kobayashi, T. Nagy, J. Glushka, F. Tayyari, D. McSkimming, N. Kannan, A. Tojo, A. S. Edison and T. Ito, *Nature*, 2017, **545**, 500-504.
3 Y. Song, C. Xu, H. Kuroki, Y. Liao and M. Tsunoda, *J. Pharm. Biomed. Anal.*, 2018, **147**, 35-49.
4 M. Tsunoda and Y. Sumida, Liquid Chromatography | Amino Acids. P. I. Worsfold, C. Poole, A. Townshend and M. Miro, (Eds.), Encyclopedia of Analytical Science, (3rd ed.). 6, 1-11, Elsevier.
5 Y. Song, T. Funatsu and M. Tsunoda, *Amino Acids*, 2012, **42**, 1897-1902.
6 S. Karakawa, R. Nishimoto, M. Harada, N. Arashida and A. Nakayama, *Chromatography*, 2019, **40**, 127-133.
7 K. Koike and M. Koike, Anal. Biochem, 1984, **141**, 481-487.
8 M. Nakamura, S. Hara, M. Yamaguchi, Y. Takemori and Y. Ohkura, *Chem. Pharm. Bull.*, 1987, **35**, 687-692.
9 Z. Wang, K. Zaitsu and Y. Ohkura, *J. Chromatogr.*, 1988, **430**, 223-231.
10 K. Pailla, F. Blonde-Cynober, C. Aussel, J. D. Bandt and L. Cynober, *Clin. Chem.*, 2000, **46**, 848-853.
11 J. Mülhling, M. Fuchs, M. E. Campos, J. Gonter, J. M. Engel, A. Sablotzki, T. Menges, S. Weiss, M. G. Dehne, M. Krüll and G. Hempelmann, *J. Chromatogr. B*, 2003, **789**, 383-392.
12 A. Hattori, T. Ito and M. Tsunoda, *Chromatography*, 2017, **38**, 129-133.
13 K. C. Olson, G. Chen and C. J. Lynch, *Anal. Biochem.*, 2013, **439**, 116-122.
14 K. Noguchi, T. Mizukoshi, H. Miyano and N. Yamada, *Chromatography*, 2014, **35**, 117-123.
15 R. Li, P. Liu, P. Liu, Y. Tian, Y. Hua, Y. Gao, H. He, J. Chen, Z. Zhang and Y. Huang, *Amino Acids*, 2016, **48**, 1523-1532.
16 D. Nguyen, G. Lee and M. Paik, *J. Chromatogr. B*, 2013, **48**, 913-914.

